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Philip A. Schwartzkroin received his Ph.D. in Neurological Sciences from Stanford University in 1972, and carried out postdoctoral work at the Institute of Neurophysiology in Oslo, Norway. He was on the faculty at University of Washington (1978–2001), in the Departments of Neurological Surgery and Physiology/Biophysics. He is currently Professor in the Department of Neurological Surgery at University of California, Davis, where he holds the Bronte Endowed Chair in Epilepsy Research, and is an Affiliate of the UC Davis Center for Neuroscience.

Dr. Schwartzkroin's research has focused on cellular mechanisms of seizures and epilepsy. He has received a number of awards for his laboratory efforts, including two NIH Javits Awards, the American Epilepsy Society Research Award, and a Guggenheim Fellowship. Dr. Schwartzkroin has published over 150 peer-reviewed research articles and numerous book chapters, and has edited several books on epilepsy topics. Although a basic scientist, Dr. Schwartzkroin has throughout his career been associated with clinical departments, and has established important interactions with both basic science and clinical colleagues. He has played a major role in the national and international epilepsy community (past-president of the American Epilepsy Society, past chair of the International League Against Epilepsy's Commission on Neurobiology), and is a recipient of the ILAE/IBE Ambassador for Epilepsy Award. He is currently co-editor-in-chief of the journal *Epilepsia*.

INTRODUCTION

Research in today's epilepsy laboratories is exciting, evolving, and full of interesting twists and turns. Epilepsy has been recognized as a common human disorder for thousands of years. But modern research into its underlying bases has been ongoing, I would argue, only since the middle of the 20th century. Nonetheless, the epilepsy research field boasts an intriguing and productive history. As with most disorders, and certainly for disorders of the nervous system, discovery of mechanisms and improvement in treatment have been a function of technical advances – which in turn have moved research efforts toward ever more detailed and revealing analysis. Because of the complexity of the nervous system, and because of the many different forms that epilepsy may take, research in this field lends itself to different conceptual and technical approaches. It attracts the involvement of those with interests at very basic levels of analysis (e.g., the "hard-core" basic scientists who are interested in "explanations" at the level of genes and molecules) as well as those whose interests are more "clinical" in nature (i.e., those interested in "net output" behaviors, both normal and pathological). Any search of current research activities in the epilepsy field confirms the breadth and variety of epilepsy-related laboratory work. In addition, even a superficial reading of the literature shows the value of epilepsy-related research for opening windows to normal brain function - through which one can begin to see the connections between, and shared mechanisms of, normality and pathology. For anyone interested in the brain, in the general area of neuroscience, and in an opportunity to explore an endlessly fascinating set of puzzles, epilepsy research provides many rewards.

My initial introduction to epilepsy research was in the laboratory. It didn't take long for me to be "hooked," but it did require some hands-on experimental time. Given the need for such exposure, I've struggled with the question of how one can convey that experimentally-generated enthusiasm to someone who's never been in a laboratory, who is in the initial stages of considering a commitment to research or is pondering a focus for his/her laboratory efforts. For a number of years, I've also been frustrated by my inability to provide a satisfying response to an all-too-common question: "Where can I find out about what's going on in epilepsy research?" This type of query comes regularly from students and fellows, from clinical colleagues, from potential colleagues and collaborators outside the epilepsy field who are seeking a reference to current basic research activities. One could, of course, simply direct these individuals to PubMed or to one of the excellent "textbooks" on epilepsy (mostly clinically oriented). But neither of these alternatives is very helpful, especially if one would like to encourage the questioner to become more interested and involved in the laboratory.

My solution to this frustration has been to try to bring many of the current studies in basic epilepsy under one roof - thus, this volume. We've called it an "encyclopedia," but it certainly isn't an encyclopedia in the classical sense. In particular, it does not pretend to be a comprehensive overview of everything (A-Z) in the epilepsy research field. Rather, the collected articles cover a broad selection of topics, contributed by some of the most active epilepsy research groups in the world. These selections were solicited specifically to answer the question "What's going on in epilepsy research today?" My goal is not to cover all potentially relevant themes, but rather to offer representation in this "encyclopedia" to those who have contributed significantly to the field – to share their goals, insights, and progress.

It might be interesting for the reader to note that my preliminary table of contents did not look like the Table of Contents that now introduces this encyclopedia. The initial version was more like something one might see in a textbook, starting with articles at the "micro" level (cellular/molecular features of cells), progressing through synaptic and circuit considerations, and ending with articles focusing on the "net output." I believed that this type of organization could present a rationally-ordered overview of the field. However, as I began to go through the articles that were eventually submitted, I was reminded of my starting motivation – to offer the reader a volume that reflects the major areas of today's research effort in the epilepsy research arena. The current Table of Contents does mirror current research emphases. And the encyclopedia offers the interested reader (including non-experts) a collection of short and focused articles that provide a real sense of the modern research field - What's exciting? What are the key technical approaches? What are the important questions?

We sent out invitations to hundreds of the most active investigators in the field. Each author was asked to frame the question or issue that drives his/her research, to provide some background that puts the research into an historical context, and to describe (briefly) some of the methods that are currently used for this line of work. Authors were then encouraged to identify recent results – from their own laboratories or from others – that are exciting and promising, and to discuss where this line of research should go in the future – the goals, challenges, and potential for clinical application. In short, I asked each author to write an article as though he/she were talking to a prospective laboratory recruit (a graduate student, a postdoctoral fellow, or a collaborative colleague) in a way that would convey the interest and excitement that draws us (as epilepsy scientists and clinicians) toward a career in this area of research. As one might predict, each author has approached this charge in a way that reflects his/her own interests, approach to research, and laboratory personality. While we've tried to make the format of these articles relatively uniform, the style and substance of each article are unique to the laboratory of origin.

Of course, as with any project of this sort, there are always hurdles, objections, and problems. One significant problem – a major concern for me in thinking about the magnitude of this effort - is that much of material collected in these articles is likely to be out of date in a few years. To address this issue, we plan to produce this Encyclopedia in an easily accessible and readily update-able electronic form. My hope from the start was that the material presented here will be updated on a regular 2–3 year cycle, with new results entered, and new areas of research described on a regular basis. Another equally thorny problem was what to include – or exclude. As you can see from the title of the encyclopedia, the emphasis has been on "basic research." But determining exactly what "basic" (and "research") means is a little tricky, especially in a field like epilepsy in which basic and clinical aspects are so intertwined; indeed, epilepsy research has always been what it is now fashionable to call "translational." The coverage of the current volume therefore expresses, in large measure, my own scope of interests; perhaps more accurately, the topics are very much a function of my circle of professional colleagues - those who agreed to participate and the topics they chose to write about. The holes in the current volume – and there certainly are some – include areas in which there is little current activity, but also topics that might have been covered by "other" sets of authors.

We anticipate that this encyclopedia will serve as a useful reference tool for a broad range of readers - laboratory scientists and clinicians, students, fellows, and well-established investigators. Students and young investigators should be able to use this volume to discover potential areas for establishing their own research efforts. Scientists and clinicians with already-established research programs can use the Encyclopedia as a tool for guiding their students and trainees, as an easy way of "keeping up," and even as a source of practical hints that will help them plan their own future studies.

I hope you find this Encyclopedia of Basic Epilepsy Research useful and informative. I've certainly learned a lot from putting it together. For those of you who have contributed to the volume, I thank you for your interest and involvement – and I look forward to your support when I approach you for updates and/or additions. For those of you who have ideas for new articles, whether stemming from your own research or from other laboratories, please contact me with your suggestions. If this Encyclopedia is to fulfill its mission, it needs to be a dynamic, evolving, interactive tool. We are committed to making it work that way.

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ABSENCE SEIZURES

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Genetic Models of Absence Epilepsy: New Concepts and Insights

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Introduction

Absence epilepsy was previously studied in chemical seizure models, in which absence seizures were pharmacologically induced either by systemic or local administration of drugs (such as low doses of pentylenetetrazole (PTZ) in rats or mice, or penicillin in cats). Pressure from society to abandon neurophysiological studies in cats, and the discovery that many (mainly albino) rats show spontaneously-occurring seizures, led to the development of genetic absence rodent models, such as the Genetic Absence Epileptic Rats from Strasbourg (GAERS) and the Wistar Albino Glaxo rats from Rijswijk (WAG/Rij). All individuals of these strains show an age-dependent increase in the expression of spike-and-wave discharges (SWDs) in their electroencephalogram (EEG), the hallmark of absence seizures. SWDs seen in these rodent models are similar to SWDs seen in absence epilepsy in humans. They are accompanied by mild clinical symptoms, which in rats include accelerated breathing, movements of the vibrissae, incidental twitches of fine facial muscles, and eye blinks. The interictal EEG of the rats appears to be normal. The SWDs appear suddenly on a normal background EEG and are bilaterally symmetrical, which is consistent with the view that absence seizures are generalized over the entire cortex of the brain, although SWDs are not well expressed at the occipital cortex. The topographical distribution of spike and wave differs: the spike is more clearly expressed in the frontal part of the cortex, while the wave is more pronounced in the occipital and parietal cortices. Differences in the topography of the spike and wave have also been described in human patients.

Background

Many early studies were designed to validate these genetic models as models of absence epilepsy. Pharmacological or predictive validity (how well outcomes of drug studies in the model predict the action of drugs in patients) was investigated by testing antiepileptic drugs (AEDs), known to be effective or ineffective against absence seizures in humans. These studies showed that specific anti-absence and broad spectrum AEDs reduce SWDs, while drugs known to aggravate absence seizures in humans do the same in these rodent models. Considering these outcomes, pharmaceutical companies now use WAG/Rij and GAERS to predict whether new compounds are effective in absence epilepsy or may aggravate absence seizures. Face validity was confirmed by the similar morphology of SWDs (clear negatively directed spikes followed by negative waves) in humans and in rats, by similarities in topographical distribution of SWDs in

rat and human, and by the frequency dynamics of long- and short-lasting spike-and-wave discharges in humans and rats. Other similarities include the occurrence of SWDs in relation to the state of vigilance, the identical circadian pattern of SWDs, the provocative effects of sleep deprivation, and the corresponding clinical manifestations. Finally, construct validity was shown by establishing the identical behavioral and concomitant electrophysiological correlates of absences in humans and rats. This demonstration focused on a key symptom of generalized epilepsy, the decrease of consciousness or responsiveness accompanying an electroencephalographic seizure. Evoked potentials are excellent indicators of the state of the brain since the amplitude and latency of various components of these potentials are modulated by sleeping and waking. Evoked potentials can be used in order to describe the state of the brain during SWDs. Auditory and visual evoked potentials recorded during wakefulness and REM sleep are similar, while slow wave sleep enhances and delays the various components. Interestingly, evoked potentials during SWDs mimic those made during slow wave sleep (but accompanied with more afterdischarges), suggesting that the state of the brain during SWDs is similar to that during slow wave sleep. Similar to the reduced responsiveness seen during slow wave sleep, the animal is less responsive during SWDs than during wakefulness and REM sleep. Comparison of the errors made in a time estimation task, by WAG/Rij rats and by children with absence seizures, show that both species underestimate the passage of time when seizures occur. These results demonstrate that decrease in the level of consciousness is a fundamental characteristic of absence seizures in both absence epileptic children and in rodent models.

Studies using genetic rodent models have been used to examine fundamental neuronal mechanisms of epilepsy. In absence models, the cortico-thalamic network and its constitutive elements have received most attention in in vitro experiments, and a few neurophysiologic single cell and intracellular studies have been carried out in vivo. The neuroanatomical pathways of the circuitry are well known. Thalamo-cortical and cortico-thalamic cells – both with collateral afferents to the reticular thalamic nucleus (RTN), and with inhibitory projections from the RTN to the thalamo-cortical cells and GABA-ergic cortical interneurons – are the primary pathways involved in the generation and propagation of seizure activity. Early neurophysiologic studies indicated a critical role of the thalamus (including the RTN), since local field potentials showing SWD-like patterns were found there. Also, in thalamic structures, multiple unit activity is synchronized with the spike of the SWD (as recorded with cortical local field potentials). Whether the thalamus, and in particular the RTN, is also the initiator of the rhythmic oscillations (as has been proposed by the cortico-reticular theory) is less clear. Critical experiments, such as surgical isolation of the RTN, have not yet been done in genetic models. Chemical and electrolytic lesions are difficult to carry out and interpret, since it is often uncertain, for example, that intra-RTN injections of drugs are restricted to the RTN. This experimental point is important considering that the RTN contains only GABA-ergic cells and that the thalamus contains mainly glutamatergic ones. In favor of the important role of the RTN (at least in rodent models) in absence seizures is the up-regulation of the T-type Ca^{2+} channels in the RTN in GAERS, the increased density of the α_1 2.1 Ca^{2+} subunit current in the RTN in WAG/Rij rats, and the complete lack of the α_3-GABA subunit immuno-reactivity at the inhibitory synapses of the RTN in these rats. The lack of immunoreactivity for a GABA subunit is particularly interesting, considering that deletion of the β_3 subunit in the RTN of knockout mice renders that part of the thalamus defective, showing a reduced inhibitory efficacy. Further, recurrent inhibitory connections act as a desynchonizer of oscillatory activity in normal RTN, but this function is much reduced in mice and rats with a defective RTN.

Methods

Absences cannot be reliably established from clinical signs only. It is imperative to record the cortical EEG in freely moving animals in order to quantify the number of seizures and to evaluate the effect of various experimental manipulations, such as systemic or local administration of drugs. The implantation of chronic epidural or subcortical electrodes for EEG recording, and of cannulas for intracerebral injections of drugs, is a standard technique. Amplifiers with a high common mode rejection ratio, differential EEG recording, and impedance modifiers placed as close to the animals head as possible, contributes to the recording of a clean, artifact-free EEG in freely moving animals.

Methodological caveat: Outcomes of strain comparative studies (epileptic vs. non-epileptic controls) are difficult to interpret since a found difference cannot directly be ascribed only to the fact that one strain has epilepsy and the other not. Only when the epileptic rat is genetically identical to its control, and the control is without seizures, the interpretation of results from these studies is straightforward. In order to circumvent the interpretation problem, one can take advantage of the fact that young adult WAG/Rij rats (c.a. 2 months of age) do not have SWDs, while all 6-month-old rats show many SWDs. A difference in findings between 2- and 6-month-old WAG/Rij rats can be due either to the presence/absence of seizures, or to the age difference. In order to control the age difference, one can include 2- and 6-month-old rats of a non-epileptic strain – for example, ACIs which show no or very few SWDs at 2 and 6 months of age. Differences between

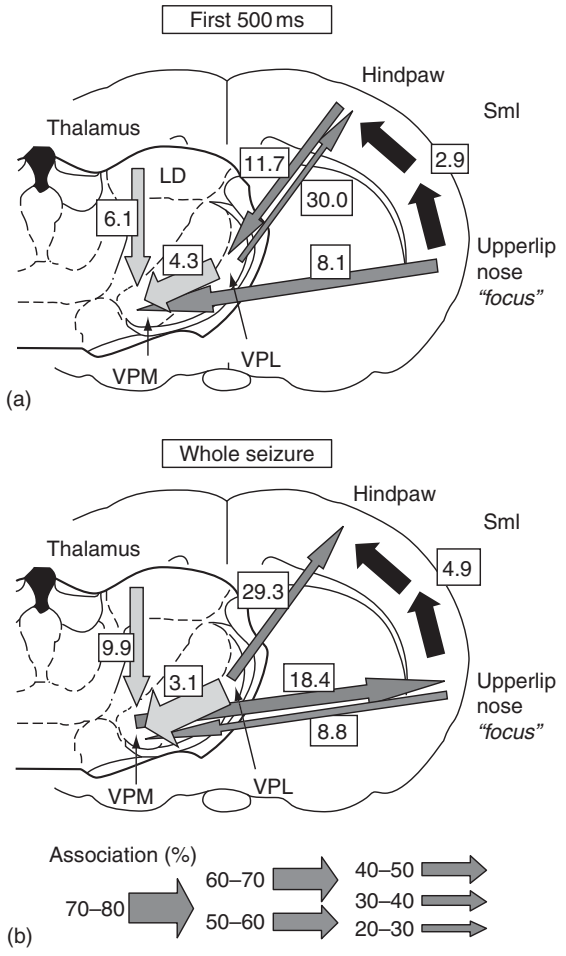


Figure 1 Summary of the cortico-cortical (represented by black arrows), intra-thalamic (light gray arrows), and cortico-thalamic (dark gray arrows) interdependencies during spontaneous absence seizures in the WAG/Rij rat as established by the nonlinear association analysis of a representative WAG/Rij rat (10 seizures were analyzed). The thickness of the arrow represents the average strength of the association, and the direction of the arrowhead points to the direction of the lagging site. The values represent the corresponding average time delays in milliseconds. (a) The relationships as found for the first 500 ms of the generalized seizure showed a consistent cortical focus in the peri-oral somatosensory cortex (SmI), because this site consistently led the other cortical recording sites. The hind paw area, for instance, was found to lag by 2.9 ms on average with respect to this focal site. Within the thalamus, the laterodorsal (LD) nucleus was found to consistently lead other thalamic sites. The ventroposterior medial (VPM) nucleus was found to lag behind the ventroposterior lateral (VPL) nucleus, with an average time delay of 4.3 ms. Concerning cortico-thalamic interrelationships, the cortical focus site consistently led the thalamus (VPM), with an average time delay of 8.1 ms. Within the somatosensory system of the hind paw, the (nonfocal) cortical site led the thalamic site (VPL) during three of 10 seizures; the thalamus led the cortex during one seizure, whereas for the other six seizures no direction of the delay could be established. (b) The relationships as found when the whole seizure is analyzed as one epoch. The same cortical focus as during the first 500 ms was found consistently. Compared with the first 500 ms, the time delay from the cortical focus with respect to the non-focal cortical sites has increased. Furthermore, the strength of association between VPL and VPM has increased. The direction of the cortico-thalamic couplings has changed. For the non-focal cortical sites, the thalamus was found to lead during all seizures. For the focal cortical site, the cortex was found to lead during two seizures, whereas the thalamus was found to lead during seven seizures. Reproduced from Meeren HK, Pijn JP, van Luijtelaar EL, Coenen AM, and Lopes da Silva FH (2002) Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. *Journal of Neuroscience* 22: 1480–1495, with permission from Society for Neuroscience.

younger and older WAG/Rij rats that are not present in the control strain can then be more readily ascribed to epileptogenesis or to the presence of seizures.

Recent Results

A Cortical Focus in Generalized Absence Epilepsy

The role of the cortex in the generation of spike-and-wave discharges has been investigated using cortical spreading depression techniques to transiently deactivate the entire cortex. During cortical deactivation, recordings have been made of the cortical and thalamic EEG in freely moving GAERS and WAG/Rij rats. In GAERS, the corpus callosum has additionally been transected. At the height of spreading depression, when cortical inactivation is complete, SWDs are abolished in both the cortex and the thalamus. Spike-and-wave discharges reappear after a long recovery period, first in the cortex and later in the thalamus. This demonstration shows that a functionally-intact cortex is a prerequisite for spike–wave generation. Moreover, after callosectomy, the vast majority of SWDs are no longer bilaterally symmetrical, although occasionally bilateral synchronous SWDs are seen. Obviously, the intra-thalamic circuitry alone is not sufficient to generate SWDs, and each hemisphere contains an oscillatory system.

Older studies in cortical slices of WAG/Rij rats showed both increased excitation and reduced inhibition in parts of the frontal cortex, and neurochemical studies show an expanded distribution of NMDA-mediated responses in the cortex of GAERS. These neurobiological studies received new momentum after the discovery of a focal zone involved in the genesis of epileptiform activity in the cortex of WAG/Rij rats. Detailed studies of interrelationships between local field potentials, quantified by means of advanced signal analysis methods of nonlinear association, yields a direct measure of the degree of correlation between the events recorded at different sites (i.e., it gives an indication of the degree of functional coupling between the underlying neuronal populations) (**Figure 1**). In addition, the method provides a time delay between signals. Together, these two parameters deliver evidence

about 'driver-response' relationships between the respective neuronal populations. The outcomes of the analyses of the cortico-thalamo-cortical, intra-thalamic, and cortico-thalamic network in WAG/Rij rats showed that absence seizures (in contrast to what has generally been assumed) have a cortical focal zone of initiation in the peri-oral region of the somatosensory cortex. The activity quickly spreads from this zone over the cortex to the thalamus. Therefore, it seems that the thalamus is only secondarily involved. Moreover, thalamo-cortical cells are only weakly active during SWDs, providing a resonant circuitry that sustains the cortical discharges. During the first 500 ms of SWDs, the cortical focus is consistently found to lead its thalamic counterpart. Thereafter, cortex and thalamus alternately lead and lag in an unpredictable way. These data suggest that the peri-oral region of the somatosensory cortex is the site at which a spike of each new cycle is originally generated. The large-scale synchronization appears to be mediated by the fast propagation of seizure activity from this focal site through cortico-cortical networks. The paroxysmal oscillation within the cortico-thalamic loop is then initiated. Once the oscillation has been set in motion, the cortex and thalamus form a unified oscillatory network in which both structures drive each other.

Several laboratories have now confirmed the existence of a focal cortical initiation zone for SWD in WAG/Rij rats and in GAERS. Pharmacological studies have shown that ethosuximide administered in the focal zone is more effective than injections in the RTN or the ventral basal complex of the thalamus. Even phenytoin, a drug known to enhance SWDs when injected systemically, reduced SWDs after local injections in the cortical focal zone (**Figure 2**). Injections of ethosuximide at other cortical locations are much less effective. Other investigators have found a specific increase in functional magnetic resonance imaging signals in the peri-oral somatosensory cortex during SWDs. These new discoveries have not only revived the classical debate about the origin of absence seizures, the identification of a specific cortical brain region from which the seizures are initiated has led to an attempt to determine why this zone is more excitable than other cortical zones. An up-regulation in the

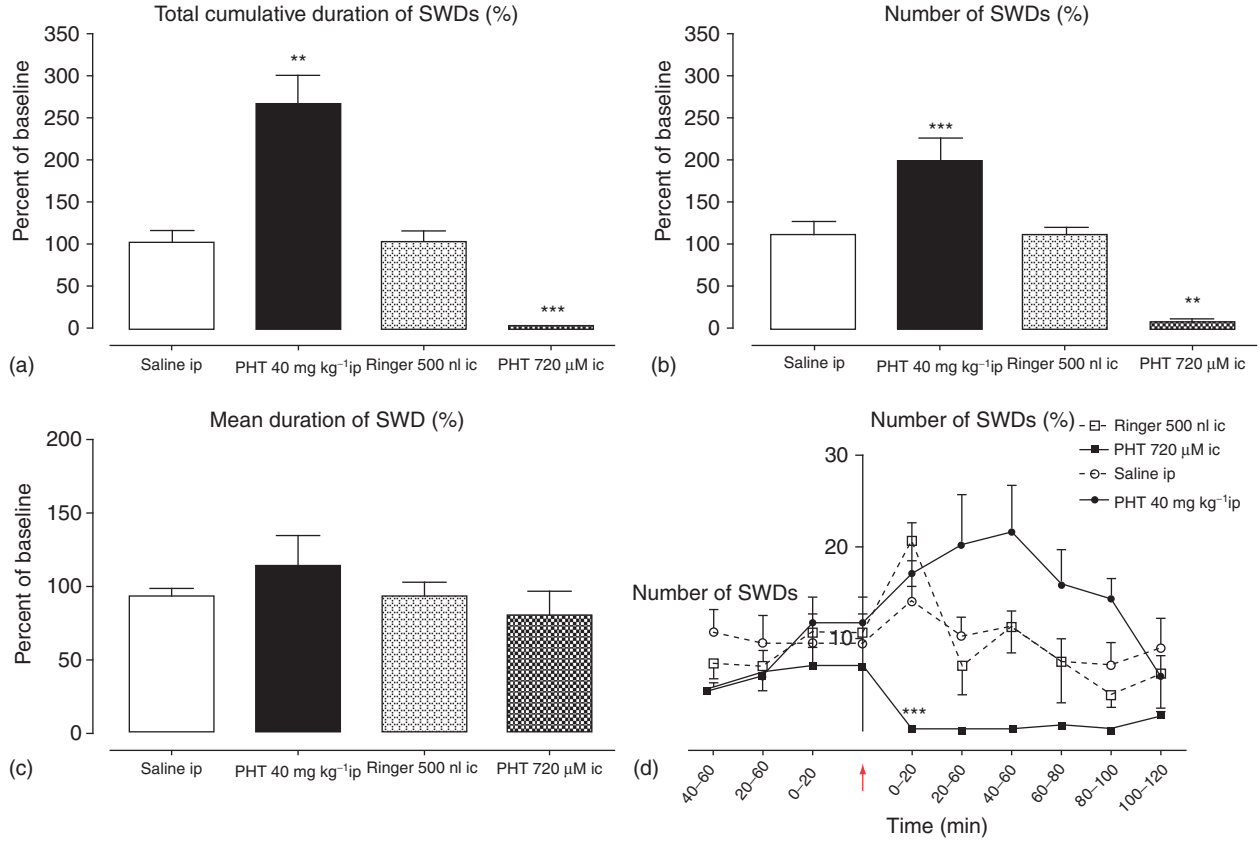


Figure 2 Effect of Phenytoin on total cumulative duration (a) number (b) and mean duration (c) of SWDs throughout 2 h post injection and the number of SWDs per 20 min intervals (d) before and after i.p. and intracortical (i.c.) injections in WAG/Rij rats in the deeper cortical cell layers ($n = 7$). $^{**}P < 0.01$; $^{***}P < 0.001$. Reproduced from Ayten A Gurbanova, Rezzan Aker, Kemal Berkman, Filiz Yilmaz Onat, Clementana M van Rijn, and Gilles van Luijtelaar, 2006. *British Journal of Pharmacology*, with permission from Nature Publishing Group.

expression of a subtype of Na^+ channels is specifically found in this region, and there are changes in I_h channels (which play a crucial role in the burst firing mode of thalamic and cortical neurons). Neurophysiologic in vivo studies in GAERS, specifically aimed at the cortical initiation zone, have shown that epileptic discharges are initiated in neurons of layers 5 and 6. These neurons, which show a distinctive hyperactivity associated with membrane depolarization, lead the firing of distant cortical cells during the epileptic discharge. Consistent with their ictogenic properties, neurons located in this focus exhibit inter-ictal and pre-ictal oscillations that are converted into an epileptic pattern. These pyramidal neurons of deep layers of the focal cortical site display an inter-ictal subthreshold and suprathreshold activity that is markedly higher than that of neurons located in the upper layers. This sustained firing, composed of a mixture of single discharges and high-frequency intrinsic bursts, has a regular pattern. Consistently, short periods (0.2–1.2 s) of small-amplitude rhythmic membrane potential fluctuations, generating periodic firing during inter-ictal epochs, are observed in deep layer neurons. Throughout an absence seizure, the firing of layer 5 and 6 neurons systematically precedes the discharge of superficial neurons. Hyperactivity of deep layer neurons is specific to the focus, and the hyperactivity of deep layer neurons in the somatosensory cortex is specific for epileptic animals.

A disregulation of inhibitory and excitatory synaptic systems may also participate in the generation of SWDs. The peak conductance of the fast component of inhibitory synaptic responses is reduced in deep layers of the focal cortical zone of WAG/Rij rats, documented in cortical-thalamic slices of WAG/Rij rats showing an increase in synaptic excitability mediated by NMDA receptors. Focal electrical stimulations elicit a late, large-amplitude, NMDA-dependent synaptic depolarization that can trigger repetitive firing. A similar stimulating protocol in control Wistar rats produces only a monophasic synaptic depolarization that can trigger only a single action potential. These recent neurophysiologic data obtained in GAERS and WAG/Rij rats provide a firm underpinning of the cortical focus theory of absence epilepsy. The excitatory cortico-thalamic inputs from cortical layers 5 and 6 (of the initiation zone) are stronger than in non-epileptic animals, and they impinge upon the RTN with its decreased propensity to dampen oscillations. These changes in intrinsic and synaptic properties facilitate resonances and spread throughout the thalamus, and propagation back to the cortex.

Circuitries

A further elaboration of the cortical focus theory requires a better understanding of the local and global mechanisms of absence epilepsy, as well as of the spread of epileptic activity over the cortex. We have hypothesized that the epileptic focus in the peri-oral region of the somatosensory cortex interacts with functionally and anatomically related regions – i.e., the adjacent parietal and frontal (sensory and motor) areas, creating an oscillatory loop that generates SWDs. If this occurs, then the onset of SWDs is associated with a significant increase of fronto-parietal coherence (and less significant changes of coherence between distant and functionally divergent areas, such as the fronto-occipital and parieto-occipital areas). Coherence, like correlation, is a measure of the interdependency of two signals; a high correlation suggests coupling of the signals. In our experiments, coherence is calculated from local field potentials recorded from various epidural and thalamic electrodes, placed intra- and inter-hemispherically in WAG/Rij rats. Coherence coefficients can also reveal intra-thalamic and cortico-thalamic synchronization, and shows whether the thalamus indeed exhibits a resonance circuitry of cortical SWDs. We have found that the onset of SWDs is associated with a strengthening of both unilateral and bilateral intracortical and unilateral thalamo-cortical interactions. A significant increase of cortico-cortical, cortico-thalamic, and thalamo-thalamic coherence is detected in frequencies from 5 to 60 Hz. The highest increase of coherence is obtained in two frequency bands – around a mean frequency of SWDs 10 (8–11.5 Hz) and of its first harmonic band of 20 (16–21.5 Hz) (**Figure 3**). Moreover, the anterior cortical areas have an intrinsic propensity to hypersynchronize, which facilitates unilateral and bilateral propagation of SWDs over anterior cortical areas. The trans-hemispheric cortical coherence is significantly higher than the intra-hemispheric cortico-cortical and cortico-thalamic and thalamo-thalamic coherence. This extensive increase of bilateral synchronization, and the peculiar profile of trans-hemispheric coherence, may imply the crucial involvement of the corpus callosum in the pathophysiology of absence seizures.

Strikingly, a SWD-related decrease of coherence is found in a narrow low-frequency band (<5 Hz), limited to cortico-cortical couples formed by the peri-oral region of the somatosensory cortex and the thalamo-thalamic pair. Since a similar pattern of coherence changes is seen in the cortical and thalamic local oscillatory networks, it appears that there is a common neurophysiologic mechanism controlling intra-thalamic and intracortical synchrony. The onset of SWDs is associated with a higher functional coupling throughout cortico-cortical, thalamo-thalamic and cortico-thalamic associative pairs. This increase of oscillatory synchrony is topographically and frequency specific, suggesting that a functional relationship explicitly distinguishes different oscillatory circuits. This finding suggests that there is nonhomogeneous spread of SWD over thalamo-cortical system, and is

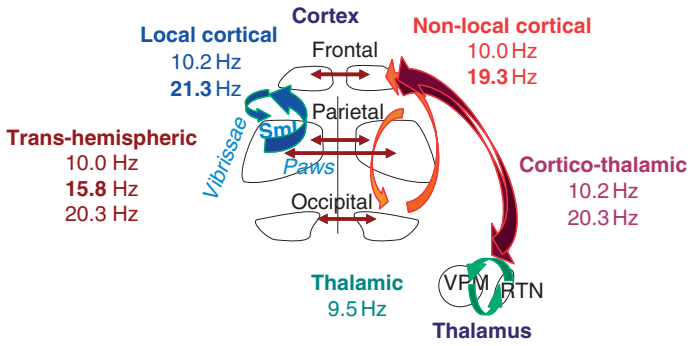


Figure 3 Outcomes of coherence analyses of local field potentials as produced in cortico-thalamo-cortical circuits. Five resonant circuits are identified during a SWD. The first circuit is at or near the site of origin, the parietal cortex. The second one is the trans-hemispheric network, it connects the two hemispheres; the third one is the nonlocal cortical network, it associates the parietal cortex with the occipital cortex; the fourth and fifth are the cortico-thalamic-cortical reverberating and local thalamic activity. The dominant oscillating frequency is around 10 Hz and its harmonic 20 Hz, the trans-hemispheric circuit has an additional 15 Hz oscillation. Reproduced from Sitnikova E and van Luijtelaar G (2006) Cortical and thalamic coherence during spike–wave seizures in WAG/Rij rats. *Epilepsy Research* 71: 159–180, with permission from Nature Publishing Group.

inconsistent with the former viewpoint that spike–wave seizures occur without localizing features.

Genotype-Environmental Interactions

Although generalized seizures (including absences) have a genetic origin, it can be clinically relevant to know how the genotype and the environment interact to modulate and shape the epileptic phenotype. To study this interaction, we subjected the WAG/Rij and a non-epileptic control inbred rat strain (ACI), which differ significantly in their genetic seizure susceptibility, to various environmental manipulations during different stages of development. We also exposed pregnant WAG/Rij mothers to PTZ, WAG/Rij pups were exposed to maternal deprivation and neonatal handling, weaned ACI and WAG/Rij rats were housed in either an enriched of impoverished condition in the period that SWDs start to emerge (0–60 days post weaning) and when they become fully mature (70–130 days post weaning). The EEGs of these ACI and WAG/Rij rats were recorded when they were adult. The outcomes of the pregnancy study show that repeated pentylenetetrazole (PTZ) injections during pregnancy protect WAG/Rij rats against SWDs and PTZ-induced seizures until about 70 days of age; older rats are more sensitive than younger WAG/Rij rats to a challenge with PTZ. Next, it was found that the occurrence of seizures was only minimally affected by housing conditions, suggesting that SWDs are mainly controlled by genetic factors. However, different seizure characteristics, such as number and mean duration of SWDs, have a different heritability and show a different sensitivity to the environment.

Kole and coworkers have established that the changes in the HCN1 subunit, a subtype of I_h channels, precede the development of SWDs, that WAG/Rij rats have more intrinsically burst firing neurons in the deep cortical layers than non-epileptic control Wistar rats, and that the loss of the dendritic I_h in WAG/Rij might form the basis for the initiation of the spontaneous seizures in the focal zone. Manipulations during the weaning period (such as maternal deprivation and neonatal handling before weaning) are accompanied by an increase in the expression of HCN1 and a decrease in SWD; notably, these manipulations during this early developmental period are more successful in changing the epileptic phenotype than the same manipulations later in life. These findings suggest that manipulations early in life, that alter seizure activity and I_h channels in the cortex, are critically involved in the pathogenesis of absence epilepsy. Further, interventions based on the prevention of, or compensation for, the loss of I_h channels might provide a new therapeutic avenue for absence epilepsy.

Interaction between Various Types of Epilepsy

The treatment of complicated mixed forms of epilepsy is one of the most challenging tasks confronting the clinical epileptologist. The suppression of one kind of paroxysms can be accompanied by the aggravation of other seizure types. Whether and how a seizure type develops in the presence of another type is completely unknown. We have taken two approaches to investigate whether there is an interaction between two different seizures types. In the first line of research, WAG/Rij and control rats were selected for their audiogenic sensitivity. Behavioral and neurochemical differences in some monoamines, as measured in extracted tissue immediately after exposure to the sound stressor, were investigated. The presence of absence seizures correlates with novelty-induced anxiety,

in which the limbic circuitry seems to be involved. Sound stress activates the 5-HT system differently in the different subject groups: audiogenic-sensitive rats and rats with only absences display predominantly a reaction in the thalamus, whereas non-epileptic rats show the highest response in the frontal cortex. The highest thalamic response occurs in rats with absence epilepsy, with much smaller changes in audiogenic sensitive control rats. We conclude that the brain 5-HT-ergic response to stress depends on the type of epileptic pathology.

A second approach to the interaction between two seizure types has made use of the kindling model. Both GAERS and WAG/Rij rats show peculiarities in the kindling process when compared with control rats. While controls are fully kindled after 12–15 electrical stimulations in the amygdala, GAERS remained at the initial stage 2 of kindling even after 30 sessions, with no motor seizures observed. Moreover, the duration of the afterdischarge after the kindling stimulus is shorter in GAERS. WAG/Rij rats are also more difficult to kindle; it takes more sessions before some of them reach the end stage 5 of kindling. Resistant rats have more SWDs, preceding the kindling sessions. Thus, it appears that kindling of genetic absence epileptic rats is strongly suppressed – perhaps because of the daily occurrence of a large number of absence seizures of these rats. This result suggests that the limbic system is changed in these genetically absence rats – which is perhaps surprising considering that SWDs occur in the cortico-thalamo-cortical circuitry and that mechanisms of its pathology and the consequences of the seizures are thought to be restricted to cortex and thalamus (without effects in the limbic system).

Behavioral Studies Show a Comorbidity with Depression

Using these genetically absence epileptic rats, we have described their behavioral characteristics, their performance in learning and memory tests, as well as their coping with stressful events Earlier studies showed only minimal behavioral differences from controls in WAG/Rij rats, some disturbances in reference memory in WAG/Rij rats, and (surprisingly) improved avoidance learning in GAERS. In experiments with graded novelty stress, WAG/Rij rats show a locomotor hyper-response to mild environmental stress, but they react with remarkable passive behavior to stronger emotional stress (from which they cannot escape). Further behavioral tests by Sarkisova showed that WAG/Rij rats exhibit depressive-like features, such as increased immobility in Porsolt's forced swim test and decreased sucrose intake (anhedonia). Interestingly, WAG-Rij rats exhibit normalization of this behavior after chronic (but not acute) treatment with antidepressant drugs. These findings validate the WAG/Rij strain as a genetic absence model with comorbidity for depression. In all, it is proposed that WAG/Rij rats are more vulnerable to stress than control rats and have depressive-like symptoms.

Behavioral and neurochemical studies have also been done in order to investigate putative causes and mechanisms of the increased vulnerability to the depressive-like phenotype. A hyper-response to amphetamine is found, similar to results in anhedonic depressive patients. The hyper-response to amphetamine might be due to a high reactivity in the mesolimbic dopaminergic system. The sensitivity for the dopamine agonist, apomorphine, is also enhanced, suggesting that the dorsal striatum is involved. Also, indices of activity of the 5-HT system are found to be reduced in the forebrain of WAG/Rij rats, while the 5-HT response to inescapable sound stress is enhanced in the thalamus. Based on these observations, it has been proposed that absence epileptic rats are more vulnerable to stress in which the 5-HT system is involved, and that they represent a model for mild depression in which the dopaminergic system also seems to be involved. The outcome is clinically relevant, considering that both the absence seizures and the depressive symptoms need to be treated in depressed epileptic patients.

Future Challenges

Progress in science is guided by theories, and a new theory on the origin of the electroencephalographic absence seizures – the cortical focus theory for absence epilepsy – has been derived from work on genetic animal models of SWD epilepsy. An immediate challenge is to test this theory in humans, with the same and other signal analytical tools as used in rats. The MEG, with its moderate spatial and high temporal resolution – alone or in combination with EEG – is well-suited for such studies of the cortical spreading of absence seizures. The fMRI technique, with it excellent spatial resolution, can be used for studying the role of the thalamus in humans. However, based on work presented here, it seems that a unifying theory is still missing. A theory is needed in which the various findings – such as the up-regulation of the various types of Ca^{2+} channels, the anomalies of I_h, and the lack of some of the GABA-subunits in the RTN – are integrated.

The genes mutated in absence epilepsy have not been identified – although different mutations in a gene termed CACNA1H have been associated with childhood absence epilepsy. Not all patients with CAE have mutations in this gene. Therefore, the hunt for the genes controlling absence epilepsy is still open. Candidate genes might come from analysis of the genetic rat models. Interestingly, linkage analyses in GAERS and WAG/Rij rats results in the identification of different chromosomal locations. Micro-array technology may help to identify the expression levels of specific genes in the focal zone of the absence models; the

specificity of the expression should be tested in the peri-oral zone of non-epileptic control rats. By including young, still not epileptic, WAG/Rij rats in such a study, it is possible to discriminate between genes that are affected by the disease rather than by the cause.

Molecular studies are helping to identify the effects of genetic deficits and its consequences, and to specify brain areas and their interconnections involved in the pathogenesis of epilepsy. The discovery of the subunits forming a large variety of GABA and glutamatergic ionophoric ion channel gated receptors with different distribution profiles, and the many subclasses of metabotropic glutamate receptors and neuromodulators, provide a broad panorama of means to target selectively and specifically the affected brain regions. Data from knockout mice are helpful and supplementary. To illustrate this point, it is worth noting that mice missing the mGlu4 receptor are completely resistant to the induction of absence seizures. These mGlu4 receptors are present presynaptically in cortico-thalamic neurons in the RTN and cortex. A positive allosteric modulator of this receptor, PHCCC, enhances dose-dependently, absence seizures, as shown by Ngomba and coworkers. This finding suggests that the mGlu4 receptor antagonist might be a new tool for targeting SWDs. Besides reducing seizures, treatment strategies that will slow or halt the progressive nature of epilepsy and prevent the development of epilepsy in susceptible individuals, are needed. One recent example was recently proposed by Blumenfeld's group, that found that ethosuximide in the drinking water of weaned animals delays the expression of the absence seizure for many weeks.

See also: **Absence Seizures**: Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; Thalamocortical Synchronization and Absence Epilepsy; **Hormones and Gender**: Hormones and Absence Epilepsy; **Sleep**: Seizure Initiation: The Transition from Sleep-Related Oscillations to Epileptiform Discharge.

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Increased $GABA_A$ Receptor-Mediated Transmission in Typical Absence Seizures

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Introduction

A typical absence episode is a nonconvulsive epileptic seizure that consists of a sudden and relatively brief impairment of consciousness occurring concomitantly with a generalized and synchronous 'spike and slow wave discharge' (SWD) paroxysm at 2.5–4 Hz in the EEG. Typical absence seizures are part of the complex clinical and EEG presentation of many idiopathic generalized epilepsies (IGEs). It is only in childhood absence epilepsy (CAE) that these seizures are the sole neurological symptom and are not accompanied by either metabolic, neuropathological, or other neurological deficits.

Typical absence seizures are genetically determined, and the classical 2.5–4-Hz SWD trait of different IGEs has been shown to be inherited in an autosomal dominant manner. Indeed, there is a strong consensus in describing the occurrence of (typical) absence seizures as a familial disease with a complex genotype, and increasing evidence suggests that they may represent a complex channelopathy of multifactorial genetic background. As far as the pathophysiology of human absence seizures is concerned, there is also a general consensus, based on some old invasive studies and more modern noninvasive imaging investigations, that the EEG manifestation of SWDs of typical absence originates from abnormal electrical activities in reciprocally connected thalamic and cortical territories – i.e., in what is generally referred to as the thalamocortical loop.

Among the various molecular genetic alterations that have in recent years provided support for the idea of IGEs as channelopathies, abnormalities in voltage-dependent Ca^{2+} channels and $GABA_A$ receptors ($GABA_A$Rs) have undoubtedly been of primary significance. In particular, different mutations in the $\gamma 2$ or δ subunits of the $GABA_A$R have been detected in different IGE cohorts, and the proteins resulting from these mutant genes have been shown to give rise to a decreased $GABA_A$R function. However, the probands in all these studies had an epileptic phenotype much more complex than that in typical absence seizures alone. To complicate the matter further, it has not been possible to detect, in every expression system, the potential alterations that these mutant proteins exert in $GABA_A$R function. It is only in linkage studies of probands with only typical absence seizures that the regions coding for $GABA_A$R subunits (namely $\alpha 5$ and $\beta 3$) have been highlighted as susceptibility loci. Thus, notwithstanding the attractive hypothesis of a decreased $GABA_A$R-dependent transmission in typical absence epilepsy, the difficulties in recruiting cohorts with a pure absence seizure phenotype and the inherent drawbacks of studying mutant proteins in expression systems that lack the normal complement of $GABA_A$R subunits might explain why direct and conclusive evidence of a $GABA_A$R loss-of-function in typical absence seizures is still missing.

Background

A decreased $GABA_A$R function in typical absence seizures has also been suggested on the basis of studies in animal models, but a critical analysis of these data indicates that the evidence in support of this hypothesis is far from conclusive. Although no change in the electrophysiological properties of cortical $GABA_A$R-mediated inhibitory postsynaptic potentials (IPSPs) had apparently been found in the feline penicillin model, indirect evidence indicating reduced $GABA_A$R function has been reported in the neocortex of two inbred models of absence epilepsy, the genetic absence epilepsy rats from Strasbourg (GAERS) and the Wistar albino Glaxo/Rij (WAG/Rij) rats.

$GABA_A$Rs in the thalamus have also been implicated in the pathophysiology of absence seizures, since $GABA_A$ antagonists applied to thalamic slices have been shown to transform sleep spindle activity into abnormal oscillatory discharges at 2–3 Hz. Furthermore, direct evidence of a role for $GABA_A$Rs in the nucleus reticularis thalami (NRT), one of the intrathalamic sources of GABAergic afferents to the main sensory thalamic nuclei, has come from the observation of hypersynchroneous 3-Hz firing in thalamic slices of $GABA_A$R $\beta 3$ subunit knockout mice. However, two major drawbacks affect these and many other experimental studies in the field. First, the phenotype of the genetic and pharmacological models that have been used for these studies does not simply show (typical) absence seizures and associated SWDs, but also includes a variety of other neurological symptoms (and in some cases neuropathological abnormalities), including ataxia, dystonia, and tonic–clonic seizures. This complexity clearly makes any attempt to draw direct and solid cause–effect relationships

between the observed genetic, biochemical, or electrophysiological abnormalities and the occurrence of SWDs or absence seizures extremely difficult. Second, the 'abnormal' low threshold Ca^{2+} potential (LTCP)-dependent, high-frequency burst of action potentials observed in thalamocortical (TC) neurons in some of these studies in thalamic slices (which contain either one of the main sensory thalamic nuclei alone or together with the NRT) has been interpreted as if it resembles SWD paroxysms. In reality, however, in both in vivo rat and cat models, the activity of the vast majority of single TC neurons in sensory thalamic nuclei during spontaneous SWDs consists of rhythmic (and in synchrony with each spike and wave complex), composite $GABA_A$ IPSPs that are superimposed on a tonic hyperpolarization, with only an occasional single, or doublet, action potential. Thus, although the overall output of the TC neuron network during spontaneous SWDs in vivo would indeed contain short synchronous discharges originating from this occasional firing, the single TC neuron does not show strong, LTCP-dependent, high-frequency bursts of action potentials.

In an attempt to overcome the interpretational difficulties associated with results originating from models with a complex phenotype, we and other groups have focused our work on GAERS, an inbred model of typical absence seizures, and on the γ-hydroxybutyric acid (GHB) model, which, when systemically injected in normal Wistar rats, also reproduces a substantially pure absence seizure phenotype. In addition, we have concentrated our investigation on the basic properties of single cells and synaptic potentials, since it has not yet been possible (to the best of our knowledge) to accurately reproduce the SWD paroxysms observed in vivo in thalamic, cortical, or thalamocortical slices. Surprisingly, our studies in both GAERS and GHB models have produced results that are consistent with a $GABA_AR$ gain-of-function in typical absence seizures.

Methods

In our laboratories, we use the GAERS rats since this is one of the best-established models of typical absence seizures with no additional neurological or neuropathological abnormalities. The epileptic phenotype of this inbred strain becomes evident only during periods of quiet wakefulness, and consists of SWDs in the EEG that are accompanied by behavioral arrest and rhythmic twitching of the vibrissae and facial muscles. Muscle tone in the neck is sometimes diminished, inducing a gradual and slight lowering of the head. The pharmacological profile of the GAERS seizures very closely matches that of the human condition: In particular, ethosuximide and valproate abolish, whereas carbamazepine and phenytoin aggravate, the seizures. Also similar to the human condition is the thalamocortical origin of SWDs in GAERS, with the hippocampus and cerebellum being devoid of any ictal activity. Another advantage of the GAERS model is the availability of a corresponding, well-characterized, inbred strain of nonepileptic control (NEC) rats, which are devoid of absence seizures. In contrast to typical absence seizures in humans (or at least to classical CAE), however, the frequency of SWDs in GAERS is 7–9 Hz, and the seizures persist during adulthood.

The attractiveness of the GHB model arises not only from the fact that it is possibly the best-established and best-characterized pharmacological model of typical absence seizures, but also that it too shows an almost pure absence phenotype when GHB is injected within appropriate doses in different species, from mice and rats to cats and monkeys. Thus, GHB-induced absence seizures consist of behavioral arrest, facial myoclonus, and vibrissal twitching; these behaviors are invariably accompanied by the EEG appearance of SWDs, which originate in thalamocortical territories and have a pharmacological profile very similar to that of the human condition. Another interesting feature of the GHB model, and one that strongly supports the study of GHB actions in thalamic slices for understanding the pathophysiological mechanisms of absence seizures, is that direct intrathalamic injection of GHB in a main sensory thalamic nucleus (the ventrobasal, VB, complex) of freely moving rats elicits a behavioral and EEG absence phenotype that is almost indistinguishable from that observed following the systemic injection of GHB. In contrast to the human condition, the GHB model clearly lacks the spontaneity and recurrent nature that are characteristic of the epileptic condition. Also, the frequency of SWDs is the same as that in humans when GHB is injected in cats and monkeys (about 3 Hz), but is 7–9 Hz in mice and rats.

Electrophysiological recordings combined with single-neuron staining are used in our laboratories to investigate the electrical abnormalities that are present in GAERS or are induced by application of GHB in normal Wistar rats. Together with EEG recordings in freely moving animals, we also investigate the biophysical properties of single neurons and their synaptic responses using brain slices obtained from the key areas of the thalamocortical loop, i.e., the somatosensory cortex or thalamic nuclei (mainly the VB and the NRT). Either patch clamp recordings are used to assess the biophysical properties of voltage- and transmitter-gated channels, or sharp microelectrode recordings are obtained to investigate the spontaneous or synaptically evoked activity in single neurons or neuronal networks. Isolation of voltage- and transmitter-gated channels is achieved using pharmacological agents. In particular, $GABA_A$ inhibitory postsynaptic currents (IPSCs) or IPSPs are isolated by adding to the perfusion medium the ionotropic glutamate receptor antagonists, DL-2-amino-5-phosphonovaleric acid (DL-APV) (50 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM), while glutamatergic excitatory postsynaptic currents (EPSCs) or excitatory postsynaptic potentials (EPSPs) are isolated by adding the $GABA_A$ antagonist gabazine (10 μM) and the $GABA_B$ antagonists CGP55845A (100 nM) or CGP35348 (1 mM).

Recorded cortical and thalamic neurons are identified by the presence of their characteristic electrophysiological or morphological features, and electrical stimulation is used to activate the synaptic pathways present in a slice.

Recent Results

Difference in $GABA_A$ IPSC Properties of GAERS and NEC Rat Neurons are Nucleus-Specific

Striking differences between NEC rats and preseizure GAERS are present in the properties of the mIPSCs recorded in NRT neurons. In this nucleus, mIPSCs have a 67% higher frequency, a 25% larger amplitude, and a 40% faster decay in GAERS than in NEC rats (Figure 1(a)–1(c)). These differences in mIPSCs are not the results of differences in either basic electrical or anatomical properties, since the passive membrane properties and the morphological features of dye-injected NRT neurons in GAERS and NEC rats are identical. In addition, differences between GAERS and NEC rats are evident in the presynaptic control exerted by $GABA_B$ autoreceptors on mIPSCs recorded in NRT neurons. Thus, although the $GABA_B$ agonist (±)baclofen (1–10 μM) reversibly decreases the frequency (but not the amplitude) of the $GABA_A$ mIPSCs in all NRT neurons tested, its potency is lower in GAERS than in NEC rats

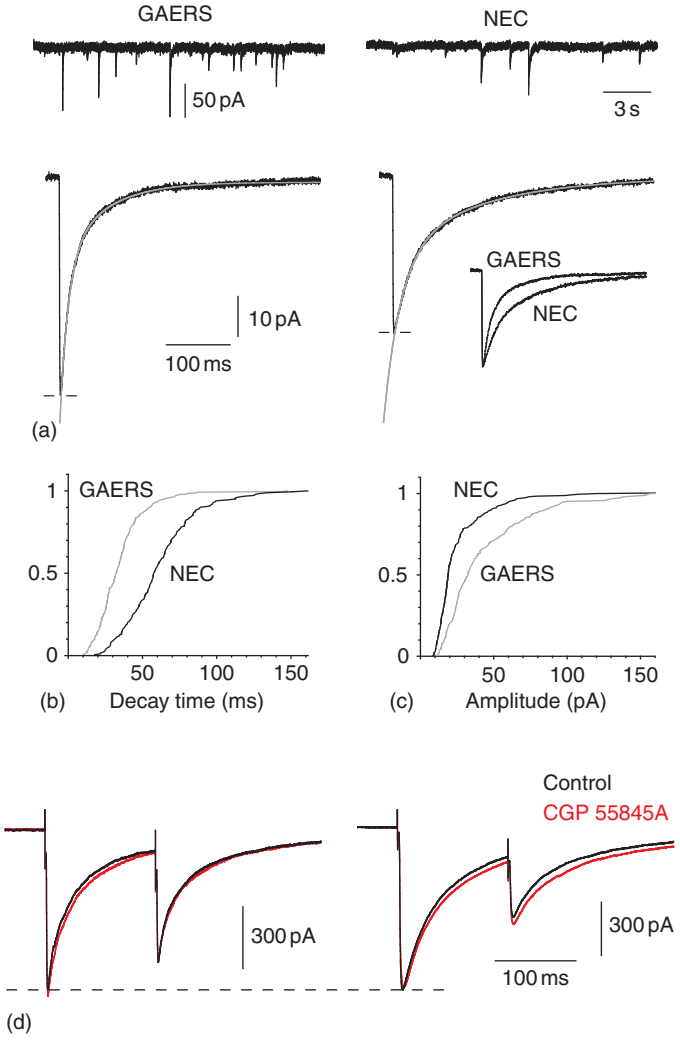


Figure 1 $GABA_A$ IPSCs in NRT neurons of GAERS and NEC rats. (a) Top: mIPSCs in GAERS and NEC NRT neurons. Note the higher frequency and amplitude of mIPSCs from GAERS than from NEC rats. Bottom: Averaged mIPSCs show the larger amplitude and faster decay of these synaptic currents in GAERS than in NEC rats. The two mIPSCs are scaled and superimposed in the inset. (b) Cumulative distribution of the decay time constant shows a faster kinetics of mIPSCs in GAERS NRT neurons (grey) than in NEC (black) NRT neurons. (c) Cumulative distribution of the amplitude shows a larger amplitude of mIPSCs in GAERS NRT neurons (grey) than in NEC (black) NRT neurons. (d) Superimposed, averaged $GABA_A$ IPSCs evoked by intra-NRT stimulation using a 140-ms paired-pulse protocol in control conditions (black) and during application of 100 nM of CGP 55845A (red) in GAERS and NEC NRT neurons. Note the smaller paired-pulse depression in GAERS than in NEC rats, and the lack of effect of CGP 55845A in both strains. Reproduced from Bessaïh T, Bourgeais L, Badiu CI, *et al*. (2006) Nucleus-specific abnormalities of GABAergic synaptic transmission in a genetic model of absence seizures. *Journal of Neurophysiology* 96: 3074–3081, with permission from American Physiological Society.

(ED_{50} of 1.6 and 0.8 μM, respectively). Moreover, baclofen maximal efficacy is 25% lower in GAERS, although no difference exists in its threshold concentration (1 μM) between GAERS and NEC rats. In both strains, the baclofen effect on mIPSCs is fully antagonized by the specific $GABA_B$ receptor antagonist CGP55845A (100 nM).

In contrast, in pyramidal and nonpyramidal cortical neurons of layers 2 or 3 and in TC neurons of the VB, $GABA_A$ mIPSCs have similar rise time, amplitude, frequency, and decay time constant in GAERS and NEC rats. Moreover, no difference between the two strains is detected in the action of (±)baclofen (1–10 μM) in VB TC neurons. As for NRT neurons, the effect of baclofen observed in every cortical and TC neuron tested is reversible and is antagonized by CGP 55845A (100 nM).

Since the $GABA_B$ autoreceptor sensitivity of NRT mIPSCs is different between GAERS and NEC rats, and because in some brain areas, including the thalamus, paired-pulse depression has been shown to be in part due to the activation of presynaptic $GABA_B$ autoreceptors, we used this stimulation protocol to investigate the strength of evoked $GABA_A$R-mediated inhibition. Indeed, paired-pulse depression of evoked IPSCs is significantly smaller (46%) in GAERS NRT neurons than in NEC NRT neurons (**Figure 1(d)**), but similar between TC neurons of the two strains. However, in both TC and NRT neurons of GAERS and NEC rats, CGP55845A (100 nM) and CGP35348 (1 mM) fail to affect the paired-pulse depression of the evoked IPSCs.

In summary, these data in a well-established genetic model of typical absence seizures indicate that one important component underlying the expression of these seizures may be related to subtle alterations in the kinetics and paired-pulse depression of phasic (i.e., IPSCs) $GABA_A$R-mediated inhibition specifically in the NRT. In this respect, our results are in agreement with the subtle abnormalities in $GABA_A$R function resulting from human mutations in genetically simple IGEs. Also, the decreased paired-pulse depression in GAERS clearly indicates that in a well-established model of typical absence seizures, there is a clear gain-of-function in $GABA_A$R-mediated transmission. This result suggests caution in extrapolating to the human condition the significance of experimental results obtained in the presence of full loss-of-function of $GABA_A$Rs that globally involves cortical and thalamic nuclei.

From a pathophysiological perspective, the smaller paired-pulse inhibition of GAERS would undoubtedly play a major role in allowing the expression of the strong, LTCP-mediated bursts of action potentials that are present in GAERS NRT neurons during SWDs. Indeed, computer simulations carried out in NRT network models that contain the $GABA_A$ IPSCs properties highlighted earlier has shown that a simulated 'GAERS NRT network' has an increased excitability compared with a simulated 'NEC NRT network,' and responds to random inputs with a resonance output at the very same frequency of the spontaneous SWDs observed in GAERS in vivo. Finally, the presence of these NRT-specific abnormalities in GAERS at a time prior to seizure onset suggests that alterations in the fine-tuning of GABAergic phasic inhibition (i.e., IPSCs) may be of epileptogenic significance.

Effects of GHB on Excitatory and Inhibitory Synaptic Potentials Recorded in VB TC Neurons

As mentioned earlier, the importance of studying the detailed mechanisms of GHB on TC neurons derives from the fact that this agent when directly injected in the thalamus of freely moving animals, elicits SWDs and behavioral components identical to those observed following systemic injection. Together with a $GABA_B$R-mediated hyperpolarization of TC neurons (**Figure 2(a)**), GHB is also capable of reversibly and dose-dependently decreasing the amplitude of sensory EPSPs and corticothalamic EPSCs in all TC neurons (**Figure 2(b)–2(e)**). This reduction is fully antagonized by CGP 56999A (100 nM) or CGP 35348 (1 mM), and is therefore likely due to activation of presynaptic $GABA_B$ autoreceptors present on these afferents (**Figure 2(f)**). The threshold concentration of GHB against the corticothalamic EPSCs is 250 μM, whereas the maximal effect (57% reduction) is obtained with 10 mM.

GHB also reversibly decreases the amplitude of NRT-derived $GABA_A$ IPSCs with a similar potency as against the EPSCs, but at low concentrations (250 μM–1 mM), it is not effective on all recorded IPSCs (**Figure 2(b) and 2(c)**). In particular, 250 μM of GHB produces an effect in <10% of neurons, whereas at 0.5–1 mM it only decreases (by 25–30%) half of the IPSCs tested (**Figure 2(d) and 2(e)**). However, application of 10 mM of GHB induces a 54% decrease in every TC neuron tested. Although the GHB-induced reduction of the NRT-derived IPSCs is always fully antagonized by CGP 56999A (100 nM), in order to exclude the possibility that the afferents generating the GHB-insensitive IPSCs lack presynaptic $GABA_B$ autoreceptors, we applied baclofen following washout of GHB in 10 of the 31 neurons that were insensitive to 500 μM or 1 mM of GHB (**Figure 2(e)**). In all these neurons, (±) baclofen (5–10 μM) decreased the IPSC amplitude by the same amount as in those neurons where GHB was active.

Controversies still exist with respect to the involvement of putative GHB receptors in the central actions of GHB, including its ability to elicit absence seizures. The results of additional electrophysiological experiments in the thalamus, however, strongly question the selectivity and usefulness of putative GHB receptor agonists and antagonists, such as NCS 356, *trans*-4-hydroxycrotonic acid, and NCS 382. Thus, the apparent block of GHB-elicited SWDs by NCS 382 in vivo should be treated with extreme caution, since it may result from any of its nonspecific actions in the thalamus. Indeed, the putative GHB agonist

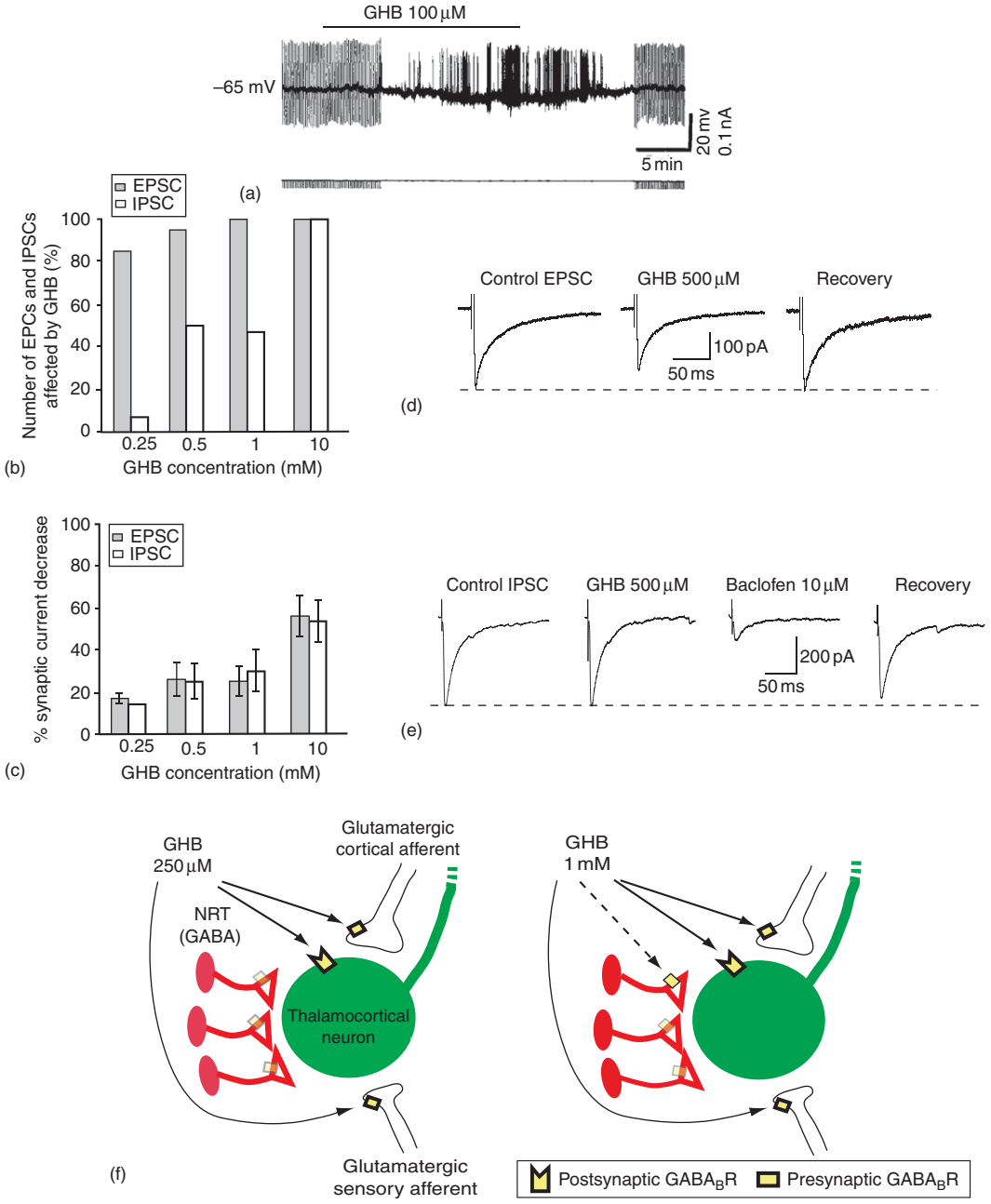


Figure 2 Effects of GHB on thalamic neuzones. (a) A small concentration of GHB hyperpolarizes this TC neuron (top trace). Downward deflections represent voltage responses to rectangular current pulses (bottom trace). (b) Percentage of corticothalamic EPSCs and NRT-derived IPSCs affected by different GHB concentrations. Note that whereas almost all EPSCs are affected by GHB (0.25–1.0 mM), a much smaller proportion of IPSCs are sensitive to these concentrations. At 10 mM, all EPSCs and IPSCs are decreased by GHB. (c) Effect of different GHB concentrations on the amplitude of EPSCs and IPSCs. No difference in the decrease elicited by GHB is observed between corticothalamic EPSCs and those IPSCs that are sensitive to GHB. (d) Example of a corticothalamic EPSC that is reversibly decreased by a low GHB concentration. (e) Example of an NRT-elicited $GABA_A$ IPSC that is unaffected by a low GHB concentration, but markedly decreased by baclofen. (f) Schematic of GHB action in the thalamus and the location of $GABA_BRs$. Presynaptic $GABA_BRs$ are negatively coupled to both glutamate and GABA release. Increasing GHB concentrations (1 mM) affects a greater number of presynaptic $GABA_BRs$ on the GABA-containing nerve terminals of nucleus reticularis thalami (NRT) neurons (highlighted yellow presynaptic $GABA_BRs$), while all presynaptic $GABA_BRs$ on the glutamate-containing cortical and sensory afferents are activated even by 250 μM of GHB. Therefore, the increased inhibition that results from this imbalance between excitatory and inhibitory synaptic drive, together with the hyperpolarization of thalamocortical (TC) neurons mediated by postsynaptic $GABA_BRs$, would increase neuronal excitability, as occurs during absence seizure paroxysms. Note that 240 μM of GHB is the threshold brain concentration for the EEG appearance of SWDs. (b)–(e): Reproduced from Gervasi N, Monnier Z, Vincent P, *et al*. (2003) Pathway-specific action of γ-hydroxybutyric acid in sensory thalamus and its relevance to absence seizures. *Journal of Neuroscience* 23: 11469–11478, with permission from Society for Neuroscience.

trans-4-hydroxycrotonic acid has been shown to be unable to elicit SWDs when injected in vivo. On the basis of all the available data, therefore, it is safer to consider the GHB model of typical absence seizures as one that involves the direct activation of $GABA_B$, and not GHB, receptors.

In summary, together with previous data from our laboratories, the present results indicate that low (250 μM to 1 mM) GHB concentrations, similar to the brain GHB levels required to elicit SWDs in freely moving rats, has two effects on TC neurons of the VB (**Figure 2(f)**): (1) the hyperpolarization of TC neurons by direct activation of postsynaptic $GABA_B$Rs and (2) the decrease in the amplitude, by a similar potency and by direct activation of presynaptic $GABA_B$ autoreceptors, of all sensory and corticothalamic EPSCs, but only 40% of the $GABA_A$ IPSCs. On the basis of these results, and since SWDs can be elicited in vivo by direct injection of GHB into the VB, we suggest that a substantial component of the mechanism underlying the generation of SWDs by systemic GHB is due to an imbalance of the physiological excitatory and inhibitory drives to TC neurons in favor of an increased inhibition. In other words, GHB would not only slightly hyperpolarize TC neurons and decrease their afferent corticothalamic and sensory excitatory volleys, but would also leave the TC neuron population with a substantial proportion of unaltered NRT phasic (i.e., IPSCs) inhibition, thus helping to impose the periodic NRT-derived inhibition that sculptures the electrical behavior of TC neurons during the SWD paroxysm.

Therefore, both in the GAERS and GHB models, a brain area-specific, $GABA_A$R gain-of-function appears to be implicated in the generation of typical absence seizures. Of course, in the GHB model, this is brought about by activation of $GABA_B$Rs (**Figure 2(f)**), whereas in the GAERS model it could be the result of genetic abnormalities of $GABA_B$Rs and/or $GABA_A$Rs or indeed of compensatory alterations in these two types of GABARs that occur as a consequence of mutations or expression in other genes or proteins.

Future Goals

It will be important to characterize the molecular substrate (s) underlying the different properties of $GABA_A$ IPSCs in the GAERS NRT, since this information may provide important leads for the genetic abnormalities that underlie the human condition. In this respect, mIPSC properties of normal animals have been ascribed by various studies to different expression levels of either α1, α3, β1, γ2L, and/or γ2S subunits of the $GABA_A$R. Interestingly, a recent study has highlighted a specific loss of the α3 subunit at the GABAergic synapses in the NRT of WAG/Rij rats. However, other parameters (i.e., receptor density, subcellular localization, occupancy, and phosphorylation) could underlie the observed IPSC differences between preseizure GAERS and NEC rats. Moreover, before conclusively excluding the presence of any alterations in cortical $GABA_A$Rs of GAERS, GABAergic transmission in deep cortical neurons should be studied, since abnormalities in these synaptic potentials have been described in deep layer pyramidal neurons of WAG/Rij rats, and pyramidal neurons in layer 5 or 6 of the somatosensory cortex have been suggested as potential initiators of the SWD paroxysm in both GAERS and WAG/Rij rats.

The sensitivity to $GABA_B$R agonists and antagonists is now considered one of the essential criteria to be fulfilled by any absence epilepsy model. It is therefore important to fully understand the mechanism underlying the differential response of GAERS and NEC rats' IPSCs to baclofen in NRT neurons, also in view of the fact that an increased expression of both $GABA_B$R1 and $GABA_B$R2 has been reported in the somatosensory cortex, VB, and NRT. Also, as far as the GHB model is concerned, it would be important to systematically investigate the actions of this agent on the basic properties and synaptic potentials of NRT and cortical neurons. In fact, any differential sensitivity of intra-NRT and/or intracortical $GABA_A$R-mediated inhibition (similar to that we have uncovered in the VB) should not be discounted when attempting to understand the full proabsence mechanism of action of GHB.

Finally, it is important to stress here that $GABA_A$R-mediated inhibition does not only consists of phasic activity (i.e., IPSCs) but also of a 'tonic' component. The tonic $GABA_A$R current that underlies this type of inhibition originates from the activation of extrasynaptic $GABA_A$Rs, and has already been shown to play a role in a number of physiological functions. Although originally described in cerebellar and hippocampal neurons, a tonic $GABA_A$ current has now been detected in TC and some cortical neurons, but it is absent in thalamic interneurons and in NRT cells. It is feasible that this tonic $GABA_A$ current might be responsible for the tonic hyperpolarization that characterizes the activity of TC neurons during spontaneous SWDs in different models of typical absence seizures, including the GAERS.

Undoubtedly, the notion that typical absence seizures involve a gain-of-function in $GABA_A$R-mediated transmission, in contrast to the traditional view of a reduced $GABA_A$R-mediated inhibition, is further strengthened by our preliminary data showing a larger tonic $GABA_A$ current in TC neurons of GAERS than of NEC rats.

Acknowledgements

Work in our laboratories is supported by the Wellcome Trust and the Fondation NRJ-Institut de France. Additional information regarding this and other published work from the Crunelli's lab is available at http://www.thalamus.org.uk.

See also: **Absence Seizures**: Genetic Models of Absence Epilepsy: New Concepts and Insights; Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; Thalamocortical Synchronization and Absence Epilepsy; **GABA**: $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; Synchrony Through GABAergic Inhibition; **Hormones and Gender**: Hormones and Absence Epilepsy.

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Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures

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Introduction

A typical absence episode is a nonconvulsive epileptic seizure that consists of a sudden and relatively brief impairment of consciousness, occurring concomitantly with a bilaterally synchronous 'spike and slow wave discharge' (SWD) paroxysm at 2.5–4 Hz in the EEG. Typical absence seizures are part of the complex clinical and EEG presentation of many idiopathic generalized epilepsies (IGEs), but in childhood absence epilepsy, these seizures are the only neurological symptom and are not accompanied by either metabolic, neuropathological, or other neurological deficits.

Typical absence seizures are genetically determined, and the classical 2.5–4 Hz SWD trait of different IGEs has been shown to be inherited in an autosomal

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Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures

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Typical absence seizures are genetically determined, and the classical 2.5–4 Hz SWD trait of different IGEs has been shown to be inherited in an autosomal

dominant manner. Indeed, there is strong consensus in describing the occurrence of (typical) absence seizures as a familial disease with a complex genotype, and increasing evidence suggests that these seizure disorders may represent a complex channelopathy of multifactorial genetic background.

There is also a general consensus about the pathophysiological nature of human absence seizures. On the basis of some old invasive studies and more modern non-invasive imaging investigations, investigators have determined that the EEG manifestation of SWDs of typical absence originates from abnormal electrical activities in reciprocally connected thalamic and cortical territories – i.e., in what is generally referred to as thalamocortical (TC) networks (see schematic circuitry in **Figure 2(a)**). However, the precise contribution of cortex and thalamus to the generation of SWDs is not known, and the detailed evolution of the membrane potential in cortical, TC, and nucleus reticularis thalami (NRT) neurons (**Figure 2(a)**) during SWDs is still ill-defined. In addition, the notion that typical absences are truly 'generalized' from the very start of the EEG paroxysm has been recently challenged by the observation that seizure onset is associated with paroxysmal activation of discrete, often unilateral, frontal cortical regions.

Many experimental investigations have tried to address some of these issues. In particular, the use of appropriate absence models and the improvements associated with both in vivo electrical recordings and drug injection in localized brain regions has started to provide some novel and interesting data. Two clear indications are now emerging: first, that the intracellular activity of TC neurons during SWDs greatly differs among various thalamic nuclei; and second, that the (oro)-facial region of the primary somatosensory cortex temporally leads other cortical areas and the thalamus in the expression of the SWDs of typical absence seizures.

Background

The proposed major contribution of the thalamus to the SWD generating mechanisms, and the potentially critical role of the GABAergic system in absence seizures, have resulted in numerous studies investigating the electrical activity resulting from the block of this neurotransmitter function in the thalamus. Bath-application of $GABA_A$ antagonists to ferret thalamic slices containing the isolated (from the cortex) TC-NRT network has been shown to lead to highly synchronous oscillatory discharges at 2–3 Hz in every TC neuron of primary sensory thalamic nuclei; these discharges are generated by the rhythmic recurrence of $GABA_B$ IPSPs and low threshold Ca^{2+} potential (LTCP)-dependent, high-frequency bursts of action potentials. Similarly, abolition of $GABA_A$ receptor function (following knockout of the $GABA_A$ β3 subunit) results in hypersynchronous burst firing at 3 Hz in mouse thalamic slices containing viable connections between TC and NRT neurons.

The abnormal membrane potential oscillations and firing patterns observed in these and similar investigations conducted in the isolated thalamus have been interpreted as if they resemble those occurring during SWDs. There is no direct evidence, however, that this is the case. Indeed, in contrast to these in vitro results, the essential contribution of $GABA_A$ receptors to the activity of TC neurones of primary sensory thalamic nuclei during spontaneous SWDs in vivo has been highlighted using localized iontophoretic application of specific $GABA_A$ antagonists in Wistar Albino Glaxo/Rij (WAG/Rij) rats, a well-established genetic model of absence epilepsy. Also, phasic GABAergic transmission (i.e., $GABA_A$-IPSPs) is unaltered in both TC and neurons of upper cortical layers in Genetic Absence Epilepsy Rats from Strasbourg (GAERS), another well-established genetic model of absence epilepsy. Moreover, intracellular recordings in the cat in vivo have clearly shown that the activity of the vast majority (about 90%) of single TC neurons in primary sensory thalamic nuclei during spontaneous SWDs consists of rhythmic sequences of composite $GABA_A$-IPSPs that occur in synchrony with each spike and wave complex, and which are superimposed on a tonic hyperpolarization; only occasionally is a single action potential, a doublet, or a burst, of action potentials present during this sequence. Thus, although the in vitro experiments demonstrate the ability of the isolated TC-NRT network to generate abnormally synchronized membrane potential oscillations and correlated high-frequency bursts of action potentials when $GABA_A$ receptors are blocked, this scenario is quite different from that observed during spontaneous SWD paroxysms in the cat in vivo.

Still, since the spontaneous SWDs in cats were suggested to be probably the consequence of multielectrode penetrations, and their pharmacological profile has not been investigated, we have now used in vivo intracellular recordings to characterize the evolution of the membrane potential of both cortical and thalamic neurons during spontaneous SWDs in GAERS. Also, following the report of a potential cortical 'initiation site' in experimental absence seizures, we investigated in the same model whether localized application of ethosuximide (ETX) in this and adjacent cortical regions is effective in abolishing SWDs, and compared these results with those obtained after ETX injection either in the NRT or in a primary sensory thalamic nucleus (i.e., the ventrobasal (VB) complex).

Methods

The GAERS is one of the best-established genetic models of typical absence seizures, which shows no additional

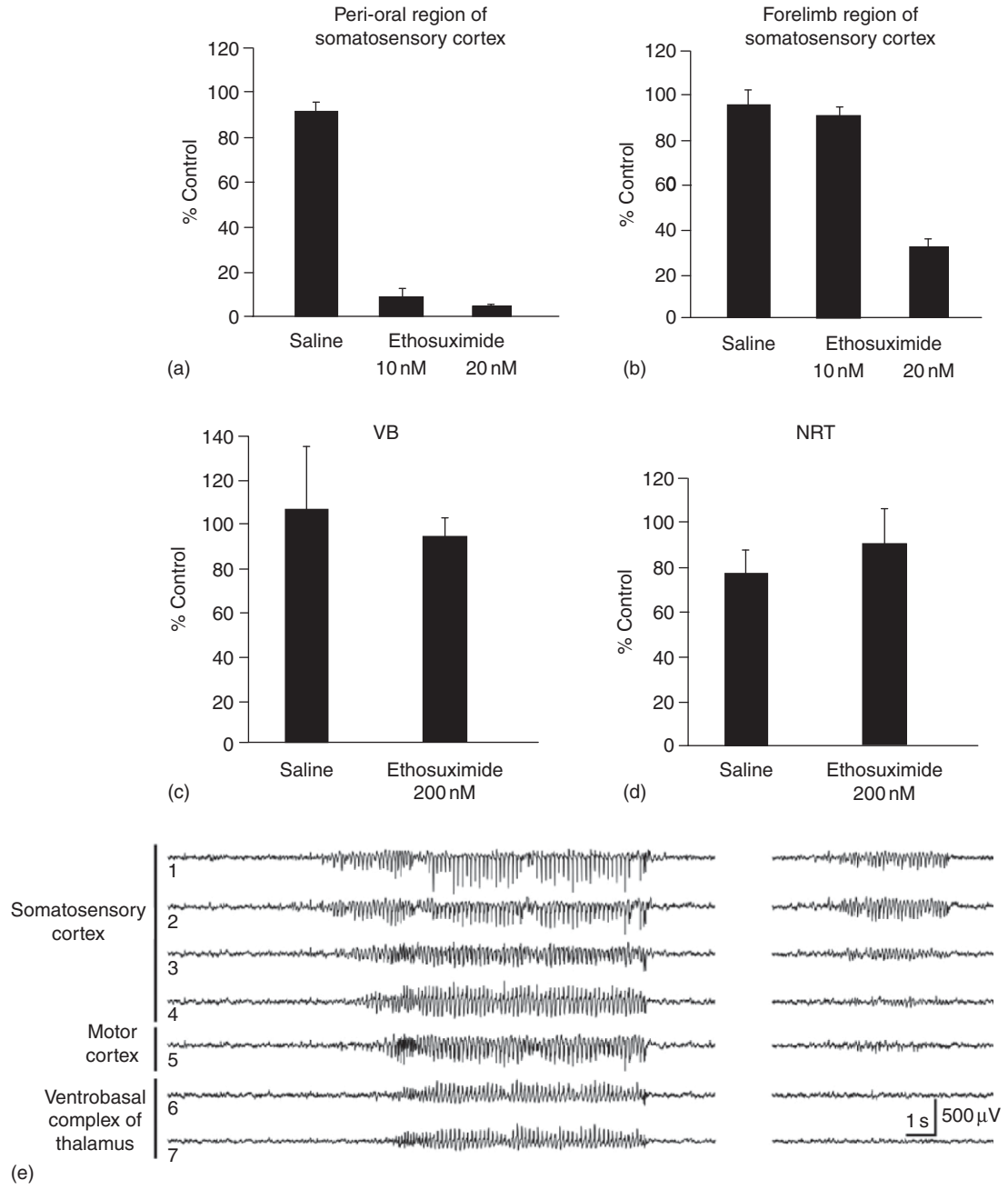


Figure 1 Cortical initiation site for SWDs in GAERS: pharmacological and electrophysiological evidence. ETX abolishes SWDs when bilaterally infused in the perioral region of the primary somatosensory cortex (a), but has no, or a much smaller, effect when it is administered either in the forelimb region of the primary somatosensory cortex (b), the VB thalamic nucleus (c), or the NRT (d). The plots show total time of SWD duration calculated over a 30 min period immediately following ETX injection as the percentage of that observed in the 30 min immediately preceding the injection. (e) Simultaneous local field potential recordings in the indicated cortical and thalamic areas during spontaneous SWDs in freely moving GAERS. The appearance of the paroxysm in the facial region of the primary somatosensory cortex (traces 1–4) precedes that in the other regions. The traces on the right show a SWD in somatosensory cortex that is not accompanied by paroxysmal activity in the VB thalamus complex. Reproduced from Polack PO, Guillemain I, Hu E, Deransart C, Depaulis A, Charpier S 2007 Deep layer somatosensory cortical neurons initiate spike-and-wave discharges in a genetic model of absence seizures. *Journal of Neuroscience* 27: 6590–6599, © with permission from Society for Neuroscience.

neurological or neuropathological abnormalities and fulfills all the accepted criteria for an absence model. The epileptic phenotype of this inbred strain only becomes evident during periods of quiet wakefulness, and consists of SWDs in the EEG that are accompanied by behavioral arrest and rhythmic twitching of the vibrissae and facial muscles. Muscle tone in the neck is sometimes diminished, inducing a gradual and slight lowering of the head.

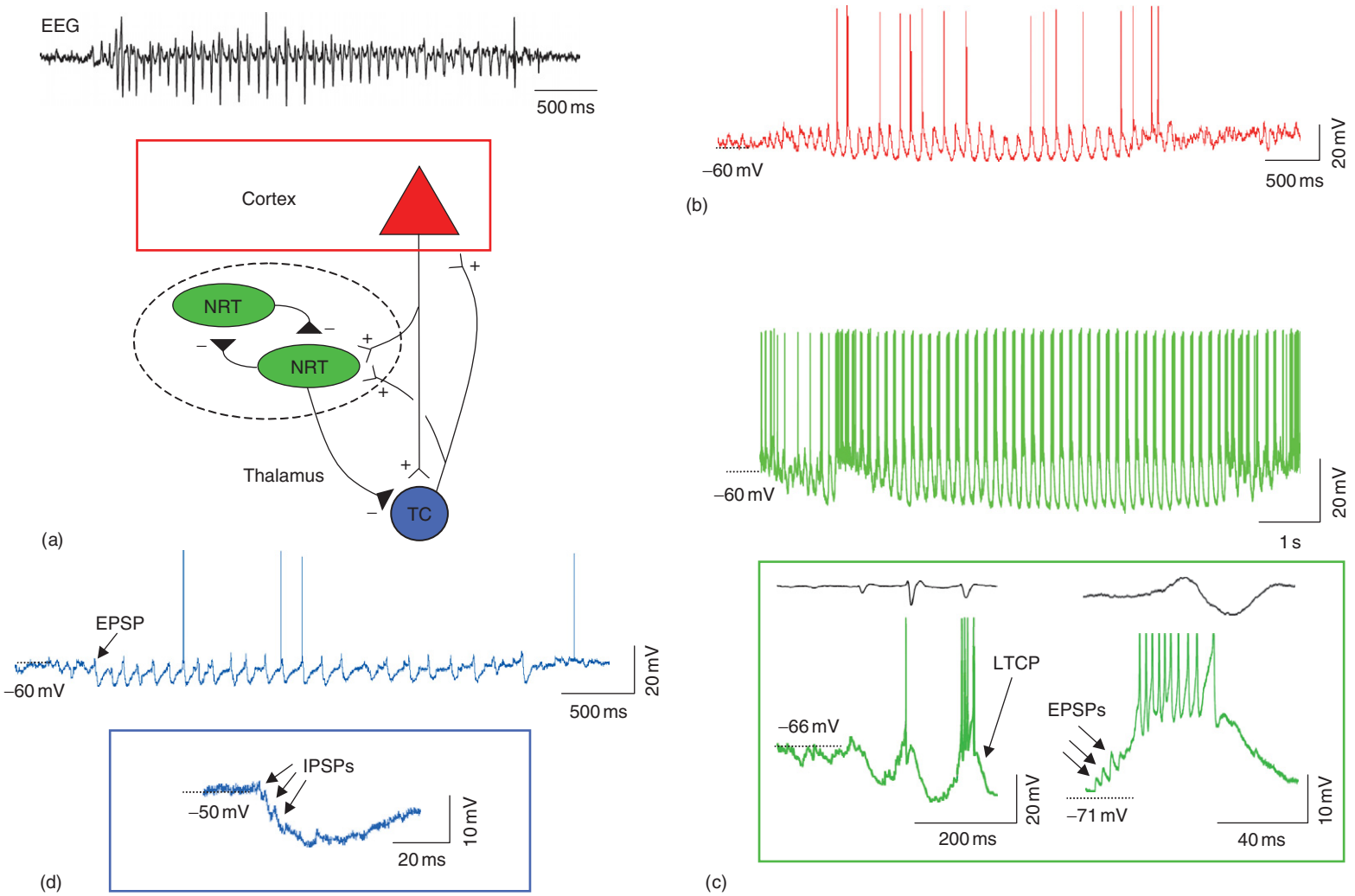


Figure 2 Intracellular activity of cortical and thalamic neurons during spontaneous SWDs in GAERS. (a) Typical SWD (top) and schematic diagram of the thalamocortical loop (bottom). The colour code of the neurons is maintained in the intracellular records of (b)—(d), which are all from GAERS. TC: thalamocortical neuron; NRT: thalamic reticular nucleus neuron; +: excitatory synapse;-: inhibitory synapse. (b) Activity of a layer 5 cortical neuron during a SWD. Note the rhythmic depolarizations that are present in synchrony with each EEG spike. (c) Activity of an NRT neuron during a SWD. Note the presence of robust, high-frequency bursts of action potentials crowing the LTCPs. Insets below are enlarged traces showing the LTCP and the EPSPs that precedes it. (d). Activity of a TC neuron in the VB thalamic complex during a SWD. Note the presence of a tonic hyperpolarization that lasts for the whole duration of the SWDs, and the presence of rhythmic sequences of composite IPSPs (inset below) in synchrony with each EEG spike. Reproduced from Crunelli V and Leresche N (2002) Childhood absence epilepsy: Genes, channels, neurons and networks. *Nature Reviews Neuroscience* 3: 371–382, with permission from Nature Publishing Group.

The pharmacological profile of these seizures very closely matches that of the human condition. In particular, ETX and valproate abolish, whereas carbamazepine and phenytoin aggravate, spontaneous seizures in GAERS. Also, similar to the human condition is the thalamocortical origin of their SWDs, with the hippocampus and cerebellum being devoid of any ictal activity. Another advantage of the GAERS model is the availability of a corresponding, well-characterized, inbred strain of nonepileptic control (NEC) rats, which are devoid of absence seizures. In contrast to typical absence seizures in humans (or at least in classical childhood absence epilepsy), however, the frequency of SWDs in GAERS is 7–9 Hz, and the seizures persist during adulthood.

We use scalp EEG recordings to monitor the occurrence of spontaneous SWDs while we simultaneously sample the evolution of the membrane potential in different types of thalamic (NRT, and TC neurons in the VB) and cortical neurons with independently mounted sharp microelectrodes. As SWDs are abolished by systemic or inhalation anesthetics, animals are kept under neuroleptanesthesia using fentanyl and haloperidol; to obtain long-lasting stable intracellular recordings, the rats are immobilized with gallamine triethiodide and artificially ventilated. In some experiments, neurobiotin is present in the extracellular and intracellular electrodes to allow subsequent morphological analysis of the recorded neuron(s).

For application of drugs in discrete brain areas in freely moving animals, we use either reverse microdialysis or microinfusion via probes or cannulae, respectively, which are positioned in the region of interest. Drugs are applied while we monitor the occurrence of spontaneous SWDs with scalp EEG electrodes in freely moving GAERS. In these experiments, each animal serves as its own control; that is, the spontaneous SWDs observed after drug injection are calculated as the percentage of those observed before drug infusion.

Recent Results

Cortical Area – Specific Action of ETX

Following the report of an initiation site of SWDs in the perioral region of the somatosensory cortex of WAG/Rji rats, we investigated whether this locus represents a preferential site for the action of one of the most commonly used antiabsence drugs, ETX. Indeed, microinfusion or injection of ETX by reverse microdialysis in this region of the primary somatosensory cortex of freely moving GAERS leads to an almost immediate (<30 min) cessation of spontaneous SWDs and the behavioral components of the seizures (**Figure 1(a)**). The onset of this effect is similar in strength and timing to the block observed following ETX systemic injection. Interestingly, a less marked response is observed when even higher concentrations of ETX are applied to the forelimb region of the primary somatosensory cortex (**Figure 1(b)**), and no effect occurs when the injection site is in the primary motor cortex. In contrast, ETX applied either in the NRT or the VB has no effect on SWDs within the first 30 min following injection (**Figure 1(c)** and **1(d)**), while a partial block (about 50%) is observed at later times (30–60 min after injection) and when using higher concentrations.

By providing solid evidence on a primary cortical site of ETX effect, these results stress the substantial importance of cortical T-type Ca^{2+} channels, persistent Na^{+} channels and Ca^{2+}-activated K^{+} channels in the anti-absence action of this anti-absence drug, compared to the too often over-emphasized role of the thalamic T-type Ca^{2+} current. As described in the next section, in fact, it is only in motor thalamic nuclei that there appears to be a substantial activation of T-type Ca^{2+} channels during SWDs, and the T-type Ca^{2+} channel-mediated LTCPs that are present in NRT neurons during SWDs are not a primary target for the action of ETX, since the block of SWDs following its localized injection in this thalamic nucleus is only partial and delayed.

Moreover, our results on ETX provide indirect support for the existence of a cortical initiation site of absence seizures in GAERS, which is located in the same region as the one directly demonstrated to exist in WAG/Rij rats. This finding is supported by recent data from another group that has now directly shown the existence of an initiation site in the facial region of the primary somatosensory cortex of GAERS (**Figure 1(b)**). Interestingly, short SWDs could be observed in this region even in the absence of any paroxysmal activity in the adjacent motor cortex or in the thalamus (right traces in **Figure 1(b)**); in these instances, the short SWDs were not associated with any behavioral abnormalities. The clear demonstration of a cortical initiation site in experimental absence seizures adds strength to the emerging observations of a similar lack of generalization at the start of human absence seizures – although in the latter case, the leading cortical areas appears to be in mesial frontal and orbital frontal regions.

Intracellular Activity of Cortical Neurons

The major component of the intracellularly recorded activity of cortical neurons of GAERS during SWDs consists of rhythmic depolarizations that occur in phase with the EEG spike and emerge from what appears as a hyperpolarizing envelope (**Figure 2(b)**). These rhythmic depolarizations probably involve the contribution of T-type Ca^{2+} channels (at least in some cortical cells), and their waveform is drastically different from the classical paroxysmal depolarizing shifts of convulsive epilepsies, West syndrome, and Lennox-Gasteau syndrome. The recent characterization of an initiation site of SWDs in the

facial region of the somatosensory cortex of GAERS and WAG/Rij has allowed comparison of the activity of cortical neurons in this area, and with those of other cortical regions. These recent results indicate that GAERS pyramidal neurons in layer 5/6 of the cortical initiation site have a higher and more regular firing rate, and a more depolarized membrane potential – compared with layer 2–4 cortical neurons in the same area and to layer 5/6 cells in the adjacent motor cortex. Indeed, a higher excitability and more depolarized membrane potential of layer 5/6 neurons in the cortical initiation site was also observed when these features were compared with layer 5/6 neurons of the same facial region of the somatosensory cortex of nonepileptic Wistar rats. Also, in the GAERS initiation site, the firing of layer 5/6 neurons clearly precedes that of both layer 4 and layer 2/3 neurons, as had already been shown by another group.

Intracellular Activity of NRT Neurons

During SWDs, the strong volley generated by the synchronous firing of corticothalamic neurons, together with the convergence of the corticothalamic input, results in bursts of EPSPs in NRT neurons. Each burst of EPSPs, with the help of a preceding hyperpolarization, generates a strong LTCP in correspondence to each EEG spike (**Figure 2(c)**). In turn, this large LTCP evokes a prolonged burst of 7–12 action potentials, with an average intraburst frequency of up to 350–400 Hz (inset in **Figure 2(c)**). An increased T-type Ca^{2+} current and a smaller paired-pulse depression of $GABA_A$-receptor-mediated IPSPs have been reported to occur in NRT neurons of preseizure GAERS, compared with NEC. It is likely that these abnormalities will also be present in GAERS later in life, when they could markedly contribute to the generation of the aberrant firing of these neurons during SWDs.

Intracellular Activity of Thalamocortical Neurons

The strong inhibitory output of the NRT arrives onto TC neurons of the VB a few milliseconds after the corticofugal volley (i.e., one synapse difference - see diagram in **Figure 2(a)**). Thus, the ictal activity of the vast majority (>90%) of TC neurons in the primary sensory nuclei consists of rhythmic sequences of composite $GABA_A$-IPSPs (in phase with the EEG spike) (**Figure 2(d)**), that are superimposed on a tonic hyperpolarization that lasts for the whole duration of the SWD. The intrasequence frequency of these IPSPs tightly matches that of the action potentials in the NRT burst. Also of interest is the fact that an EPSP can at times be seen to closely precede the sequence of $GABA_A$-IPSPs (inset in **Figure 2(d)**), probably because in these instances the corticofugal volley arriving onto the TC neurons is not fully abolished by the NRT inhibition. This sequence of intracellular events is supported by two findings: the more powerful excitatory action exerted by the corticofugal afferents on NRT than on TC neurones, and the strong (up to 12 action potentials), high-frequency burst observed in NRT neurons at each EEG spike (inset in **Figure 2(c)**). Only very rarely are LTCPs observed during SWDs in the intracellular records of TC neurons in primary sensory thalamic nuclei (i.e., the VB) of GAERS, and also rare are the instances of extracellularly recorded bursts of action potentials with characteristics similar to those mediated by LTCPs.

Similar findings have been recently reported by another group for TC neurons of the ventrolateral nucleus of GAERS. The extracellularly recorded firing of TC neurons in this nucleus is characterized by occasional single action potentials and almost no bursts. In contrast, extracellular recordings in TC neurons of the ventromedial nucleus show short, high-frequency bursts of action potentials, reminiscent of those generated by LTCP-mediated bursts. Indeed, intracellular recordings in TC neurons of the ventromedial nucleus highlight clearly evident LTCPs in correspondence to a substantial number of EEG spikes.

The presence of a LTCP in TC neurons of the ventromedial nucleus could at least in part explain why mice with selective knockout of α1G, the gene that encodes for the most prevalent T-type Ca^{2+} channel subunit that is present in TC neurons, are resistant to pharmacologically induced SWDs. More likely, however, is the possibility that the inability to elicit SWDs in α1G knockout mice results from the lack of T-type Ca^{2+} channels in those cortical layer 5/6 neurons which, as indicated previously, temporally lead thalamic and other cortical neurons in the expression of the SWD paroxysm.

Future Goals

Although the discovery of a cortical initiation site for spontaneous SWDs in the two best-established genetic models of typical absence seizures (i.e., GAERS and WAG/Rij) stresses the cortical origin of this experimental paroxysm, the precise contribution of subcortical structures to the full generalization of SWDs remains to be elucidated. In this respect, the presence of clear differences in the intracellular activity of TC neurons among thalamic nuclei during SWDs warrants systematic investigations of the potential alterations in intrinsic and synaptic conductances that might underlie these differences. It would also be of interest to characterize the activity of intralaminar thalamic nuclei during SWDs, as they provide important subcortical and thalamocortical loops for the spreading of the absence paroxysm.

Another goal, both in GAERS and WAG/Rij, would be to carry out a systematic characterization of the biophysical properties of the intrinsic and synaptic membrane

currents of neurons in the initiation site of the primary somatosensory cortex, in particular in those cortical layer 5/6 neurons where the abnormal activity associated with a SWDs appears to precede that of layer 2–4 neurons. Some of these data is already available (at least for WAG/Rij) from the work of other groups, including an increased excitability mediated by NMDA receptors, a smaller I_h, a reduced $GABA_B$ receptor expression, and a decrease in paired-pulse depression of $GABA_A$-IPSPs. Unfortunately, only the latter two findings were recorded in the cortical initiation site. Moreover, these studies should ideally be carried out both prior and after seizure onset to determine whether any alteration in synaptic or intrinsic conductances are either the consequence, or the cause, of the absence paroxysm.

Finally, it would also be important to assess why different cortical regions, i.e., somatosensory vs. frontal cortex, are the initiating sites in rodent vs. humans, respectively. In both human and experimental studies addressing this issue, it will remain important to consider that the primary genetic alterations could not be necessarily confined to the cortical initiation site. In this respect, uncovering a cortical initiation site as well as studying the intracellular activity of cortical neurons in genetic mouse models with SWDs (i.e., stargazer, lethargic, tottering, etc.), though their phenotype also includes other neurological deficits, might be of help as the underlying primary genetic abnormality of these mouse models is well characterized.

Acknowledgements

Work in our laboratories is supported by the Wellcome Trust and the Fondation NRJ-Institut de France. Additional information regarding this and other published work from the Crunelli's lab is available at http://www.thalamus.org.uk.

See also: **Absence Seizures**: Genetic Models of Absence Epilepsy: New Concepts and Insights; Increased $GABA_A$ Receptor-Mediated Transmission in Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; Thalamocortical Synchronization and Absence Epilepsy; **Networks**: Interactions between Cortico-Thalamo-Cortical and Limbic Seizures.

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Thalamic Neurons and Networks Related to Absence Epilepsy

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Introduction

Oscillations in the thalamocortical network are central to a number of neurocognitive functions, including attention, cognition, perception, memory, wakefulness, and sleep. Several apparently unrelated neurological and psychiatric conditions, including depression, dystonia, neurogenic pain, Parkinson tremor, spasm, tinnitus, and epilepsy, are characterized by the occurrence of slow resonant interactions between thalamus and cortex. These interactions seem to be based on increased inhibition, deafferentation, and/or discrete changes in cellular properties (e.g., ion channel expression) of thalamic neurons, and have been attributed to a new class of syndromes called thalamocortical 'dysrhythmia.' Since natural and pathological oscillations are based on the same network architecture and cellular elements, a key question is why naturally occurring oscillations transform (either by hypersynchronization of physiological oscillations or by gradual evolution from physiological oscillations) into pathological oscillations.

A prominent example of these syndromes is absence seizure activity. Absence epilepsy is an idiopathic, generalized, and non-convulsive form of epilepsy with an as yet unknown polygenic background. A typical absence episode consists of a sudden epileptic seizure associated with severe impairment of consciousness. While the interictal electroencephalogram (EEG) appears normal, the ictal EEG is characterized by waxing and waning phases of bilateral, synchronous 2.5–4.0-Hz spike and slow-wave discharges (SWDs). These ictal episodes can be accompanied by minor motor clonic movements or automatisms. Although SWDs appear to have sudden onset and offset, gradual modulation in α-frequency has been observed in patients just prior to SWDs, indicating a transition state. Absence-related SWDs occur mainly during immobile, inattentive wakefulness and drowsiness. Normal consciousness returns immediately following the cessation of SWDs. Absence seizures have a unique pharmacological response profile, with effective seizure control with ethosuximide (ETX) or valproate. In contrast, drugs effective in focal seizures (such as carbamazepine and vigabatrin) are either ineffective or aggravate absence seizures. As defined by the current International League against Epilepsy (ILAE) classification, typical absence seizures are an integral part of several types of idiopathic generalized epilepsies, including childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME), and generalized tonic–clonic seizures (GTCS) – all of which display complex, partially overlapping phenotypes. It should be noted that the following considerations primarily relate to CAE.

Background

CAE has a Genetic Basis

CAE is genetically determined with incomplete penetrance and complex autosomal dominant inheritance of the SWD trait. Linkage studies, twin studies, and genetic defects in rare families all point to a predominant genetic etiology of CAE. A number of gene mutations localized to the voltage-gated Ca^{2+} channel $Ca_V2.1$, the $GABA_A$ receptor γ2 subunit gene, and the low-threshold (T-type) Ca^{2+} channel $Ca_V3.2$, have been identified in CAE families. A number of additional susceptibility loci and polymorphisms in various types of ion channels, transmitter receptors, and other proteins have also been described, although the linkage to CAE is not always clear (see **Table 1**).

The Thalamocortical Network – General View

The thalamocortical network is the neuronal substrate for the generation and maintenance of SWDs. Defined types of thalamic neurons possess intrinsic pacemaker properties and are synaptically interconnected in functional loops which facilitate recurrent, synchronized network oscillations (see **Figure 1**). The neuronal elements of the thalamic network are composed of three basic cell types, excitatory thalamocortical relay (TC) neurons and two classes of inhibitory cells, namely, neurons of the thalamic reticular nucleus (NRT) and local circuit interneurons (not illustrated here). TC neurons and NRT neurons have extensive synaptic connections between each other, thereby creating a recurrent intrathalamic loop. Relay nuclei are subdivided into two major types, often termed 'specific' and 'higher order.' The specific nuclei contain first order relay neurons which carry signals from the sensory periphery and lower brain centers to the neocortex, mostly in a topographically organized fashion. One example is the dorsal lateral geniculate nucleus (dLGN), which receives afferents from the retina and sends axonal projections to the visual cortex. 'Higher order' nuclei may, in addition or exclusively, contain higher order relay neurons, which

Table 1 Gene, transcript and protein alterations associated with CAE

	Mutation of human genes	*Human linkage and genome scan studies*[a]	*Animal models*[b]
Ion channels	Voltage-gated Ca^{2+} channels $Ca_V2.1$, $Ca_V3.2$	Voltage-gated Ca^{2+} channel $Ca_V2.1$	Voltage-gated Ca^{2+} channels $Ca_V2.1$, $Ca_V3.1$, $Ca_V3.2$
		Voltage-gated Na^+ channel $Na_V1.1$	Two-pore domain K^+ channel TASK-3
		Voltage-gated K^+ channel KCNQ2	Hyperpolarization-activated and cyclic nucleotide gated cation channel HCN1
		Two-pore domain K^+ channel TASK-3	
Membrane receptors	$GABA_A$ receptor $\gamma2$ subunit	$GABA_A$ receptor subunits $\alpha1$, $\alpha5$, $\beta3$, $\gamma2$, $\gamma3$	$GABA_A$ receptor subunits $\alpha3$, $\gamma2$
		Ionotropic glutamate receptor GluR6	$GABA_B$ receptors 1, 2
		nAChR $\alpha4$ subunit	
		μ-Opioid receptor type 1	
Membrane transporters		Glutamate transporter 1 GLT1	Glutamate transporters GLT1, GLAST, EAAT1
		Dopamine transporter 1 DAT1	
		Serotonin transporter 5-HTT	
Other		Monoamine oxidase type A	Lactate dehydraogenase
		Paired-box 6	Cytochrom oxidase
		Activity-regulated skeleton-associated protein	
		Signal-transduction-associated, RNA-binding 3	

[a]Alterations mainly relate to polymorphisms including nucleotide exchange and insertion.
[b]Alterations typically relate to changed mRNA and protein expression in epileptic rat models.
Some changes could be defined for specific thalamic cell types. Note that some changes are only found in young animals before the onset of seizures. GABA, γ-aminobutyric acid; nAChR, nicotinic acetylcholine receptor.

receive incoming messages from the cortex and relay these messages from one cortical area to another. One example of an 'higher order' thalamic nucleus is the lateroposterior nucleus. Furthermore, 'unspecific' nuclei include the intralaminar nuclei (containing, for instance, the centromedial, paracentral and centrolateral nucleus), which represent a mixed group of cells receiving ascending afferents from the anterolateral system (concerned with thermal and nociceptive stimuli) or from motor cortex, and sending their axons to widespread cortical and striatal areas. They also receive massive monosynaptic input from the brainstem, and thereby are considered to mediate widespread neuronal excitation and arousal.

Thalamic Cell Types

TC neurons

TC cells are a population of excitatory neurons (see Figure 1) that are segregated in specific thalamic nuclei, each associated with different aspects of arousal and consciousness (e.g., sensorimotor processing and higher order and associational roles like attention, cognition, and perception). Mutual excitatory connections between relay cells are rare within and between thalamic nuclei. TC cells possess an intrinsic predisposition for oscillatory activity or resonant activity. The characteristic 'rebound' spiking in TC neurons following inhibition, associated with a Ca^{2+}-dependent low-threshold spike (LTS) (post inhibitory rebound potential) whose activation requires a conditioning membrane hyperpolarization, has been known for a long time. The LTS is a long-lasting depolarization, carried by a low-threshold activated T-type Ca^{2+} current (I_T), which triggers a burst of conventional Na^+/K^+ action potentials and can drive oscillatory activity in the thalamocortical network (see Figure 1, inset). Only recently, genes for three members of a family of T-type Ca^{2+} channels were identified and cloned – $Ca_V3.1$, $Ca_V3.2$, and $Ca_V3.3$. In addition, a number of splice variations have been found that give rise to Ca^{2+} currents with different voltage-dependent properties. One splice variant of $Ca_V3.1$, namely, $Ca_V3.1a$, is highly expressed in TC neurons. The crucial role of T-type Ca^{2+} channels in the generation of reciprocal thalamocortical burst firing and LTS generation in TC neurons is emphasized by knock-out mice lacking $Ca_V3.1$. TC neurons of $Ca_V3.1$ null mutants fail to show rebound burst activity, and unlike wild-type controls, are insensitive to pharmacologically induced SWDs. Moreover, cross-breeding of $Ca_V3.1$ knock-out mice with mice revealing absence epilepsy with ataxia abolishes SWDs in the progeny. This result indicates that a reduction of the T-type Ca^{2+} current – in this case produced by reducing the gene dosage – has an antiepileptic effect.

A second membrane current is of crucial importance for oscillatory activity in the thalamocortical system.

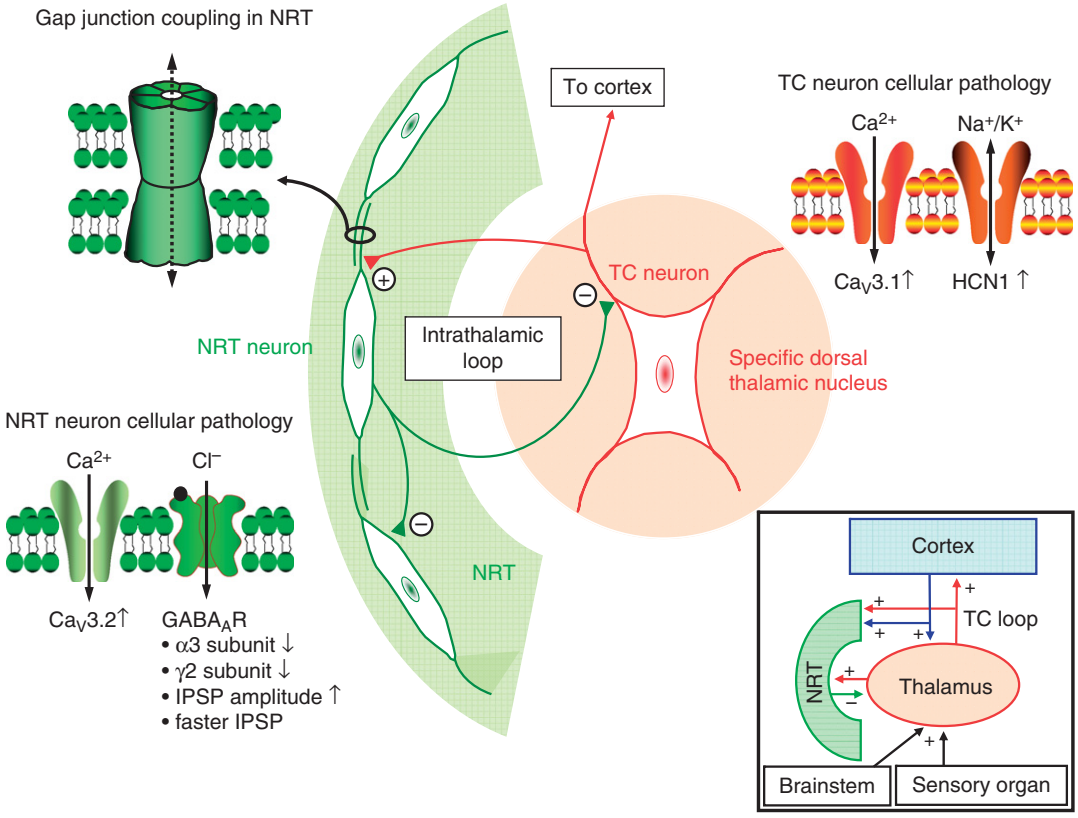


Figure 1 Simplified scheme of the thalamic network. The inset shows the general connectivity of the thalamocortical network including driving inputs from sensory organs and modulating inputs from the ascending brainstem system. Neurons of the nucleus reticular thalamic (NRT) are shown in green. A thalamocortical relay neuron is shown in red. Some of the cellular pathologies found in rat models are shown as membrane sketches near the cells. NRT neurons reveal reciprocal inhibition and electric coupling via gap junctions (see upper left membrane sketch). The latter is regarded as a pro-oscillatory mechanism. +, excitatory connection; −, inhibitory connection; GABA, γ-aminobutyric acid; IPSP, inhibitory postsynaptic potential; TC, thalamocortical.

The hyperpolarization-activated cation current (also called pacemaker current), I_h, possesses unique properties – including dual gating by membrane voltage and cyclic AMP (cAMP) – which make it a candidate for regulation of neuronal activity in a functional range not easily covered by other membrane conductances. In the thalamic synaptic network, there is an active contribution of I_h to the generation of rhythmic activity. The properties of I_h have been analyzed in detail in TC neurons of specific thalamic nuclei (e.g., the dLGN), where it has been shown to act as a cellular pacemaker, and through interplay with the T-type Ca^{2+} current generates slow-rhythmic burst discharges at delta frequencies (1–4 Hz). Furthermore, activation and de-activation of I_h in TC cells are critical for the temporal patterning of low threshold spikes interacting with GABAergic synaptic inputs from the NRT, thereby contributing to the typical waxing and waning of thalamic network oscillations, evident as sleep spindles or SWDs. Regulation of the voltage-dependence of I_h activation through the intracellular adenylyl cyclase/cAMP system, therefore, is considered an important mechanism for the control of oscillatory activity during the sleep-wake cycle. Four members of a gene family encoding mammalian hyperpolarization-activated, cyclic nucleotide-gated cation channels (HCN1–4) have been cloned; they are thought to assemble to form homomeric and/or heteromeric functional channels. The kinetics, voltage-dependency, and cAMP-sensitivity of the channels and the corresponding I_h are largely determined by the particular subunit composition.

A third membrane current with an important role in the integrative properties of TC neurons during burst-firing patterns is a persistent, non-inactivating component of the Na^+ current (I_{NaP}), which influences amplitude and latency of low-threshold Ca^{2+} potentials.

Interneurons

About 20–25% of the cells in some thalamic nuclei (e.g., dLGN) of most species are local circuit interneurons, which contain the inhibitory transmitter GABA. Although these cells are endowed with critical oscillation-related membrane currents such as I_T and I_h, and reveal intrinsic oscillations, the role of thalamic interneurons in thalamocortical rhythmicity is not clear. Because of limited

synaptic connectivity with other thalamic elements (i.e., they receive little input from either TC neurons or NRT neurons), investigators have hypothesized that interneurons have no major role in generating recurrent thalamocortical oscillations but contribute primarily to the processing of sensory inputs. Indeed, the specific expression of the splice variation $Ca_V3.1$-bc and the $Ca_V3.3$ isoform seems to give rise to an I_T with voltage-dependent and kinetic properties largely overlapping with a fast transient outward current (I_A), thereby limiting the generation of an LTS in these interneurons.

NRT neurons

The third neuronal population involved in thalamic rhythm generation is the population of NRT neurons, which are confined to a shell-like nucleus that surrounds mainly the anterior and lateral aspects of the dorsal thalamus (see Figure 1). The NRT is composed entirely of GABAergic neurons that express $Ca_V3.2$ and $Ca_V3.3$ T-type channels. The properties of NRT T-type Ca^{2+} currents are specifically different from those expressed in TC neurons and interneurons, in that they show much slower current kinetics and more depolarized voltage-dependency. These properties allow NRT neurons to generate burst firing patterns from resting membrane potential. The T-type channels in NRT neurons seem to be primarily localized in dendrites and thus can boost efferent signals to these cells, including those arriving from TC neurons or cortical neurons. Knockout mice for $Ca_V3.2$ and $Ca_V3.3$ have not yet been reported, so the primary mediator of burst firing in NRT neurons remains unknown. Activation of the T-type channels results in an electrogenic depolarization; functional interaction with Ca^{2+}-dependent membrane channels (like Ca^{2+}-activted nonspecific cation channels (I_{CAN}) and K^+ channels (I_{KCa})) and rhythmic oscillations of the membrane potential trigger bursts of Na^+/K^+-mediated spikes. Compared with TC cells, the burst generated by NRT neurons is not so stereotyped and is often of a longer duration and higher frequency.

Neurons of 'unspecific' intralaminar nuclei

Data are limited regarding the intrinsic and synaptic properties of intralaminar thalamic neurons, particularly in relation to SWDs. Experimental animal studies have shown burst firing of intralaminar neurons correlated with SWDs on the EEG, similar to the bursts seen in TC neurons of specific thalamic nuclei and the NRT, although firing is not so stereotyped and is often delayed. Clinical studies have demonstrated that electrical stimulation of defined intralaminar thalamic nuclei (in particular, the centromedial nucleus) can reduce or aggravate absence seizure activity, depending on the stimulation paradigm.

The Intrathalamic Loop: TC Neurons – NRT

Reciprocally connected excitatory TC neurons and inhibitory NRT neurons give rise to a synaptic loop consisting of GABAergic inhibitory contacts (from NRT axons onto TC neurons) and axon collaterals from TC neurons onto NRT neurons (that form excitatory synaptic contacts with NRT neurons) (see Figure 1). One important function of this loop is to generate and sustain network oscillations. Inhibitory output resulting from rhythmically bursting NRT neurons leads to activation of $GABA_A$ and/or $GABA_B$ receptors on TC neurons and the generation of postsynaptic inhibitory potentials (IPSPs). The membrane hyperpolarization of the IPSP deinactivates $Ca_V3.1$ channels, and upon termination of the IPSPs, a rebound burst of action potentials is generated. Reciprocal connectivity in the circuit ensures that the burst output of TC neurons is transmitted back to NRT, to excite NRT cells via ionotrophic glutamate receptors, thereby renewing the recurrent network activity. The strong activity induced by burst firing in both cell types ensures robust synaptic output with every oscillatory cycle. This type of network oscillation can occur spontaneously in an isolated thalamic slice preparation. Furthermore, pharmacological manipulations that block synaptic excitation, synaptic inhibition, or T-type channel function completely abolish intrathalamic oscillations in slices. During absence epilepsy, NRT neurons and TC neurons have a propensity to fire in a synchronous, phase-locked manner during every SWD complex. However, both the probability of firing and the number of action potentials during the SWD complex are much lower in most TC neurons than in NRT neurons. The SWD-related inhibition of TC neurons is thought to represent the neuronal substrate for the drop in sensory responsiveness during absence seizures. During absence-related inhibitions, TC neurons show very limited responsiveness to prethalamic signals and so cannot relay peripheral signals to the cerebral cortex.

Synchronized Thalamocortical Oscillations

The thalamus is capable of initiating and sustaining network oscillations. However, the intrathalamic network is considerably more complex than the paired inhibitory–excitatory connectivity described above (and shown in Figure 1). There is considerable synaptic convergence and divergence at all the cellular connections, such that individual NRT neurons inhibit multiple TC neurons and receive inputs from several TC neurons. With this extensive lateral spread of activity, a small local network oscillation can recruit neighboring cells, leading to the generation of widespread thalamic synchronous oscillations in slices. Synchronized oscillatory electrical activity of groups of neurons occurs in vivo during various behavioral states, with sleep and seizures being prominent examples. These synchronous

oscillations can be detected with EEG recordings; two EEG rhythms that reflect this type of activity of thalamocortical networks are sleep spindles and SWDs. During sleep spindles a limited number of the neurons participate in the network oscillation, thereby generating individual spindle waves which are variable in duration and amplitude, and recruit variable populations in diverse cortical areas. By contrast, SWDs are characterized by spiking in the underlying cellular networks that is strongly synchronized within a given cycle of the oscillation and across multiple cortical areas. Thus, bilaterally synchronous SWDs reflecting widespread thalamocortical synchrony are the characteristic sign of generalized absence epilepsy.

Methods

A number of experimental animal models of absence epilepsy have been described. They can be roughly divided into models of seizure generation and models of epilepsy. In the former, seizures are induced in nonepileptic animals by applying chemical or electrical stimuli resulting in SWDs accompanied by behavioral arrest. Important examples are the feline generalized penicillin epilepsy model (FPGE; involving the intramuscular injection of large doses of penicillin to cats) and the γ-hydroybutyrate (GHB) model (involving the application of GHB or the prodrug γ-butyrolactone to thalamic nuclei). Models of epilepsy make use of animals with spontaneous, recurrent seizures. These models seem to better account for the integral nature of various types of absence epilepsies, the polygenic background, and the pathophysiology of the underlying synaptic network. Two genetic rat models, termed Genetic Absence Epilespsy Rats from Strasbourg (GAERS) and Wistar Albino Glaxo Rats from Rijswik (WAG/Rij), were independently derived from inbreeding of the Wistar strain. Their genotypes are not known, although there is some evidence of an autosomal-dominant inheritance of the SWD trait and a polygenic background that may modify seizure characteristics. The absence phenotype is similar in both strains (although subtle differences have been reported for the developmental and electrophysiological profile of the seizures) and largely resembles that in human CAE. The SWDs occur spontaneously on a normal EEG (mostly during quiet wakefulness) and are associated with a severe reduction in sensory responsiveness and a mild facial myoclonus; there are no major neuropathological abnormalities. Furthermore, classic absence antiepileptic drugs (ethosuximide, valproate) suppress SWDs, whereas drugs specific for convulsive or focal seizures (carbamazepin, phenytoin) are ineffective or aggravate SWDs in these rats. Differences from human CAE relate to the higher frequency of the SWDs (7–11 Hz), the relatively late appearance of absence seizure activity during development, and the persistence of SWDs through rat 'adolescence.'

A number of mutant mice models, involving the genes encoding different subunits of high-threshold Ca^{2+} channels (e.g., ducky, leaner, lethargic, rocker, stargazer, tottering), the Na^{+}/H^{+} antiporter (slow-wave epilepsy mouse), adapter-like proteins (mocha), and SNAP-25 (coloboma) are considered less relevant to the human situation because of their monogenetic mode of inheritance and the presence of significant neurological deficits normally not present in CAE (e.g., ataxia, hyperactivity).

Because the relative roles played by the different components of the thalamocortical system in the generation of SWDs are still a matter of debate, experimental models with different network connectivity (i.e., thalamic, cortical, and thalamocortical models) have been developed, thereby helping to assess their specific contributions and to investigate the mechanisms underlying the generation and spread of SWD-like activity in this brain structure. The ferret dLGN and the associated section in the NRT nucleus have proved particularly useful. A sufficient part of the synaptic network is preserved in a slice preparation in vitro to generate spontaneous, recurrent, spindle-like activity, and this pattern of activity can be acutely transformed into SWD-like activity upon pharmacologic manipulation of $GABA_A$ receptors. Similarly, a rodent in vitro thalamic slice preparation has been developed that contains interconnected neurons of the ventrobasal thalamic complex (VB) and the NRT, and which is capable of generating sustained low-frequency (2–4 Hz) oscillations. This horizontal thalamic slice provides a preparation that allows straightforward analyses of genetic mutations or pharmacologic interventions on intrathalamic rhythmicity. To further account for the joint contribution of both thalamus and cortex to the generation of SWDs, in vitro preparations including both parts of the forebrain (i.e., thalamus and cortex) have been developed. The rodent thalamocortical slice was introduced as a suitable system for studying the physiology and pharmacology of the thalamocortical synapse and for exploring the synaptic circuitry of the somatosensory cortex. This thalamocortical slice preparation is a reproducible model that enables investigators to analyze thalamocortical synchronization and to understand the pathogenesis of absence epilepsy.

Recent Results

Pathophysiological Mechanisms of CAE

Clinical evidence indicates that absence seizures are associated with states of decreased vigilance, such as drowsiness. In the early FPGE model, it was observed that SWDs on the EEG developed gradually from sleep spindles during early stages of slow-wave sleep. More recent studies in GAERS suggest that SWDs may originate in a cortical focus and

develop from a more wake-related natural thalamocortical sensorimotor oscillation (medium-voltage, 5–9 Hz oscillations in the somatosensory system of rats during quiet immobile wakefulness). In addition to these different observations according to which 'naturally-occurring' activity represents 'embryonic' SWDs, there have been extensive studies of mutant mice, genetic rat models, and the in vitro slice preparation that have generated the following scenario:

1. The neurons and synaptic interconnections of the thalamocortical system that normally sustain rhythmic activities during slow-wave sleep or sensorimotor oscillations are also critically involved in the generation of SWDs.
2. Seizure activity arises from a concerted interaction within this network, with initiation sites most likely residing in the cortex. The cortico-thalamocortical loop plays a pivotal role in the generation and synchronization of SWDs at an extended spatiotemporal scale. Importantly, neither cortical nor thalamic networks alone can generate or sustain SWDs, and influences exerted by brain structures other than the thalamocortical system can modulate SWD generation. Studies of various models, including GAERS and WAG/Rij, have indicated that spontaneous SWDs occur first in the somatosensory cortex and then invade other cortical and thalamic areas. Pathophysiological mechanisms in the cortex may involve an increase in excitatory synaptic transmission mediated via *N*-methyl-D-aspartate (NMDA) receptors, a decrease in GABAergic inhibition, and/or an altered expression of HCN1.
3. Cortical SWDs reach the NRT via corticofugal fibers. The response in NRT neurons consists of bursts of excitatory postsynaptic potentials (EPSPs) and a regenerative LTS crowned by a burst of fast action potentials. The overall response is synchronized burst activity in NRT neurons, which coincides with the SWDs on the EEG. Pathophysiologic alterations (see **Figure 1**) that have been reported with respect to SWD generation include: an increase in strength of corticofugal inputs; an increase in I_T amplitude associated with an increased expression of the $Ca_V3.2$ subunit in NRT neurons; increased and faster $GABA_A$ IPSCs; decreased expression of the $GABA_A$alpha3 subunit; and changes in the strength of $GABA_A$ receptor- and gap junction-mediated synaptic interactions involved in synchronization of local synaptic networks.
4. Circuitry within the thalamus promotes recurrent network activity as described above. An imbalance of $GABA_B$ over $GABA_A$ receptor-mediated inhibition has been reported to shift activity in TC neurons toward SWD-like discharges, although $GABA_A$ receptor-mediated influences dominate during spontaneous SWDs in rat genetic models, and the balance between $GABA_A$ and $GABA_B$ seems to relate to the prevailing frequency of the SWDs (3–5 Hz in humans versus 7–11 Hz in most animal models). Slightly different conclusions have been reached by investigators studying different models with respect to the relative contribution of LTS-related burst firing and of $GABA_A$ potentials to the SWDs in TC neurons. Further pathophysiological mechanisms in TC neurons may be related to an increased HCN1 and $Ca_V3.1$ expression, resulting in prolonged and enhanced burst activity (see **Figure 1**).
5. Thus, genetically determined, absence-related SWDs are the result of hypersynchronized, rhythmic excitations and inhibitions that result from complex intrinsic, synaptic, and network mechanisms in thalamus and cortex. A number of alterations found in genetic rat models characterize CAE as a 'transcriptional channelopathy.'

Pharmacology of CAE

The pharmacological target of typical absence drugs, especially ETX, has been a matter of long-standing controversy. In line with the I_T dependence of thalamocortical slow oscillations, the mechanism of action of ETX had initially been attributed to a reduction of this current in TC neurons. Subsequent work has failed to reproduce the finding of ETX induced I_T reduction by therapeutically relevant concentrations (0.25–0.75 mM). Instead, the mechanism of ETX action has been attributed to a reduction of I_{NaP} and the Ca^{2+}-activated K^+ current (I_{KCa}). Recently, the issue was readdressed by showing that a specific I_T antagonist was able to inhibit thalamic oscillations in vitro, and by showing that ETX blocks cloned I_T channels expressed in HEK-293 cells. A systematic comparison of ETX effects between thalamic cell types from epileptic and nonepileptic rat strains points to a possible solution to this controversy: (i) Combined effects of ETX on several membrane currents (I_T, I_{NaP}, I_{KCa}) may account for the full anti-absence action of therapeutically relevant ETX concentrations. (ii) The inhibition of I_T in several cell types, especially those important for network synchronization, must be considered. (iii) The dependency of ETX action on membrane potential and stimulation frequency may enhance I_T inhibition.

Future Challenges

Further studies are needed to address a number of important issues: (1) The genetic basis of CAE must be further elucidated – for instance, through identification of candidate genes that convey a predisposition to the generation of SWDs. (2) The mechanisms that lead to the altered expression of otherwise normal ion channels remain to be

identified. (3) Of special interest is the investigation of the role of intralaminar thalamic nuclei to the SWDs generation, as these nuclei are ideally positioned to spread activity within the thalamocortical system. (4) An interesting question is whether the mechanisms of SWD generation are characteristic of, and/or critical for, other types of thalamocortical dysrhythmia. (5) In more general terms, future studies must unravel the mechanisms that transform normal brain activity into SWDs. These mechanisms likely involve widespread neuronal changes that predispose epileptic individuals to develop hypersynchronous phenomena in the thalamocortical system.

See also: **Absence Seizures**: Genetic Models of Absence Epilepsy: New Concepts and Insights; Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamocortical Synchronization and Absence Epilepsy; **GABA**: Synchrony Through GABAergic Inhibition.

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Thalamocortical Synchronization and Absence Epilepsy

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Introduction

Primary generalized epilepsies occur in ~0.2% of the population under 25 years of age and are characterized by a major genetic component. The ictal manifestations in these patients show some degree of variability. However, the common clinical marker is a short (<30 s) period of loss of contact with the surrounding environment (i.e., the 'absence') accompanied by a stereotyped electro-encephalographic (EEG) discharge of spike-and-wave (SW) complexes at approximately 3 Hz (**Figure 1** (**a**)). This discharge has several distinctive features, including (i) generalized onset with bilateral synchrony; (ii) non-progressive pattern (i.e., similar SW complexes occur throughout the discharge); and (iii) sudden termination with no ensuing postictal depression. SW discharges can diminish the prospects of a satisfactory life, and may in some individuals develop into tonic–clonic seizures that

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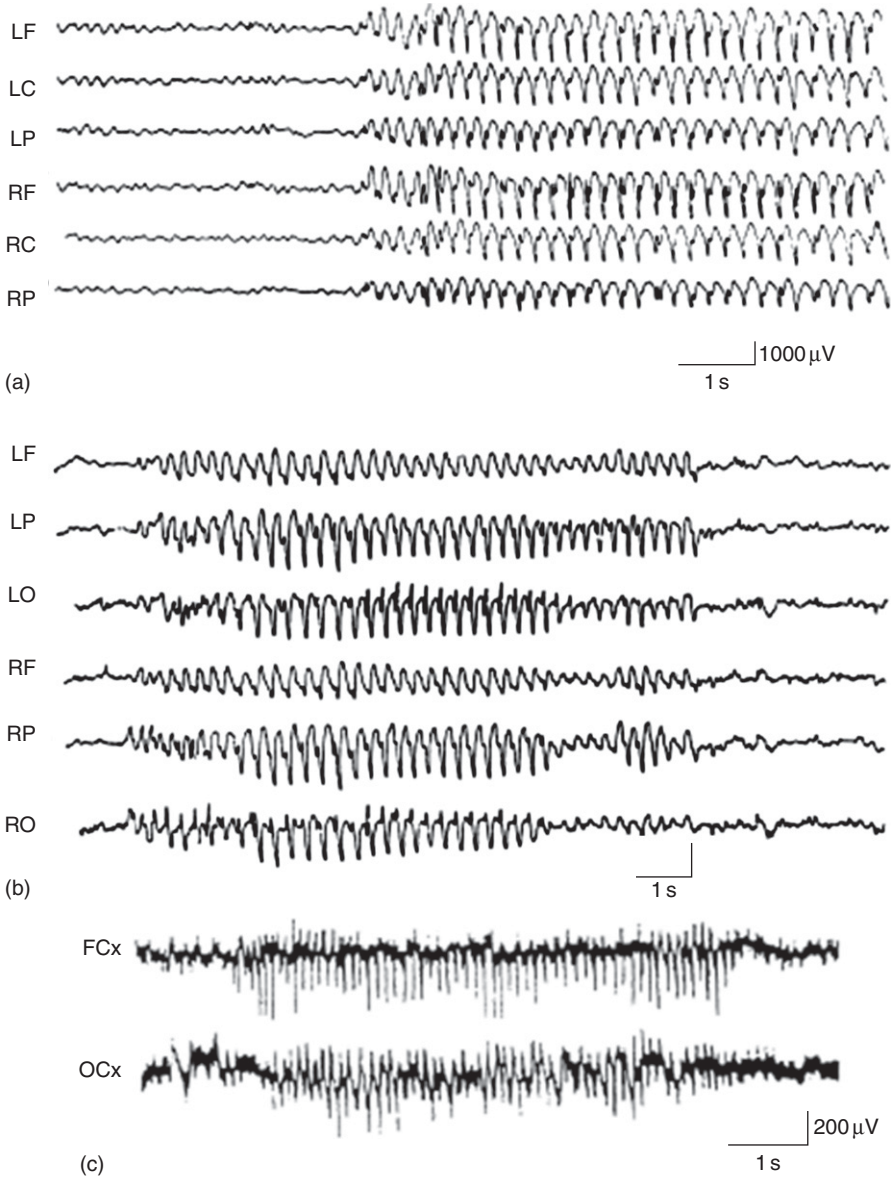


Figure 1 EEG traces of generalized SW discharges obtained from a patient (a), from a cat injected with i.m. penicillin injection (b) and from a WAG/Rij epileptic rat (c). These recordings are similar for the generalization at onset and throughout the discharge, the nonprogressive feature of the pattern and the absence of postictal depression. Abbreviations in this figure are: C, central; Cx, cortex; F, frontal; L, left; P, posterior; O, occipital; R, right. Part (a) reproduced from Gloor P and Fariello RG (1988) Generalized epilepsy: Some of its cellular mechanisms differ from those of focal epilepsy. *Trends in Neurosciences* 11: 63–68; Part (b) reproduced from Guberman A, Gloor P, and Sherwin AL (1975) Response of generalized penicillin epilepsy in the cat to ethosuximide and diphenylhydantoin. *Neurology* 25: 785–764; Part (c) reproduced from Inoue M, van Luijtelaar EL, Vossen JM, and Coenen AM (1992) Visual evoked potentials during spontaneously occurring spike-wave discharges in rats. *Electroencephalography and Clinical Neurophysiology* 84: 172–179.

are resistant to medications. Hence, understanding the pathophysiogenesis of SW discharges remains an important challenge in epilepsy research.

Background

Generalized SW discharges were reproduced experimentally by Jasper and Droogleever-Fortuyn over 60 years ago. The discharge was recorded during low-frequency stimulation of midline and intralaminar thalamic nuclei in the cat, suggesting that the SW pattern may have a subcortical origin ('centrencephalic hypothesis'). However, this view was later challenged by the discovery that bilateral application of convulsants to the frontal cerebral cortex of cats and monkeys could produce a similar pattern of generalized, 3 Hz SW activity. In addition, in the photosensitive baboon (*Papio papio*), investigators identified a cortical origin of the electrographic activity characteristic of this genetic model of generalized

epilepsy. This evidence, along with an extensive review of clinical data, led Gloor in 1968 to propose the term 'generalized corticoreticular epilepsy' to reconcile the centrencephalic and cortical hypothesis.

Choosing the proper animal model is of paramount importance in the pursuit of the mechanisms of absence seizures. SW discharges have been widely studied in normal brains treated with either convulsants or electrical stimuli. The EEG and behavioral patterns resulting from these manipulations reasonably approximate the human condition (**Figure 1(a)** and **1(b)**). However, the genetic component of primary generalized epilepsies suggests that genetic animal models are more likely to give insight into the brain alterations leading to SW discharges. Interestingly, rat genetic models (not yet completely defined in terms of their genetic background) present ictal behavioral and EEG manifestations similar to those seen in absence seizure patients (**Figure 1(c)**). These models include the Genetic Absence Epilepsy in Rats from Strasbourg (GAERS) and the Wistar Albino Glaxo rats of Rijswijk (WAG/Rij). SW discharges recorded in these rodents have electroclinical (**Figure 1(c)**) and pharmacological profiles that closely resemble absence epilepsy in humans.

Methods

Feline Generalized Penicillin Epilepsy

Acute experiments were performed under neuroleptic analgesia in awake cats immobilized with gallamine triethiodide. Surface EEG was recorded from both hemispheres while 'local' EEG signals and single-unit activities were simultaneously obtained by each of two tungsten microelectrodes, one placed in the cortex (anterior part of the middle suprasylvian gyrus) and the other in the thalamus (nucleus lateralis posterior/pulvinar). Stimulating electrodes were positioned in the deep cortical layers to identify thalamic neurons as thalamocortical (i.e., antidromically activated) or as cortical targets (i.e., orthodromically activated). The activity of pairs of single neurons, one located in cortical areas 5 and 7 and the other in the dorsal thalamus, was studied during generalized SW discharges induced by intramuscular (i.m.) injection of large penicillin doses (350 000 IU kg^{-1}). All data (i.e., cortical surface, intracortical and thalamic EEG, and cortical and thalamic single-unit activity) were fed to a computer after analog-to-digital conversion. Thalamic and cortical action potentials were processed through window discriminators.

The WAG/Rij Rat Model of Generalized Epilepsy

Rats of the inbred WAG/Rij strain display hundreds of spontaneous seizures per day characterized by bilaterally generalized SW discharges arising abruptly from normal background EEG. At the time of surgery, rats were 16–22 month old. Epidural and depth electrodes for EEG recordings were stereotactically implanted during surgery under pentobarbital anesthesia. Spontaneous field potentials were recorded in freely moving WAG/Rij rats until a large number of spontaneous SW discharges were obtained. These data were used for the signal analysis. To estimate the degree of association between two signals and the corresponding time delay, the nonlinear correlation coefficient was calculated as a function of time shift between the two signals. Field potential recordings were also obtained from nonepileptic control rat thalamocortical slices maintained in vitro and superfused with medium containing 4-aminopyridine.

Results

Contribution of Cortical and Thalamic Networks to SW Discharges

Studies of cats injected intramuscularly with penicillin have demonstrated that functional and anatomical integrity of thalamus and cortex is required for the generation of generalized SW discharges. Depression of thalamic activity transiently abolished both normal spindle and SW activity in this model of absence seizures. Further, SW discharges did not occur when the thalamus was deprived of connections with the neocortex following surgical decortication. Finally, penicillin-induced SW discharges in the intact cat brain were blocked (and replaced by spindles in both thalamus and neocortex) when cortical excitability was reduced by spreading depression. Similar evidence has been obtained from studies with genetic rat strains of absence seizures. Experiments performed in cats in Steriade's laboratory have also identified a major role of neocortical networks in the generation of slow SW discharges typical of the Lennox–Gastaut syndrome.

Single unit extracellular and intracellular recordings have provided further insight into the mechanisms underlying the SW discharges. Both in cats treated with penicillin (**Figure 2(a)**) and in genetic rodent models (**Figure 2(b)** and **2(c)**), the spike of the SW complex is associated with a marked increase in firing probability in cortex and thalamus, while a reduction in firing probability (to virtually zero) is present during the wave component. In addition, intracellular recordings from cortical projection cells in the feline penicillin model have shown that the spike of the SW complex reflects summated EPSPs induced by incoming thalamocortical volleys, while the wave component is mirrored by depression of firing because of a long-lasting, membrane hyperpolarization (**Figures 2(b)** and **3**). A similar sequence of intracellular potentials also occurs during SW discharges in GAERS (**Figure 2(c)**) and WAG/Rij rat cortical and thalamic cells. It is, however, unclear

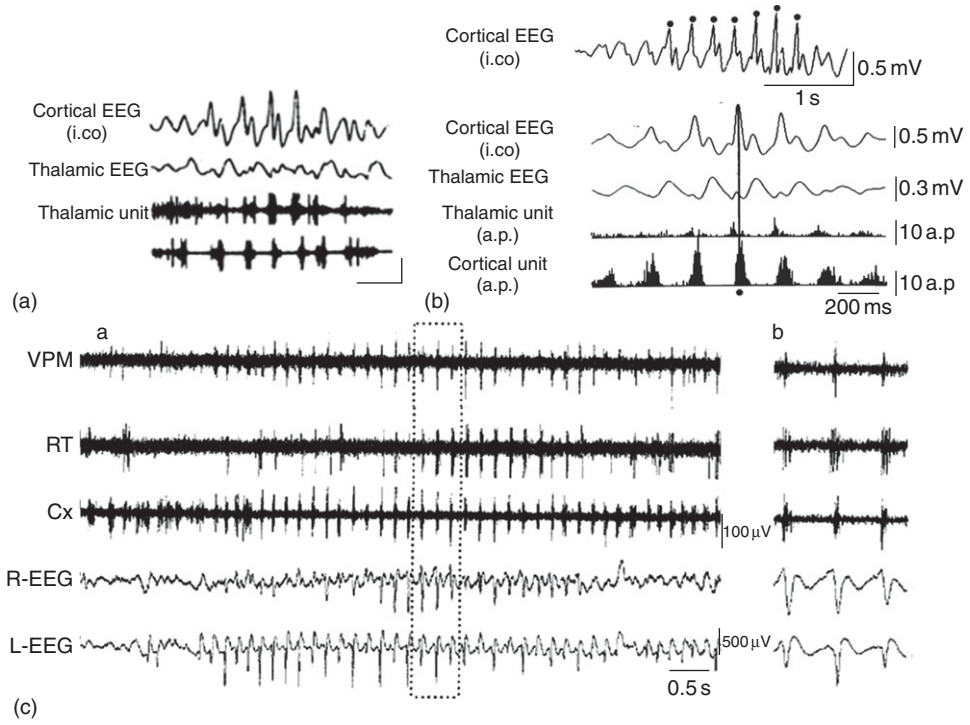


Figure 2 (a, b) EEG-unit correlation for a cortical and a thalamocortical neuron during SW discharges recorded in a cat injected with i.m. penicillin. In (a), raw data of the EEG and single-unit activities; in (b), EEG averages and histograms of single unit activity triggered by the negative peaks of the cortical EEG spikes. Note that both cortical and thalamic units correlate with oscillations between marked increase in firing probability during the *spike*, and a reduction of this probability of firing to virtually zero during the *wave* component of the SW discharge. (c) SW discharges recorded in a GAERS rat from units in the thalamus (RT, rostral reticular nucleus and VPM, ventroposteromedialis) and in the cortex (Cx) and bilaterally from the EEG. Cb: Inset demonstrates the temporal relationship of SW discharges and burst-like unit activity at an enlarged time scale. Parts (a, b) reproduced from Avoli M, Gloor P, Kostopoulos G, and Gotman J (1983) An analysis of penicillin-induced generalized spike and wave discharges using simultaneous recordings of cortical and thalamic single neurons. *Journal of Neurophysiology* 50: 819–837; Part (c) reproduced from Seidenbecher T, Staak R, and Pape HC (1998) Relations between cortical and thalamic cellular activities during absence seizures in rats. *European Journal of Neuroscience* 10: 1103–1112.

whether the hyperpolarization occurs through conventional GABA inhibitory mechanisms.

Thalamic Networks and SW Discharge Rhythmogenesis

The corticothalamic projection to the ventrobasal thalamus consists mainly of small modified pyramidal cells located in layer VI, and in part by larger pyramidal cells located in the lower layer V. Anatomical studies have demonstrated that layer VI cortical neurons, but not those in layer V also send collaterals to the reticular thalamic nucleus. Most of the inhibitory control exerted on thalamocortical relay cells originates from the reticular thalamic nucleus, which is composed entirely of GABAergic neurons that are excited by corticothalamic and thalamocortical afferents. Reticular thalamic neurons are endowed with a pronounced low-threshold (termed also T-type) Ca^{2+} current that causes rhythmic action potential bursting. The volley of action potentials, traveling along the reticular cell axons, induces $GABA_A$ and $GABA_B$ receptor-mediated hyperpolarizing IPSPs in thalamocortical neurons. In thalamocortical neurons, another T-type Ca^{2+} current triggers bursts of action potentials as the membrane potential hyperpolarization (associated with the IPSPs induced by the reticular thalamic nucleus input) decays toward the resting membrane level. In addition, a noninactivating Na^{+} current acts synergistically with the T-type Ca^{2+} current in these cells, contributing to an increased frequency of burst firing. Bursts of action potentials in thalamic principal neurons then excite neocortical cortical cells that in turn re-excite nucleus reticularis neurons via corticothalamic connections.

It is well established that these events lead to the rhythmic activity that paces sleep spindles, and a similar hypersynchronous thalamo-cortico-thalamic mechanism may pace burst firing in thalamocortical cells to frequencies characteristic of the SW discharges of absence epilepsy. The synchronization of thalamocortical and corticothalamic networks can be easily appreciated in vitro by stimulating with 4-aminopyridine slices in which the reciprocal cortical and thalamic connections are preserved (**Figure 4**). A malfunctioning of these networks in

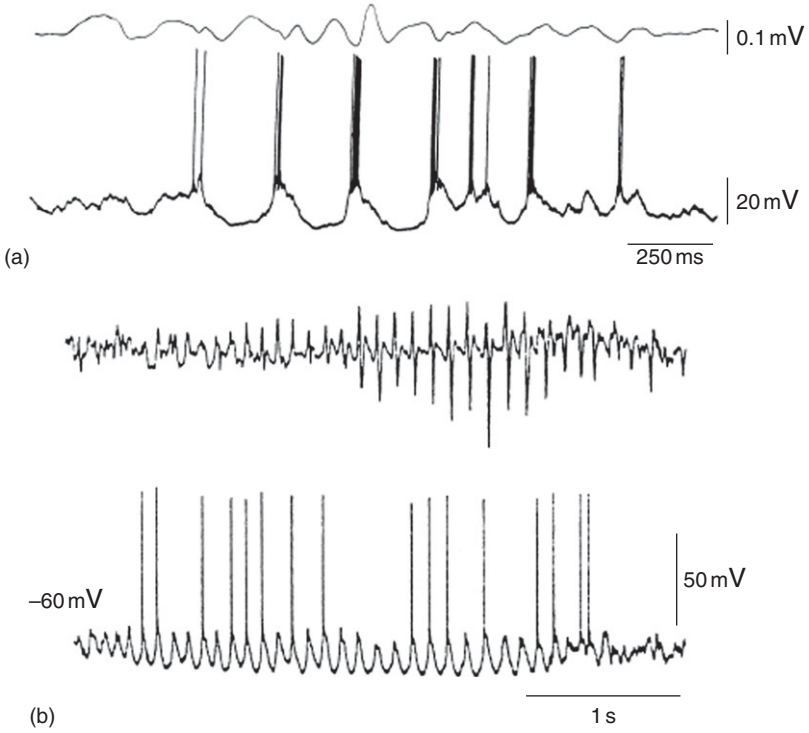


Figure 3 (a) EEG (uppertrace) and intracellular potentials (lower trace) recorded during a SW discharge in a cat injected i.m with penicillin. Note that the *spike* is associated with a depolarization that triggers action potentials, while the *wave* of the SW complex is associated with a hyperpolarization of the membrane. (b) Rhythmic depolarizations are present in the intracellular recording (bottom trace) performed at resting membrane potential from a cortical neuron during the occurrence of a SW discharge recorded in the EEG (upper trace) from a GAERS rat. Part (a) reproduced from Giaretta D, Avoli M, and Gloor P (1987) Intracellular recordings in pericruciate neurons during spike and wave discharges of feline generalized penicillin epilepsy. *Brain Research* 405: 68–79; Part (b) reproduced from Charpier S, Leresche N, Deniau JM, Mahon S, Hughes SW, and Crunelli V (1999) On the putative contribution of $GABA_B$ receptors to the electrical events occurring during spontaneous spike and wave discharges. *Neuropharmacology* 38: 1699–1706.

generalized epilepsy is supported by several pieces of evidence. First, as already mentioned, spindles and SW discharges disappear in cats treated with i.m. penicillin when the thalamic activity is transiently depressed. Second, selective lesioning of reticular thalamic nucleus permanently abolishes SW activity in GAERS animals. Third, the low-threshold Ca^{2+} current is overexpressed in isolated reticular nucleus neurons isolated from GAERS. Fourth, microinjection of $GABA_A$ or $GABA_B$ receptor agonists into thalamic relay nuclei increases the duration of SW discharges in the GAERS model and in other genetic models of primary generalized epilepsies, while microinjection of $GABA_B$ receptor antagonists abolishes SW discharges. Finally, deletion of the $GABA_A$ receptor β_3 subunit – which is mainly restricted to the reticular nucleus in rodents – markedly reduces $GABA_A$ receptor-mediated inhibition in reticular (but not in thalamocortical relay) cells, and causes a marked increase of the oscillatory synchrony of thalamocortical relay neurons. The mutual recruitment of thalamic and cortical networks during generalized SW rhythmogenesis has also been investigated in computational models. In these studies, investigators have shown that the burst responses of thalamic neurons, as well as the properties of $GABA_B$ receptor-activated conductances, are critical in generating SW discharges.

Role of Cortical Networks in SW Discharge Initiation

According to the evidence outlined in the previous section, the interaction between reticular and thalamocortical cells projecting to the cortex, along with the reciprocal corticothalamic connections, provides the anatomical and functional substrate for SW discharges. It remains, however, to be established where the initiation of these epileptic discharges resides. Are there initiation sites in the cortex, or does neocortex just act as a passive by-stander to a rhythm elaborated within the thalamus? Although the evidence obtained in the feline generalized penicillin model indicates that the occurrence of generalized SW discharges require hyperexcitable neocortical networks, a definitive answer to how absence seizures are initiated, and then generalize within the brain, has been provided by studying WAG/Rij rats. By employing nonlinear association analysis of the EEG signals recorded from thalamus and neocortex, investigators have demonstrated that SW activity originates

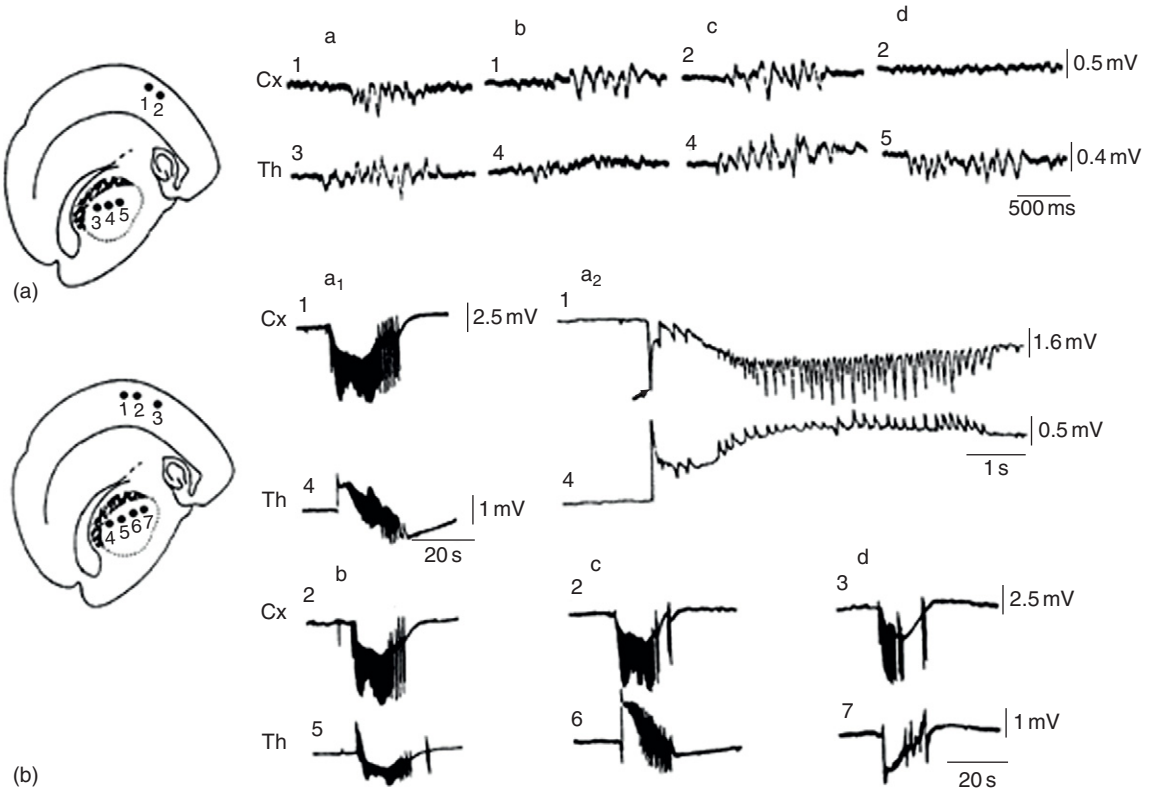


Figure 4 Spontaneous field potential activity induced by low (a) and high (b) concentrations of 4-aminopyridine in the thalamocortical slice preparation. Position of the recording electrodes in the frontoparietal cortex (Cx) and in thalamus (Th) is numbered in the two inserts. (a) Rhythmic field potential oscillations occur in cortex and thalamus during application of low (0.5 μM) concentrations of 4-aminopyridine. Note that these field potential oscillations are synchronized in cortex and thalamus in panels a and c, while synchronization is no longer observed when the recording electrode is moved by <500 μm in either structure (panels b and d). (b) Spontaneous ictal discharges induced by large doses (100 μM) of 4-aminopyridine. These prolonged epileptiform events are characterized in both cortex and thalamus by an initial tonic phase that is followed by recurring rhythmic discharges (a1 and a2). This pattern is characterized by a high degree of synchronicity between thalamus and cortex even when the recording electrodes are repositioned at distances >1.5 mm (panels b, c, and d). In a2, the ictal discharge is illustrated at a faster time base showing that the onset of the discharge is characterized by a negative- and positive-going potential in cortex and thalamus, respectively. Reproduced from Biagini G, D'Antuono M, Tancredi V, *et al*. (2001) Thalamocortical connectivity in a rat brain slice preparation: Participation of the ventrobasal complex to synchronous activities. *Thalamus and Related Systems* 1: 169–179.

from a 'consistent cortical focus' that overlaps with the perioral region of the somatosensory cortex (**Figure 5**). These findings were later confirmed also in GAERS rats. Consistent with this view, in slices of the ferret lateral geniculate nucleus, other investigators have shown that the frequency of the rhythmic oscillations generated by geniculate neurons is controlled by the patterns of activation of corticothalamic inputs (which in these experiments were mimicked by electrical stimulation of corticothalamic fibers).

Mechanisms of Cortical Hyperexcitability in Absence Seizures

Given the earlier results, an obvious question is: 'What mechanisms contribute to neocortical hyperexcitability in genetic rodent models of absence seizures?' Recent studies have begun to shed light on this issue, revealing that both nonsynaptic and synaptic mechanisms are altered in absence epileptic animals as compared with nonepileptic controls. SW discharges have been identified in mice carrying spontaneous mutations in genes belonging to the family of high-threshold voltage-gated calcium channels, such as *Cacna1a*^{tg} ('tottering' mouse), *Cacnb4*^{lh} ('lethargic'), *Cacna2d2*^{du} ('ducky'), and *Cacng2*^{stg} ('stargazer'). Single point mutation in the sodium/hydrogen exchanger (NHE1) gene (*Slc9a1*^{swe}), abolishing its transcription, also causes SW discharges and absence seizures. Interestingly, impairment in the expression (−50%) of the synaptic vesicle-associated protein SNAP25 is also associated with SW discharges in 'coloboma' mice (*Cm*, *Snap25*). In addition, the deletion of the *Hcn2* gene, which abolishes the I_h current, also leads to an absence epilepsy phenotype.

Recently, a new mouse model harboring the R43Q mutation of the $GABA_A$ subunit γ_2, found in an Australian family affected by absence epilepsy and febrile seizures,

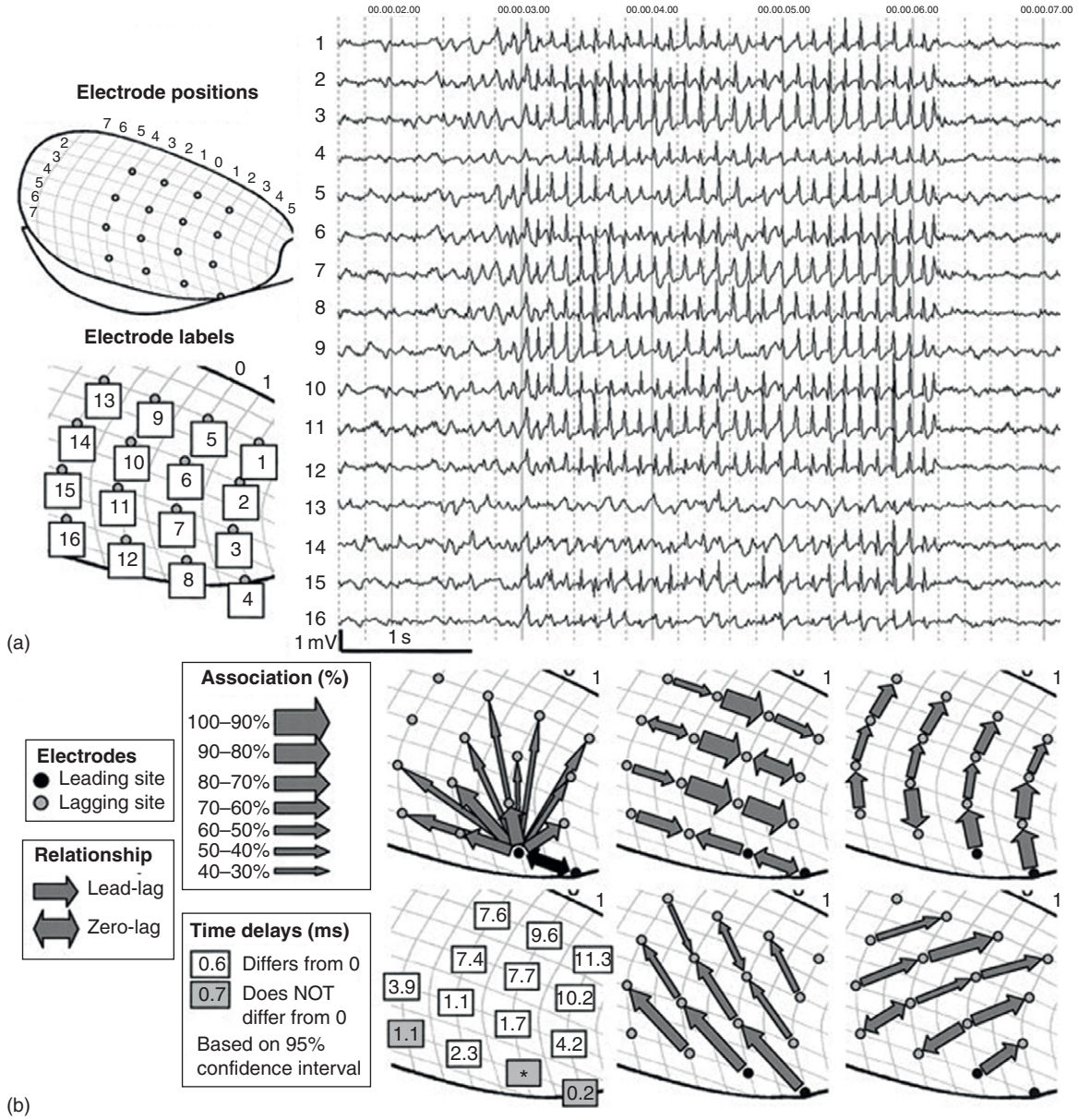


Figure 5 Identification of neocortical epileptic foci in WAG/Rij rats. (a) Electrographic seizure recorded with a cortical grid that covers the lateral convexity of the neocortex. The position of the electrodes and their labels are illustrated on the left. Note that the discharges are visible in each tested position. Note also that the potentials present with larger amplitude in the rostroventral locations, whereas they are minimal in the caudal positions (13–16). (b) Representations by arrows of the results of the nonlinear association analysis from five different perspectives. The thickness of the arrow represents the strength of the association, and the direction of the arrowhead points to the direction of the lagging site. Electrodes corresponding to positions 4 and 8 (black dots) identify the leading sites. The numbers depict the average time delays (in milliseconds) over 10 seizures with respect to electrode 8. Reproduced from Meeren HK, Pijn JP, van Luijtelaar EL, Coenen AM, and Lopes da Silva FH (2002) Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. *Journal of Neuroscience* 22(4): 1480–1495.

was developed. Seizures in this model appear at approximately 3 weeks of age and are mirrored by reduced $GABA_A$-mediated currents in cortical pyramidal neurons. No changes were found in reticular and ventrobasal thalamic neurons, pointing to a selective reduction of neocortical inhibition. Accordingly, GABA receptor-mediated inhibition is slightly reduced in the neocortex of WAG/Rij rats, where deep layer neurons generate hyperexcitable synaptic responses that appear to be caused by NMDA receptor activation. Paired-pulse depression of excitatory and inhibitory potentials is decreased in epileptic WAG/Rij rat neocortical slices, suggesting a process involving

activation of presynaptic $GABA_B$ receptors. Molecular and immunohistochemical analysis has also demonstrated a reduced expression of the mRNA encoding some $GABA_B$ receptor subunits, as well as the respective proteins. Therefore, diminished function of presynaptic $GABA_B$ receptors may make epileptic WAG/Rij neocortical networks hyperexcitable by increasing inhibitory and, perhaps to an even greater extent, excitatory transmitter release. Interestingly, $GABA_{B(1)}$ knockout mice also generate various types of epileptiform activity, including absence seizures. However, early studies in GAERS and WAG/Rij rats have shown that injecting $GABA_B$ receptor antagonists into the thalamus (or introducing it systemically) can block absence seizures. It is unclear whether these results reflect changes in the molecular properties of $GABA_B$ receptors within the GAERS and WAG/Rij thalamocortical circuits or rather a pharmacological effect based on the ability of $GABA_B$ receptor antagonism to reduce slow hyperpolarizing IPSPs (and thus low-threshold Ca^{2+} currents) in thalamocortical relay cells. Attempts to exploit these findings in order to develop therapeutic procedures in humans have thus far failed.

Pharmacological Treatment of Absence Seizures

Drugs used for treating absence seizures fall into two classes: broad spectrum and specific antiabsence agents. The broad spectrum antiepileptic drugs are effective in the treatment of generalized absence seizures, in addition to their better known activity in partial and generalized tonic–clonic seizures. In contrast, specific antiabsence drugs have little or no effect on other seizure types. Broad spectrum antiepileptic drugs such as lamotrigine, topiramate, and valproate act, at least in part, through state-dependent block of voltage-gated Na^+ channels. Interestingly, a similar mechanism of action is shared by antiepileptic drugs such as phenytoin and carbamazepine that are effective in partial epilepsies but ineffective in absence seizures.

The basis for a distinct spectrum of clinical efficacy for lamotrigine, topiramate, and valproate is unclear, although one may consider (i) subtle differences in the way these drugs modulate voltage-gated Na^+ channels, (ii) region-specific differences in sensitivity to the various agents, or (iii) mechanisms other than Na^+ current modulation. Specific antiabsence drugs, such as ethosuximide, methsuximide, and dimethadione, are known to act by reducing T-type Ca^{2+} currents in thalamocortical relay neurons and in neurons of the nucleus reticularis thalami, thus preventing burst firing and abolishing the rhythmic thalamocortical synchronization that paces SW discharges. However, ethosuximide also reduces bursting in thalamocortical neurons while increasing their tonic firing, an effect accompanied by inhibition of noninactivating Na^+ currents (but no effect on transient Na^+ currents, and a decrease in the activity of Ca^{2+}-activated K^+ channels).

It is also established that barbiturates lack antiabsence activity whereas benzodiazepines are highly effective as antiabsence agents. This differential efficacy may be due to differences in $GABA_A$ receptor subtype expression in thalamocortical relay and thalamic reticular neurons. Indeed, $GABA_A$ receptors on thalamocortical neurons fail to respond to clonazepam, whereas $GABA_A$ receptors on inhibitory cells of the thalamic reticular neurons are sensitive. As a consequence, clonazepam enhances recurrent inhibitory strength within the reticular nucleus, leading to reduced inhibitory output from these cells. This reduction, in turn, causes a decreased propensity of neighboring inhibitory neurons in thalamic reticular nucleus to fire synchronously and produce the powerful inhibitory synaptic responses that are presumably required for SW synchronization. Barbiturates do not show such a differential action, which may account for their lack of antiabsence activity.

Conclusions and Directions for Future Research

Overall, the expression of generalized SW discharges seen in animal models of absence seizures requires the function of reciprocally interconnected thalamic and cortical networks. This experimental evidence is consistent with data obtained from patients with absence seizures, in whom SW discharges were seen to occur simultaneously in cortex and thalamus. It is reasonable to propose that enhanced corticothalamic outputs make GABAergic cells in the thalamic reticular nucleus fire action potential bursts that are more intense than under normal conditions; these increased bursts cause extended IPSPs in thalamic relay cells, thus slowing their pacing frequency and producing recruitment of a larger number of cortical and thalamic neurons – which leads to SW activity. While the rhythmicity of SW appears to arise from properties of thalamic neurons, the initiation of the absence seizure discharge appears to reside in specific cortical neuronal populations.

Evidence from animal models suggests that $GABA_B$ receptors are dysfunctional in absence seizures. The resultant weakened presynaptic control should lead to an augmented release of inhibitory transmitter, which in turn may depress interneuron excitability and thus cause disinhibition of corticothalamic neurons. In addition, this condition is associated with, and perhaps may cause, the functional overexpression of NMDA receptor-mediated mechanisms. The increased release of GABA should activate postsynaptic $GABA_B$ receptors, thus producing robust

hyperpolarizing IPSPs that contribute to de-inactivate low-threshold Ca^{2+} conductances, which in turn facilitate rhythmic bursting. In line with this view, $GABA_B$ receptor antagonists injected either into the thalamus or systemically, block absence seizures in both GAERS and WAG/Rij rats. It remains unclear whether the changes in $GABA_{B(1)}$ subunit expression, and thus the decreased function of $GABA_B$ receptors, are genetically determined (and thus developmentally regulated) or whether they are the consequence of on-going SW discharge activity. The development of radioligands that can be imaged by positron emission tomography (PET) could allow a better understanding of these mechanisms both in the animal models and in patients. The confirmation of the present findings, by applying PET analysis to generalized seizures, could lead to further experiments aimed at establishing whether antiepileptic drug pretreatment (i.e., before the appearance of SW discharges) can influence the interplay between GABAergic and glutamatergic control systems. When proved to be of pathophysiological importance in patients, $GABA_B$ and NMDA receptors could be rationally targeted by the respective specific drugs.

See also: **Absence Seizures**: Genetic Models of Absence Epilepsy: New Concepts and Insights; Increased $GABA_A$ Receptor-Mediated Transmission in Typical Absence Seizures; Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; **GABA**: $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; **Networks**: Interactions between Cortico-Thalamo-Cortical and Limbic Seizures; Network Interactions in the Limbic System and Epileptiform Synchronization; **Sleep**: Seizure Initiation: The Transition from Sleep-Related Oscillations to Epileptiform Discharge.

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AGING

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Effects of Aging on Seizures and Epilepsy
Epilepsy and Seizure Susceptibility in the Aging Brain
Poststroke Epilepsy and the Aged Brain

Effects of Aging on Seizures and Epilepsy

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Introduction

The prevalence and incidence of epilepsy increase significantly in the elderly (people older than 60 years). In addition, the aging population has a significantly higher chance of developing status epilepticus. These, and other related factors, may be expressed in two ways: (1) The expression of existing seizures and epilepsy (both inherited and acquired) may change as a function of the changes associated with aging; and (2) Seizures and epilepsy may occur de novo, sometimes as a result of conditions associated with aging such as stroke, brain trauma, tumors, and neurodegenerative disorders. Since aging is not a condition of the CNS alone, aging-associated changes in other organ systems (such as in liver metabolic capacity and renal clearance) may have important effects on the way a patient's antiepileptic medication is metabolized or cleared, thus affecting seizure expression.

The population of elderly persons worldwide, especially in developed countries, is increasing at a high rate. Trends predict that by 2050, the population over 60 years of age will comprise more than 30% in many countries (Figure 1). This increase calls for new and innovative diagnostic and treatment options for elderly patients with epilepsy. Increased needs in patient care should also be reflected in enhanced research in this area. Yet, there remains an alarming gap between the increasing numbers of elderly people with either new or modified seizures and basic research in the field. A recent study suggested that between 1965 and 2003, there were only 30 scientific articles investigating the condition of seizures in the aging brain. From 2003 through 2006, a few additional studies pushed up the number of papers up to about 50. Finally, in 2007, a volume of International Review of Neurobiology appeared that was devoted to this topic. Even with this modest surge in research, a critical mass of knowledge of basic mechanisms leading to significant improvements in seizure diagnosis and treatment in elderly patients has not been achieved. The need is pressing.

Seizures in the elderly are further confounded by the fact that age cannot be simply used as a covariant or grouping variable. Physical conditions of elderly people may be very different even within the same age group. A 60-year-old person with poor health and physical condition may be at a higher risk of seizures than an 80-year-old person in excellent health. Thus, it seems that, as in the case of the early postnatal development, aging studies need to be carried out in several distinct age groups of experimental animals to fully appreciate different conditions associated with aging in humans with seizures. Models of seizures in aged animals should also include some of the underlying conditions frequently occurring in the elderly. That is, the models should include stroke or brain injury within the context of an aging brain, to better mimic the human situation.

Background

Studies on animal models performed so far have investigated, both in vivo and in vitro, mechanisms of seizures and epilepsy in aged rats with normal as well as impaired brain. Unfortunately, some of these studies have sometimes generated inconsistent findings.

To make studies on rodent models relevant to human aging, it is important to define 'aged' in rats. With a life expectancy between 750–850 days, rats beyond 600 days of age can be considered aged. In studies using the kainic acid model to induce seizures in adult and aged rats, there were no differences in peak severity of status epilepticus. However, the progression through seizure stages was

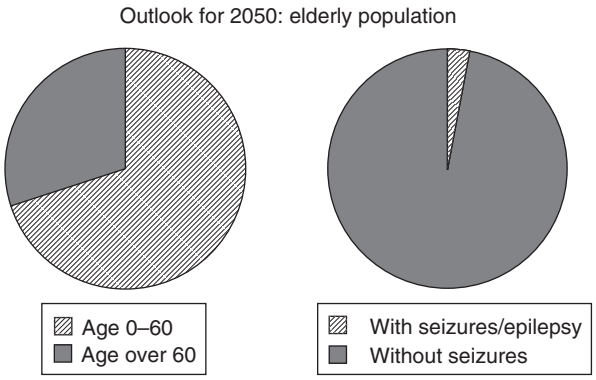


Figure 1 Projected proportion of the elderly population in 2050, along with the projected number with seizures and epilepsy. The proportion of persons 60 years old and older is estimated to exceed 30% of the world's population by the year 2050 (left). The average incidence of seizures and epilepsy ranges from 1.5% in 60 year olds to 4.5% in 80 year olds. Thus, the average incidence can be estimated at 3% of the population older than 60 years (right). As the world's population will reach 9.5 billion by 2050, there will be about 85 million elderly people with seizures worldwide, representing a major diagnostic and treatment challenge.

different; aged rats reached each seizure stage earlier than adult rats, suggesting an increased susceptibility in the aged brain. In contrast, in the hippocampal kindling model, aged rats kindled more slowly (i.e., required more kindling stimulations to reach the criterion of five generalized stage 5 seizures) than adult rats. Despite this slower kindling rate, aged rats displayed longer afterdischarges and faster propagation of epileptiform activity to the contralateral hemisphere.

An in vitro model focused on the features of the hippocampal circuitry to account for age-related differences in susceptibility to kainic acid (KA)-induced seizures. In hippocampal slices, the frequency of inhibitory postsynaptic potentials in granule cells is significantly decreased in aged rats than in adult rats. Further, perforant path stimulation at 5 Hz for 10 s produces multiple population spikes in granule cells as a response to a single stimulus in about 40% of aged rats but in none of adult rats, indicating an impairment of dentate gyrus filtering as a function of age.

Some studies focused on the effects of stroke (using the model of middle cerebral artery occlusion: MCAO) on the development of seizures with aging. One year after after MCAO, there was no evidence of behavioral or EEG seizure activity in the region of the occlusion. More recent studies have employed photothrombotic brain vessel occlusion, with Rose Bengal stain and transcranial illumination of a relatively restricted area of the neocortex. Spontaneous seizures in rats subjected to this injury developed only in few cases (7/36) over a period of 10 months after the insult. However, other studies have reported that 35–100% animals exhibit seizures 4–10 months after cortical photothrombosis, with seizures occurring up to several times per day.

An in vitro model system of stroke-induced epilepsy used glutamate-induced injury in hippocampal neuronal cultures. Neurons that survived yet incurred damage (a correlate of penumbra neurons in stroke) developed epileptiform discharges and network oscillations that persisted throughout their life in culture. This model has potential for studying the mechanisms associated with stroke-induced epilepsy, since the culture model is easily accessible for electrophysiologic, biochemical, and imaging studies.

Methodology

Both mice and rats are used in seizure models. Mice are more commonly used to study genetic modifications that alter seizure susceptibility, while rats are more appropriate for acute seizure models. However, none of the models described in the following sections have been explored and utilized systematically in aged animals.

Mice

One of the convenient mouse models for studies of seizures and epilepsy in aging may be the senescence-accelerated mouse (SAMP8). This mouse is often used in geriatric research, and information gleaned from such studies might aid investigations of epilepsy during aging. Another mouse strain potentially useful for aging studies is the metallothionein-III (zinc-binding protein) knockout mouse. Of course, aged common mouse strains (such as BDF1 mice and C57BL/6J) should also be investigated in epilepsy research.

Rats

Aged (i.e., >600 days old) Sprague-Dawley, Long-Evans, and Fisher 344 rats are more sensitive to KA-induced seizures than adult rats. These findings are consistent with the human condition of increased seizure susceptibility with aging. Other convulsants, such as pentylenetetrazole and strychnine, have also been used to study seizure susceptibility in aged rats, and invariably indicate that aged rats develop seizures at lower thresholds than younger adult animals.

KA-Induced Seizures

Seizures are elicited usually using systemic injection of aqueous solution of KA (with pH adjustment), a neurotoxic analog of glutamate with preferential effects on the limbic system. To elicit clonic seizures without loss of righting (sometimes termed 'limbic seizures') that evolve

into status epilepticus, a bolus dose of KA is administered intraperitoneally. In adult rats, the effective dose is between 10–20 mg kg^{-1}, while mice usually require a higher dose (between 20–40 mg kg^{-1}). An alternative approach is to infuse graded doses of KA into the tail vein, usually 2.5–5 mg kg^{-1} in rats every 5–20 min until seizures occur.

Pentylenetetrazole (PTZ)-Induced Seizures

Seizures are elicited most commonly by a bolus dose of subcutaneous aqueous solution of 50–100 mg kg^{-1} in both rats and mice. This dose results in blockade of the $GABA_A$ receptor via the TBPS (*t*-butylbicyclophosphorothionate) site in the chloride channel. If the dose used is at the lower end of the range, PTZ induces bilateral clonic (myoclonic) seizures of face and forelimbs without loss of righting. With higher doses, these seizures progress to tonic-clonic seizures of all four limbs with loss of righting. As in the case with KA, graded PTZ doses can be delivered intravenously, allowing the determination of seizure threshold.

Strychnine-Induced Seizures

Seizures are elicited by injection of strychnine solution (aqueous; 1 mg ml^{-1}), usually intraperironeally, at a dose range of 1–4 mg kg^{-1} in both rats and mice. Strychnine induces tonic-clonic convulsions with loss of righting and prominent hyperextension, including the tail ('Straub-tail').

Kindling

Kindling is a process by which subconvulsant electrical stimuli, delivered at intervals (usually daily) to various brain structures (e.g., olfactory bulb, amygdala, perforant path), eventually cause local seizure activity (afterdischarge) that can progress to limbic seizures and finally to generalized seizures. The classic protocol for kindling uses electrical stimuli at 50–60 Hz for 1–2 s delivered via electrodes in the amygdala or hippocampus, with 24-h interstimulus intervals. The stimulation that evokes a kindled seizure will cause seizures even weeks or months later.

Results

Data from experiments performed on aged animals provide information about the seizure propensity of the aged brain. Studies carried out so far have been consistent with observations made on the human population, attesting to the increased incidence and prevalence of seizures and epilepsy as a function of age. These studies will also provide information about the conditions leading to these age-related changes in seizure susceptibility.

Mice

The SAMP8 mouse displays increased susceptibility to KA seizure-induced damage compared with the animals of the same age without the 'aging mutation,' but few studies have used this model to study seizures and epilepsy in aging. Similarly, aged metallothionein-III knockout mice are more susceptible to KA seizure-induced damage than the wild-type age-matched controls. Finally, within common mouse strains (BDF1 and C57BL/6J), aged animals display increased susceptibility to PTZ- and KA-induced seizures.

Rats

In aged Long-Evans rats, administration of KA elicits increased release of aspartate, glutamate, and norepinphrine compared to adult controls. This enhanced neurotransmitter release in response to KA might underlie the increased seizure susceptibility seen in aged subjects (compared adult control rats). Other differences seen in the aged rats' response to KA include a delayed c-fos expression and altered regulation of microtubule associated protein and axonal-growth associated protein. However, more research is necessary to determine whether these changes are a consequence of seizures themselves. Future studies should concentrate not simply on whether the aged brain is more susceptible to seizures (we already know this to be the case), but why the aged brain is more seizure susceptible. Such information might improve treatment options for elderly patients.

Future Goals

Several goals should be addressed promptly in research on aging and epilepsy. First, we need to collect significantly more descriptive information about seizures in aged animals, to correlate with human data. Second, experiments need to move beyond description into mechanism. Only at that point will we be able to identify potential therapeutic interventions that might target age-related modifications of brain structure and function. In that regard, the efficacy of antiepileptic drugs needs to be studied in parallel with the earlier-mentiond experiments, always keeping in mind the need for appropriate adult controls.

There are some big challenges to achieving these goals. Research on aged animals is expensive because of the steep costs of purchasing aged animals or maintaining adult rats into senescence. The distinction between causation and association must be considered carefully in experiments on epileptogenesis during aging. Investigators must keep in mind the difference between reactive

seizures (possibly with lower seizure threshold as a function of various age-related neural changes) versus the development of the epileptic state. Data must be interpreted in the context of ongoing age-related brain changes as well as aging in other organ systems. For example, is it possible that the higher susceptibility to KA-seizures in aged rats partially reflects age-related liver metabolic changes?

Despite those challenges, there is an enormous opportunity to explore and define age-related changes in seizure susceptibility and epileptogenesis, as this field has been ignored (with a few notable exceptions) in experimental laboratories. With the burgeoning population of elderly citizens, the need to understand and develop new treatment modalities is urgent.

See also: **Aging**: Effects of Aging on Seizures and Epilepsy; Epilepsy and Seizure Susceptibility in the Aging Brain; Poststroke Epilepsy and the Aged Brain.

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Epilepsy and Seizure Susceptibility in the Aging Brain

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Introduction

Epidemiological studies reveal that the elderly (individuals $\geq$ 65 years of age) exhibit the highest incidence/prevalence for seizure disorders, with estimates suggesting that 4–5% of the geriatric population is affected compared to 1% of the total population. Seizure disorders are the third most common neurological disorder in the elderly and a major factor in aging-related mortality and morbidity. Moreover, the incidence/prevalence of geriatric epilepsy is likely to be underestimated; differences in the behavioral manifestations and electrographic characteristics of seizures observed in the elderly, compared to those seen in the younger adult population, can complicate diagnosis, and epileptic seizures can be misdiagnosed as several other common age-related conditions if EEG-video monitoring is not employed. While available antiepileptic drugs (AEDs) are generally effective in the elderly age group, complications frequently arise due to increased drug sensitivity, altered pharmacokinetics, complex drug interactions due to multidrug regimes, and a decreased reliability of the elderly to accurately self-report symptoms. Taken together, these facts are alarming since demographic data indicate that the elderly are the most rapid growing segment of the population. In response to these data, the Epilepsy Foundation released a position paper in 1996 detailing this impending public health crisis.

To elucidate the mechanisms that contribute to aging-related epileptogenicity, it is first important to assess whether specific forms of epilepsy are observed preferentially in the elderly (since different forms of epilepsy entail different neural networks), and whether specific antecedents precede the onset of these seizures. Data suggest that

seizures (possibly with lower seizure threshold as a function of various age-related neural changes) versus the development of the epileptic state. Data must be interpreted in the context of ongoing age-related brain changes as well as aging in other organ systems. For example, is it possible that the higher susceptibility to KA-seizures in aged rats partially reflects age-related liver metabolic changes?

Despite those challenges, there is an enormous opportunity to explore and define age-related changes in seizure susceptibility and epileptogenesis, as this field has been ignored (with a few notable exceptions) in experimental laboratories. With the burgeoning population of elderly citizens, the need to understand and develop new treatment modalities is urgent.

See also: **Aging**: Effects of Aging on Seizures and Epilepsy; Epilepsy and Seizure Susceptibility in the Aging Brain; Poststroke Epilepsy and the Aged Brain.

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To elucidate the mechanisms that contribute to aging-related epileptogenicity, it is first important to assess whether specific forms of epilepsy are observed preferentially in the elderly (since different forms of epilepsy entail different neural networks), and whether specific antecedents precede the onset of these seizures. Data suggest that

complex–partial seizures are most commonly seen in the elderly. Complex–partial seizures generally involve temporal lobe onset (e.g., hippocampal formation). As for etiology, a number of common aging-related complications, including cerebrovascular insult, metabolic disturbance, and Alzheimer's disease, have been identified as precipitating antecedents; these epilepsy cases are referred to as symptomatic. However, in more than 40% of cases, no proconvulsive antecedent can be identified. These cases are referred to as idiopathic/cryptogenic geriatric epilepsy, and have led to the hypothesis that aging of the central nervous system (CNS) itself may be epileptogenic.

Based on these clinical findings, our laboratory has begun to test the hypothesis that anatomical and electrophysiological changes occur in the CNS during aging that can directly increase seizure susceptibility. We have chosen the hippocampal formation as our model system, given the predominance of complex–partial seizures in the elderly. We postulate that these aging-related changes occur normally, but are benign in the majority of individuals. However, if a secondary insult (e.g., strokes and ischemia, pathological aging) occurs, it can allow these usually benign aging-related changes to manifest more robustly, resulting in abnormal neuronal synchronization (i.e., a seizure). Further, in some subjects these aging-related changes may be more severe, and thus could subsequently contribute to spontaneous seizure generation. This later scenario could contribute to the aging-related increase in idiopathic/cryptogenic epilepsy. There are many other questions regarding geriatric epilepsy that are currently unanswered and are clinically very important; for example, why do seizure characteristics (behavior and EEG activity) change with age?

Background

The rationale for our central hypothesis is based on data from numerous laboratories that have studied the neurobiology of aging. These studies revealed that a myriad of physiological factors that can affect neuronal synchronization and excitability are altered with age. For example, changes have been reported in: (1) dye coupling (i.e., an anatomical change that could represent an increase in electrotonic coupling), (2) extracellular volume, (3) synaptic connectivity, (4) receptor and channel properties, (5) the number and/or functionality of specific types of neurons, (6) potassium and calcium buffering, and (7) the efficacy of neuromodulatory systems. Many of these changes appear to be proconvulsive in nature (e.g., decreased extracellular volume, decrease in the number of glutamic acid decarboxylase immunoreactive neurons), while others appear to be antiepileptogenic (e.g., increased afterhyperpolarization, decreased NMDA receptor-mediated efficacy/activity). However, minimal research has focused on determining the net effect of all these aging-related changes on seizure susceptibility, or how each specific aging-related change affects neuronal synchronization and excitability.

To date, in vivo and in vitro studies examining age-related susceptibilities to epileptiform activity have described apparently contradictory findings (**Table 1**). For example, while repetitive afferent stimulation in vivo (kindling) leads to prolonged afterdischarges in aged animals, the rate of kindling is retarded. Further, while aged animals are more susceptible to systemic kainate treatment, they are more resistant to nicotine-induced seizures. These examples illustrate the need for caution when interpreting data, since the various manipulations used to induce epileptiform activity can activate different neuronal networks, resulting in different types

Table 1 The effect of aging on convulsant induced epileptiform activity

Proconvulsive stimulus	*Affect of Age*
In vivo	
Electroshock	Increased threshold for minimal and maximal evoked seizures in BDF1 mice
Electrical stimulation/ kindling	Discharges are of longer duration; propagation to contralateral side is increased; however rate of kindling is slower
Kainic acid	Decreased threshold; preseizure behavioral manifestations are exacerbated; shorter latency to onset for all classes of seizures/ status
Nicotine	Delayed onset; seizures are abbreviated
Pilocarpine	Decreased threshold
Pentylenetetrazol (PTZ)	Threshold in mice is increased; in rats, a higher incidence and longer duration of generalized convulsions can occur; whether seizure onset differs in rats is controversial
Strychnine	Increased sensitivity and increased mortality
Aminooxyacetic acid (AOAA)	Decreased responsiveness; threshold for convulsions is higher
In vitro	
Zero-Mg^{2+}	Spread and conduction velocity of epileptiform activity is decreased
4-aminopyridine; 4-AP	Spread and conduction velocity of epileptiform activity is decreased
Kainate	Reduced threshold and longer duration events
Repetitive stimulation	Increased susceptibility for generating epileptiform activity in hippocampal slices
Bicuculline	Increased susceptibility for paroxysmal depolarizing shifts in dentate granule cells

of seizures (e.g., complex–partial versus generalized) that involve different mechanisms. In this regard, aging does not affect all types of seizures comparably. For example, while complex–partial seizures are observed commonly in the aging clinical population, generalized seizures are uncommon. Additionally, the underlying mechanism(s) whereby a specific treatment elicits seizure activity may be altered with age in either a pro- or anticonvulsive fashion. Thus, it is not surprising that the rate of kindling and the rate of propagation of epileptiform activity in vitro (in zero-Mg^{2+} artificial cerebrospinal fluid) are retarded in aging animals, since aging is associated with a decrease in NMDA receptor-mediated activity.

Methods and Results

In Vivo Electrophysiology: Susceptibility to Kainate-Induced Seizures and Status Epilepticus

In our laboratory we chose to focus on how aging affects the susceptibility to kainic acid-induced seizure/status epilepticus, since: (1) aging humans also exhibit increased susceptibility to kainate analogs (i.e., domoic acid) and (2) the hippocampus, which is often associated with complex–partial seizures, is highly susceptible to kainate. Additionally, previous studies report that aged rats (>20 months), compared to adults, were more likely to: (1) generate generalized seizures at lower doses of the convulsant and/or (2) display a shorter latency to the onset of stereotypical behaviors associated with nongeneralized seizure activity. While these findings are consistent with aged rodents exhibiting an enhanced seizure susceptibility, such data are not straightforward since generalized seizures do not directly reflect temporal lobe activity, and the stereotypical behaviors associated with complex–partial limbic seizures (wet-dog shakes, head bobbing, teeth gnashing) are difficult to identify accurately with behavioral monitoring if concurrent EEG recordings are not performed. Consequently, experiments were performed to examine the behavioral and electroencephalographic progression of seizures and status epilepticus in adult and aged Fischer 344 rats, using a modified repetitive-kainate treatment protocol coupled with concurrent video and EEG monitoring. These studies revealed that aged rats, compared to adults, exhibit: (1) a greater propensity for wet-dog shakes, a behavior that requires dentate and limbic activation, (2) a shorter latency to onset for all classes of seizures/status epilepticus, including those associated with limbic activation, and (3) a reduction in the power of higher frequency EEG activities (12.5–35 Hz) during the progression of kainate-induced status. These findings are consistent with conclusions from earlier studies, that aged subjects have an enhanced susceptibility to kainate-induced complex–partial seizures and that the temporal lobe may become more epileptogenic with age. Furthermore, the change in EEG activity could reflect a change in network dynamics associated with seizure onset and susceptibility. In addition to the finding that aging affects the susceptibility to kainate-induced seizures, several groups have recently demonstrated that the consequences of kainate-induced status are also dramatically altered with age. For example, aged rodents exhibit a greater degree of cell loss and oxidative stress following kainate-induced status than adult animals. Additionally, the level of status-induced gene expression (e.g., for voltage gated calcium channel subunits) may also be altered in aging animals.

In Vitro Electrophysiology: Susceptibility to Kainate-Induced Epileptiform Activity

Conclusions that can be drawn from these in vivo studies are limited, however, since the relative contribution of aging-related changes in pharmacokinetics (i.e., altered blood–brain barrier, renal clearance) versus pharmacodynamics (altered CNS network activity) cannot been delineated. Subsequently, additional preliminary in vitro experiments were performed to assess whether the intrinsic capacity of the aged CNS (hippocampus) to generate epileptiform activity is increased. In one series of experiments, we examined whether the concentration of kainate required to elicit spontaneous and evoked epileptiform activity differed in hippocampal slices from adult (6–8 months) versus aged (22–30 months) Fischer 344 rats. Field potential recordings were obtained in stratum pyramidale of CA3 while sequentially increasing kainate concentrations (12.5–100 nM) were applied. Hippocampal region CA3 was examined in this study due to its high susceptibility to kainate-induced epileptiform activity. These experiments revealed that lower kainate concentrations were required for the generation of evoked and spontaneous epileptiform activity in slices from aged animals. Moreover, when the characteristics of the spontaneous epileptiform events were compared between groups (at 50 nM, a kainate concentration that elicited epileptiform activity in an identical percentage of aged and adult slices), aged slices exhibited longer duration discharges. Additionally, preliminary data from power spectral and wavelet analyses revealed that dramatic difference also exists in the power and time course of certain spectral components during an epileptiform discharge (**Figure 1**). These preliminary data suggest that network dynamics may differ between age groups during the generation of epileptiform activity and illustrate the need for more rigorous quantitative analysis. Taken together, these in vitro experiments suggest that aging-related pharmacodynamic changes in the hippocampus contribute to both the increase in susceptibility to kainate-induced seizures in vivo, and (potentially) to the differences in EEG

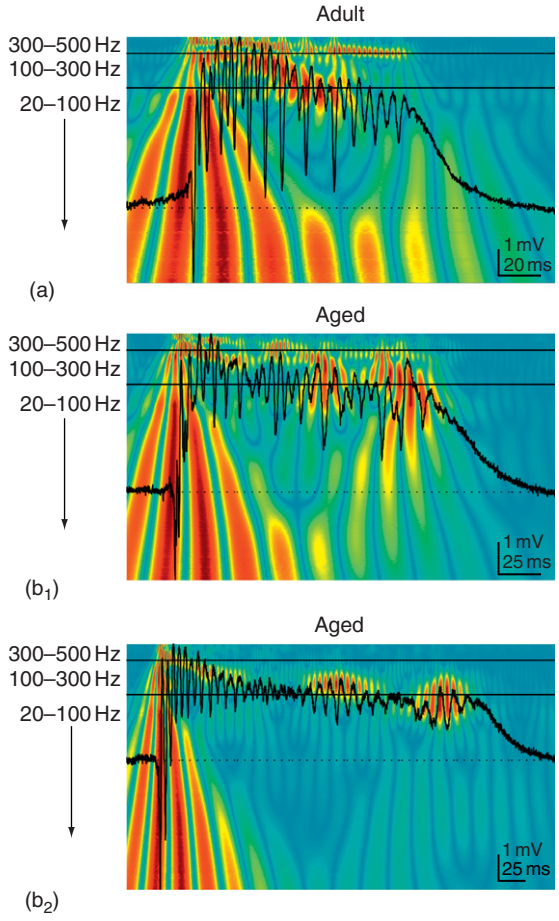


Figure 1 Preliminary time-frequency analysis indicates marked differences in both the time course and composition of the spectral components of epileptiform discharges in a subpopulation of aged slices compared to adults. Shown are representative scalograms from continuous wavelet transforms of individual discharges from adult and aged F344 rats. (a) In the adult, robust high frequency oscillations (HFOs > 200 Hz) are prominent throughout the course of the discharge. (b_1) A subset of aged slices shows a similar profile of HFOs. (b_2) However, in the remaining slices from aged rats, HFO activity is dramatically reduced during an epileptiform discharge.

characteristics. Since kainate activates both glutamatergic and GABA-ergic networks, these results are likely to reflect an aging-related change in the balance of excitatory/inhibitory circuits in the hippocampus, the net effect of which reflects the extent to which these local circuits are activated. Whether aging-related changes in pharmacokinetics (renal clearance and blood–brain barrier permeability) also contribute to the in vivo phenomenon cannot be ruled out and requires further investigation.

In Vitro Electrophysiology: Dentate Gating

In addition to these in vitro kainate studies, we were interested in determining whether more physiological manipulations could also reveal an enhanced susceptibility to epileptiform activity in the aged CNS. For these experiments, hippocampal slices from adult and aged Fischer 344 rats were used. A stimulating electrode was placed in the molecular layer of the dentate gyrus to activate the perforant path and field potential activity was monitored in stratum pyramidale of CA3 in order to assess whether the flow/propagation of neuronal activity through the entorhinal–dentate–CA3 pathway was altered. Several groups have shown that a disruption in the capacity of the dentate gyrus to gate/filter afferent activity impinging onto the hippocampus is associated with converting a normal hippocampus into an epileptic one. In this regard, recent data indicate that the aged dentate appears to be hypometabolic, a condition also observed in patients with temporal lobe epilepsy where dentate gating is thought to be altered.

After an initial characterization of this pathway with single stimuli, a 5-Hz stimulus train (10-s duration) was delivered to the molecular layer with evoked activity monitored during and after the train. As shown in **Figure 2**, slices from adult rodents and the majority of the slices from aged rats exhibited minimal change in field potential activity during the train; only small amplitude (<1 mV) secondary and/or tertiary population spikes were occasionally seen. In contrast, in 30% of the aged slices, prolonged field postsynaptic potentials were generated that were superimposed with significant population spike activity (population spikes >1 mV). These events resembled interictal-like epileptiform activity (**Figure 2**). Moreover, when spontaneous activity was monitored in slices following the train, nearly all of these hyperexcitable aged slices generated interictal-like discharge (**Figure 2**); either no spontaneous activity or only small amplitude positive field potential shifts (that were typically devoid of superimposed activity) were observed in slices from adult animals and the remaining aged animals. Thus, it appears that the propagation of information through the dentate gyrus is altered in a proconvulsive manner during aging in some animals, and can result in the generation of epileptiform activity in hippocampal region CA3.

This finding is fascinating since the percentage of aged rats that exhibited epileptiform activity following molecular layer stimulation is similar to that of aged animals with cognitive decline. It is plausible that a decline in dentate gating function can result in the generation of epileptiform activity in CA3 of aged subjects. Since the entorhinal-dentate-CA3 pathway is involved with the encoding of afferent input, such aberrant activity could potentially interrupt the processing of novel information. In this regard, it is interesting to note that place cell recordings in rodents report a preferential increase in basal activity in CA3 of aged animals with cognitive decline, as compared to adult and aged cognitively intact animals. It is possible that the aberrant activity we observe in hippocampal slices from a subpopulation of aged rats could be an in vitro correlate of this

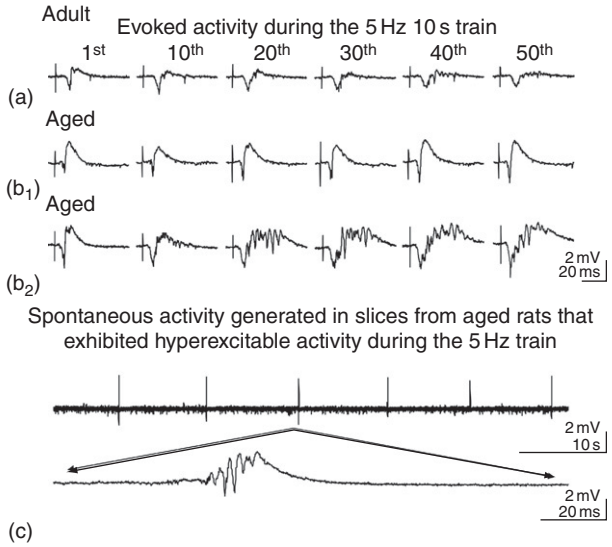


Figure 2 In a percentage of aged slices, the capacity of the dentate to filter afferent input is altered. (a) In slices from adult Fischer 344 rats, repetitive stimulation in the molecular layer of the dentate elicited minimal change in the field potential response of CA3. (b_1) The majority of slices from aged rats exhibited a similar response to 5 Hz stimulation as was observed in adult slices. (b_2) However, in approximately 30% of aged slices multiple population spikes evolved in CA3 during the train. (c) Further, nearly all of the aged slices that exhibited hyperexcitable activity during the 5-Hz train developed spontaneous interictal like events.

in vivo phenomenon. Experiments are currently underway in our laboratory to assess this possibility and determine the relative contribution of aging-related changes in the dentate gyrus and CA3 to this decline in dentate gating during aging.

In Vitro Electrophysiology: Intrinsic and Synaptic Properties of Dentate Granule Cells

To examine whether aging-related changes in dentate physiology contribute to the breakdown in dentate function, experiments were performed using field potential and whole cell patch recordings in slices from adult and aged Fischer 344 rats. Field potential recordings revealed comparable responses to single and paired lateral perforant path stimuli (10–100 ms interpulse interval) in both age groups. In contrast, repetitive stimulation at 5 Hz for 10 s revealed a dramatic aging-related difference. Specifically, while the slices from adult animals and the majority of the slices from aged animals (60%) exhibited a suppression of population spike amplitude during the train, the remaining 40% of the slices from aged animals generated hyperexcitable activity consisting of multiple population spikes. This response pattern was seen in almost the same percentage of aged animals that exhibited epileptiform activity in CA3 during and after molecular layer stimulation, and thus further supports the contention that dentate gating is compromised with age.

While the properties that underlie the capacity of the dentate to filter afferent input are unclear, several factors are likely to be involved. First, granule cells have a relatively hyperpolarized membrane potential and strong spike frequency adaptation. Second, the dentate gyrus has an extensive inhibitory system and a paucity of recurrent excitatory interconnections. Third, there exists a unique relationship between the mossy fibers (granule cell axons) and the CA3 region; the axons of the granule cells preferentially synapse onto inhibitory (versus excitatory) neurons in CA3. Given these possibilities, we examined whether the intrinsic and/or network properties of the dentate granule cells change with age. In general, the intrinsic membrane properties that we examined were preserved, including resting membrane potential, action potential width, time constant, and input resistance. Further, paired-pulse field potential recordings and whole cell recordings of polysynaptic evoked inhibitory postsynaptic potentials in granule cells suggested that the efficacy of short-loop feedback inhibition – provided by the basket and chandelier interneurons – was not significantly altered. In contrast, a decrease was noted in the frequency of spontaneous inhibitory postsynaptic potentials, which was interpreted to reflect a decrease in the degree of inhibitory input onto the distal dendrites of dentate granule cells. This phenomenon could, in part, explain the change in responsiveness of the aged dentate gyrus to 5 Hz lateral perforant path stimulation. Specifically, the interneurons that project onto the distal dendrites of granule cells are localized in the dentate hilus and become activated by the granule cells following afferent input. In turn, these interneurons release GABA onto the distal dendrites of granule cells; this effect would be expected to decrease input resistance and thereby shunt/suppress subsequent afferent input. If this 'long-loop' feedback inhibitory circuit in the dentate is impaired, repetitive afferent input could summate in granule cells and elicit a hyperexcitable response. In agreement with these electrophysiological findings, anatomical data on the dentate gyrus of aged rats indicate that a subclass of interneurons in the hilus is either decreased in number or exhibits a decline in glutamic acid decarboxylase (GAD) levels which could dramatically affect interneuron function since GAD is the major enzyme involved with GABA synthesis. Further, this affected subclass of interneurons (which appear to include those that contain somatostatin and/or neuropeptide y) can regulate afferent input and have been proposed to play a major role in information processing. This subclass of interneurons has also been reported to be lost and/or decreased in number in temporal lobe epilepsy. Ongoing experiments in our laboratory are focusing on understanding the mechanisms associated with this decrease in inhibitory tone.

In addition to observing aging-related changes in inhibition in the dentate gyrus, our experiments revealed that

the degree of recurrent excitatory interconnections also was enhanced with age. Specifically, following disinhibition (the $GABA_A$ receptor antagonist bicuculline was added to the bathing medium), aged granule cells preferentially exhibited prolonged large amplitude excitatory postsynaptic potentials that resembled epileptogenic paroxysmal depolarizing shifts (PDSs). Although the exact cellular players contributing to this aberrant circuit are unknown, current source density studies indicate that hilar stimulation can elicit an additional inward current flow in the vicinity of the inner molecular-granule cell layer – a location which is consistent with the presence of an additional excitatory input. Further, several studies suggest that mossy fiber distribution can change with age. While additional experiments are required to identify the cellular players that are involved with creating this novel recurrent excitatory activity, these data indicate that the balance of excitation vs. inhibition is disrupted during aging. These changes are likely to contribute to the aging-related decline in dentate gating function and subsequently could result in enhanced seizure susceptibility and potentially a decline in cognitive function.

Future Directions

The experimental data presented in this article support the hypothesis that proconvulsive changes occur normally in the CNS during aging and can contribute to an enhanced susceptibility for epileptiform activity in the hippocampal formation. However, whether such aging-related changes also contribute to the increased incidence of symptomatic geriatric epilepsy is unclear. What data are available on this question come from a thrombosis study, using the photosensitive dye rose Bengal to induce experimental infarcts. These experiments revealed that while all postmature Fischer 344 rats can develop seizures, regardless of age or infarct location, aged animals were more likely to develop seizures following a comparable insult than adults. This scenario could arise if the balance of excitation/inhibition is already altered in aged animals and the ischemic/hypoxic episode further aggravates this balance (e.g., due to a disconnect of excitatory input onto inhibitory interneurons). Additional support for our central hypothesis comes from studies on transgenic murine models of Alzheimer's disease. A number of such models exists that express human amyloid precursor protein with or without other transgenes that express anomalous proteins found in humans with Alzheimer's disease. An increase in seizure susceptibility and/or spontaneous epileptiform activity has been reported in several of these models, and data from at least one model suggest that the increase in seizure susceptibility can occur prior to the development of amyloid plaque deposits (one of the histopathological hallmarks of Alzheimer's disease). If Alzheimer's disease is viewed as 'accelerated aging' as some have proposed, then it is plausible that changes in local neuronal networks arise in these animals at an abnormally early age, and contribute to the change in seizure susceptibility prior to plaque formation. Additional experiments are however required to directly test this possibility. Moreover, as Alzheimer's disease progresses, it is plausible that more detrimental changes arise in local neuronal circuitry which further increases seizure susceptibility.

In future, additional experiments are needed to rigorously test the hypothesis that aging-related changes in local excitatory and inhibitory circuitry underlie the increase in cryptogenic and symptomatic epilepsy in the elderly. These studies can take advantage of the vast array of animal models that exhibit enhanced or decreased life span, as well as models of pathological aging. Further, it will also be critical to examine how aging-related changes in neuronal dye coupling (electrotonic coupling), extracellular volume, ionic buffering, and intrinsic neural properties contribute to the generation of epileptiform activity in models of normal and pathological aging.

See also: **Aging**: Effects of Aging on Seizures and Epilepsy; Poststroke Epilepsy and the Aged Brain.

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Poststroke Epilepsy and the Aged Brain

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Introduction

The prevalence and incidence of unprovoked seizures and epilepsy increase in the elderly, and first occurrence rates for epilepsy in the elderly are higher than for any other age group. Etiologies associated with this increase include cerebrovascular disease, brain tumors, toxic or metabolic disturbances, dementia, and trauma. Of these etiologies, ischemic cerebrovascular disease is the dominant cause of epilepsy in the elderly, accounting for 25–50% of all cases; approximately 15–20% of the elderly who experience a stroke will develop epilepsy. The most important risk factor for developing epilepsy after an ischemic stroke is the involvement of the cerebral cortex. Lacunar, subcortical, and posterior circulation infarcts are rarely associated with the development of epilepsy. In general, poststroke epileptic seizures are considered focal in onset and frequently arise in close proximity to the area of infarcted tissue. To date, numerous clinical studies have detailed the epidemiology of late-onset epilepsy and have underscored the importance of stroke as the primary cause of new-onset epilepsy in the elderly. In contrast, there is little understanding of the pathophysiological mechanisms of aged brain following stroke that cause or predispose to the development of epileptic seizures. The reason for this poor mechanistic insight is clear-the limited development of standard animal models of poststroke epilepsy.

Background

Despite the longstanding and widespread use of arterial occlusion models of cerebral ischemia to study postlesional pathophysiology, neuroprotection, and functional outcomes, these studies typically have not reported evidence of seizures in lesioned animals. This apparent lack of seizure activity may be related to relatively brief periods of observation (from the time of ischemia to animal sacrifice is typically hours to days), and little or inadequate behavioral monitoring (seizures may occur but be nonconvulsive). Middle cerebral artery occlusion (MCAO) is a standard in vivo large artery occlusion model employed by numerous laboratories to cause focal cerebral ischemia and infarction. MCAO is most commonly performed by insertion of a filament in the artery to cause its occlusion (intraluminal filament model). Another technique, injection of endothelin-1 near the MCA, induces spasm of the artery and resultant ischemia. Similar to MCA-distribution strokes in humans, MCAO can produce a large cortical and subcortical infarct ipsilateral to the occlusion, which is characterized by an infarct core and a surrounding volume of partial cellular injury and death (i.e., the ischemic penumbra). In most rat strains, distal MCAO does not generate a consistent size or distribution of infarct because of the animals' overall vascularity. Importantly, the hippocampus of rats can lie within the region of ischemic injury because it is perfused by the posterior cerebral artery, which can be blocked by MCAO; in humans, the hippocampus is supplied by the anterior choroidal and posterior cerebral arteries, which are typically unaffected by occlusion of the MCA. In order to produce infarction of the cerebral cortex that spares subcortical structures and hippocampus, investigators have used a combination of transient unilateral MCA and common carotid artery (CCA) occlusion (MCA/CCAO). This technique in young Long-Evans rats typically results in moderately large cortical infarcts ($\sim$100 mm^3) of reproducible size and location, while sparing injury to the hippocampus. For these reasons, MCA/CCAO may be a preferred technique for large vessel occlusion applied to the study of poststroke epilepsy.

An alternative to large vessel occlusion models of stroke is the model of cortical photothrombosis, which induces cortical infarcts with reproducible area, depth, and location. The photothrombosis model is based on a class of light-sensitive molecules and the reactions they undergo following photic stimulation. One such molecule is rose bengal, a derivative of fluorescein, which can be injected directly into the circulation. Molecules of rose bengal become excited following absorption of photons (peak absorption, 562 nm), typically activated in brain vasculature in the photothrombosis model by an external light beam that is focused on and penetrates the animal's translucent skull and underlying cerebral cortex. This excitation stimulus results in the transition of the molecules from their ground state to a triplet state. Direct reaction of the triplet state molecules with molecular oxygen creates an excited singlet state species of molecular oxygen; singlet oxygen can directly peroxidize certain cell membrane components, such as unsaturated fatty acids and proteins, and likely cause discontinuities (microruptures) in the endothelia of the pial vasculature thereby triggering platelet aggregation at those sites. These defects promote vasogenic edema, which can compress the underlying cortical vasculature and thereby increase

the size and extent of the evolving infarct. The typical infarct is a sharply circumscribed, wedge-shaped necrotic lesion occupying the entire depth of the cortex. This method is well characterized, relatively non-invasive, produces highly reproducible infarcts, and allows for the selective placement of infarcts in well-defined cortical areas. For these reasons, photothrombosis is an attractive alternative to large vessel occlusion techniques as a means to study poststroke epilepsy.

Methods

Photothrombosis

Photothrombosis of young adult and aged rats is performed after the animals are anesthetized with ketamine and xylazine and placed in a stereotaxic frame. A midline scalp incision is made, and the scalp is retracted laterally. Rose bengal (20 mg/kg) is injected through a catheter into the left femoral vein over 2 min, as the brain is stimulated through the intact skull for 10 min by an argon laser-activated light beam. The laser is turned on immediatedly before the rose bengal is injected. The injected volume is based on the weight of the animal (typically about 250 μl); laser time is based on the half-life of the rose bengal ($\sim$ 6 min). The incident beam is $\sim$5 mm wide and focused 1.8 mm posterior to the bregma and 2.8 mm lateral to the midline, corresponding to the area of the left sensorimotor cortex. The laser stimulation protocol generates cortical infarcts of approximately 25 mm^3. The skull is cooled by continuous airflow from a fan to prevent heat-mediated tissue injury. Body temperature is monitored and maintained at 37 °C using a thermoregulated pad. After stimulation, the catheter is removed, and the wound is sutured. Animals receive a subcutaneous injection (5 ml) of lactated Ringer's solution immediately after surgery and each day postoperatively until adequate hydration and nutrition are ensured.

MCA/CCAO

Focal cortical ischemia in young adult and aged rats can be induced by transient or permanent occlusion of the ipsilateral MCA and CCA. For transient MCA/CCAO, after surgical exposure of the MCA, a wire is placed underneath it, the artery is then lifted, and the wire is rotated clockwise; the CCA is occluded using atraumatic aneurysm clips. After 3 hours, reperfusion is established by first removing the aneurysm clips from the CCA, and then rotating the wire counterclockwise and removing it from beneath the MCA. Animals that display reduction of cerebral profusion following ischemia, to less than 15% of the pre-ischemic value, typically demonstrate relatively reproducible cortical infarcts in the distribution of the left MCA. The technique of permanent MCA/CCAO is essentially the same as that of transient MCA/CCAO except that the arteries are ligated with silk sutures followed immediately by surgical closure of the animal. Postoperative supportive measures similar to those used in the photothrombosis model are utilized.

Video-EEG

Three recording screws are placed parasagitally, on each side of the skull, to sample neocortical activities symmetrically. Screw placement are: +2.0 mm from bregma/2.25 mm lateral; −3.0 mm from bregma/275 mm lateral; and −7.0 mm from bregma/2.75 mm lateral. Qualitative video-EEG analysis is performed off-line by a complete visual examination of the EEG recordings, and ictal behaviors are classified by a modification of the Racine scale. EEG interictal activities are distinguished from ictal activities based on waveform morphology and frequency, and the associated absence or presence of behavioral change, respectively.

Recent Results

Photothrombosis

Beginning in the late 1990s, our laboratory sought to determine whether a rodent model of cerebral infarction could result in epileptic seizures; at that time, no model of poststroke epilepsy existed. Because large cortical strokes are most likely to be associated with poststroke epilepsy clinically, we wanted to produce large isolated neocortical infarcts (intentionally not including hippocampus so as to keep the analysis as straightforward as possible) that were relatively easy to generate and reliably reproducible in size and distribution. We chose to explore use of the photothrombosis technique - i.e., photochemically-induced cortical thrombosis and brain infarction, using the photosensitive dye rose bengal. In our initial exploratory study, we used photothrombosis to determine whether animal strain or age, or infarct size or location, were critical variables for the development of epileptic seizures. Fischer 344 (F344) rats at 2, 6, 12, 24, and 30 months of age, and Sprague-Dawley (SD) rats at 2 and 6 months of age underwent photothrombosis creating either large or small focal infarcts overlying left hemisphere anterior frontal, midfrontal, frontoparietal, or parietal areas. The consequences of these lesions were analyzed by video-EEG and morphological techniques. Our main finding was that epileptic seizures were recorded in young adult rats ($<$ 2 months of age) 2 months after lesioning the frontoparietal cortex with large photothrombotic infarcts that extended to the cortical-subcortical interface. These seizures were characterized behaviorally by motor arrest and appeared to originate electrically in the peri-infarct area. Small cortical lesions were essentially ineffective in producing seizures.

Importantly, class 3 convulsive seizures (Racine scale) were observed in a small number of 12- and 24-month old animals lesioned with large infarcts in anterior frontal and frontoparietal areas.

Based on the electrobehavioral findings obtained in our initial study, we designed a parallel study to assess potential long-term changes in cortical $GABA_A$ receptors following photothrombosis. Cell death is largely confined to the volume of the wedge-shaped infarct created by photothrombosis (i.e., there is little cell death beyond the margins of this area). Therefore, we were able to examine the entire hemisphere from both sides (each side separately) for this analysis. Using young adult SD animals, hemispheric cortical $GABA_A$ receptor α_1, β_1, and γ_{2S} subunit mRNA expression was quantified by RT-PCR; these studies indicated that large cortical infarcts resulted in a long-lasting differential expression of the subunit mRNAs in ipsilateral cortex; since some animals became epileptic and others didn't, this mRNA expression in ipsilateral cortex was variably associated with the epileptic state. In a subsequent study designed to assess the early time course of cortical $GABA_AR$ α_1 subunit mRNA and protein expression following photothrombosis, we found that $GABA_AR$ α_1 subunit mRNA levels were decreased ipsilateral and contralateral to the infarct at 7 days, but were increased bilaterally at 30 days, whereas $GABA_AR$ α_1 subunit protein levels were unchanged compared with shams; however, at 30 days, $GABA_AR$ α_1 subunit protein expression in lesioned animals was significantly increased within proximal and distal regions of perilesional cortex compared with distal cortical areas contralaterally. Taken together, these exploratory studies suggested that the photothrombosis model could be used confidently to tailor specific neocortical infarcts to study molecular, cellular, and network alterations, such as short- and long-term changes of $GABA_ARs$, as potential formative mechanisms of poststroke epileptogenesis.

Based on the results of our early studies, we standardized our photothrombosis protocol and characterized the electrobehavioral properties of young adult SD rats during 6-month video-EEG monitoring following lesioning of the sensorimotor cortex. We initially focused on young adult animals in order to validate and standardize the model upon which subsequent aging studies are now performed (studies in progress). With our standardized protocol, 50% of lesioned animals developed focal epileptic seizures ipsilateral to the cortical infarct, characterized by rhythmic spike-wave discharges with or without behavioral change; class 4 seizures were observed in 1 lesioned and 1 naïve control animal as well. Epileptic animals demonstrated increased delta, theta, and low beta range power ipsilateral to the infarct that reliably distinguished them from lesioned nonepileptic and control animals. In a follow-up study, we used neuropeptide Y (NPY) protein expression to assess anatomical changes in the brains of both epileptic and nonepileptic animals after the 6-month monitoring period, and found that lesioned epileptic animals with frequent seizure activity demonstrated significant increases of NPY protein expression in the cortex, CA1, CA3, hilar interneurons, and in granule cells of the dentate gyrus. Additionally, activated astroglia were detected in the cortex and hippocampus of animals exhibiting seizure activity. In general, NPY protein expression and morphological changes after cortical photothrombosis were region- and pathological state-dependent, indicating that significant anatomical changes extended beyond the lesioned cortex in epileptic animals. These observations confirm at least histological evidence of hippocampal involvement; preliminary EEG monitoring with depth electrodes suggest that seizure activity (e.g., class 2 and 3 seizures) is of limbic origin.

In a recent study by a different group, late seizures were assessed in adult Sprague-Dawley rats following photothrombosis. Video-EEG recordings were obtained around-the-clock for 7 to 14 days, at 2, 4, 6, 8, and 10 months post-lesioning. They recorded convulsive seizures in 18% of lesioned animals, with an average seizure frequency of 0.39 seizures/day and a mean seizure duration of 117 seconds. Latencies to the first recorded seizure ranged from 71 to 297 days. Histological analysis of lesioned brains revealed that infarct depths varied from cortical layer I to VI in epileptic animals; epileptic animals demonstrated light mossy fiber sprouting in the inner molecular layer of the dentate gyrus, both ipsilateral and contralateral to the lesion. These findings provided clear evidence of convulsive seizure activity that developed over long periods of time following cortical photothrombosis and were associated with anatomical changes in the hippocampus, similar to changes observed in models of status-epilepticus-induced limbic epilepsy. These results have substantially extended the findings from previous studies of the photothrombosis model and provide clear justification for continued use of the model to explore mechanisms of poststroke epileptogenesis. Because of the aging-related findings obtained in our initial study using the photothrombosis model, ongoing studies have been designed to use young adult and aged F344 rats to determine whether age is a critical or contributing factor in generating epileptogenesis after lesioning. **Figure 1** demonstrates use of an argon-activated laser beam to generate a cortical infarct in an aged animal.

Arterial Occlusion Models

Recent studies using young SD rats and video-EEG recordings have shown that multiple ipsilateral or generalized electrographic seizures (provoked seizures from the acute effects of lesioning) could be recorded within the first 2 hours following transient intraluminal filament-induced MCAO without change in the animal's behavior. In separate studies, using similar techniques adapted to long-term recordings, lesioned young animals

were monitored up to 1 year post-lesioning; these animals did not show evidence of either EEG or behavioral seizures. However, a subsequent study using endothelin-1-induced MCAO demonstrated epileptic seizures in 1 of 26 lesioned animals, associated with an infarct of the ipsilateral basal ganglia. To date, no study using the MCAO model has demonstrated evidence of reproducible poststroke epilepsy.

Over the last several years, our laboratory has sought to develop an arterial occlusion model of poststroke epilepsy.

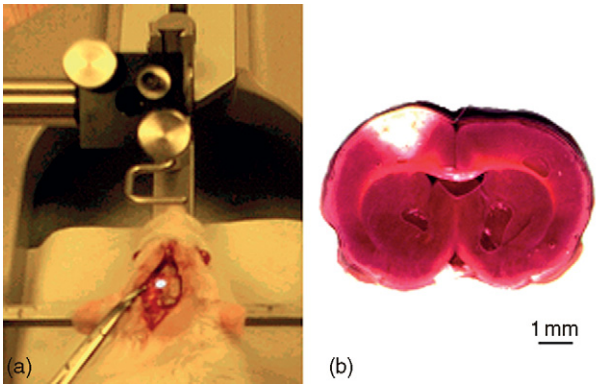


Figure 1 Photothrombosis. (a) Placement of an argon laser beam on the exposed skull of a rat overlying the left sensorimotor cortex. (b) TTC-stained coronal slice of brain performed 24 hr after lesioning of a 20 mo old animal. From Kelly (2006).

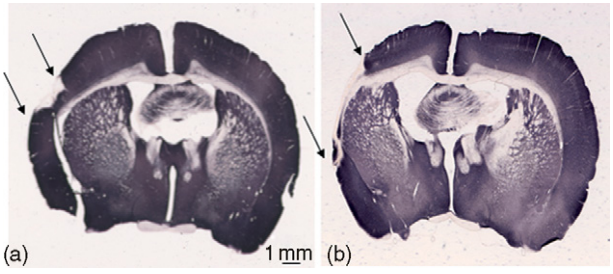


Figure 2 MCA/CCAO. MAP-2 immunostained coronal sections of a 4-month old (a) and a 20-month old (b) animal 2 months after lesioning. Arrows demarcate a smaller infarct in (a) compared with (b). From Kelly (2006).

Our efforts were guided, in part, by the results of previous studies using MCAO. Our initial study consisted of MCA/CCAO and sham-operation in young Long-Evans rats, followed by intermittent video-EEG monitoring for 6 months. Disappointingly, these studies failed to demonstrate poststroke epilepsy in any animal. Because of this result, and similar to our ongoing studies using the photothrombosis model, current studies in our laboratory using the MCA/CCAO model have focused on a comparison among 4-, 12-, and 20-month old F344 rats to determine whether age is a critical or contributing factor in generating poststroke epilepsy. Figure 2 illustrates cortical infarcts created by the MCA/CCAO technique; infarct morphometry indicates a trend to larger infarct volumes in aged animals compared with young adult animals. Figure 3 demonstrates a class 5 seizure in an aged animal followed by sudden death (probably caused by cardiorespiratory failure during the prolonged seizure.

Future Goals

Animal models of cerebral ischemia have had relatively limited development for the study of epileptogenesis and the epileptic state. Goals and challenges for the future broadly include: 1) elucidation of the elements of available experimental models that are necessary and sufficient for them to work reliably for the study of poststroke epilepsy; 2) development of new and improved animal models; and 3) bringing new investigators into the field of study. Basic methodological issues that require exploration and refinement for an improved understanding of poststroke epileptogenesis include the role of: 1) critical brain areas for ischemia/infarction; 2) animal age; 3) MCAO with or without CCAO, and transient vs. permanent occlusion; and 4) different types of recording techniques (e.g., depth, epidural, or skull electrodes, and cabled vs. telemetry systems). Advances in these areas will enable and inform more focused studies to determine: 1) the principal elements of lesion-induced network changes; 2) how excitatory and inhibitory neurotransmission changes during epileptogenesis; 3) the functional

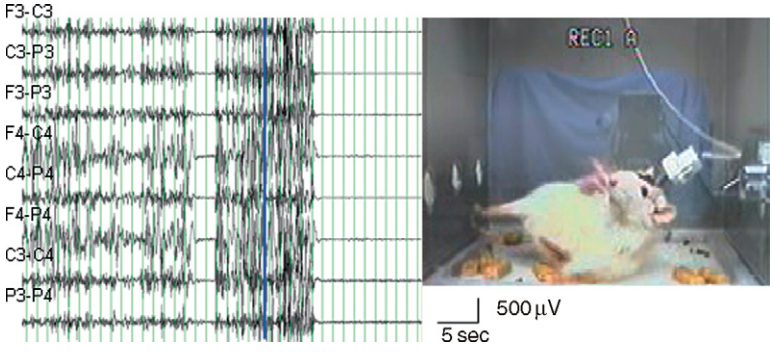


Figure 3 MCA/CCAO. EEG record representing continuous convulsive activity (Racine class 5) prior to the sudden death (flatline EEG) of a 20-month old animal 1 month after lesioning. From Kelly (2006).

anatomy of seizure initiation and propagation; and 4) the electrobehavioral properties of a potentially dynamically evolving epileptic state.

See also: **Aging**: Effects of Aging on Seizures and Epilepsy; Epilepsy and Seizure Susceptibility in the Aging Brain; **Inflammation**: Cerebrovascular Diseases, Seizures and Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders.

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ANTIEPILEPTIC DRUGS

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Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models
Preclinical Drug Development in Epilepsy

Anticonvulsant Drugs as Neuronal Network-Modifying Agents

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Introduction

Despite decades of research, it is still not really understood how anticonvulsant drugs act to control epilepsy. The action of anticonvulsants on neurons observed in vitro likely contributes to the ability of anticonvulsants to prevent seizures. However, exactly how to translate these findings from the dish (in vitro) to the intact brain is problematic. One reason for this quandary is that the mammalian brain contains billions of neurons, and a true

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understanding of the complexity of drug action requires studying the intact brain. It is now agreed that neuronal networks play important roles in seizures, including those seizures that were considered to be strictly localized (focal) in nature. Neuronal networks in the intact brain have the capability to exhibit 'additional' properties that arise from neuronal interactions between nuclei within the network. These 'new' properties that arise from the intact network are called 'emergent properties,' and recent findings indicate that anticonvulsant drugs can act selectively on these properties to exert their therapeutic effects. Therefore, CNS drugs – including those agents that are currently classified as anticonvulsants – are well described as '*neuronal network-modifying agents*.' A major reason for proposing such a new drug classification is that many anticonvulsants are also effective in chronic pain disorders and certain psychiatric disorders, such as anxiety and mania, as well as epilepsy. Each of these disorders involves neuronal networks in the brain.

Background

Approaches to Understanding CNS Drug Mechanisms

Early in the development of CNS drugs, investigators realized that most agents exert therapeutic effects by selective actions on neurons in a specific brain site rather than by acting globally on all neurons. An example of this neuropharmacology paradigm is the theory that sedative-hypnotic drugs produce sleep by selective actions on the brainstem reticular formation, a wakefulness center. This theory has not been disproved. However, it was superseded by a new paradigm, based on the fact that most CNS drugs affect neurotransmitter receptors or ion channels, which consist of several distinct subunits. Receptors and channels consist of different subunit combinations that are distributed differentially in different brain nuclei. The current neuropharmacology paradigm indicates that drugs interact most strongly with specific subunit combinations and exert their effects on the brain regions that contain a high concentration of neurons expressing that specific subunit combination. However, many of the reported selective subunit effects of drugs have been hard to confirm, and the therapeutic relevance of subunit selectivity remains to be established. An example is the anticonvulsant drug, gabapentin, which selectively affects a specific subunit combination in Ca^{2+} channels (P/Q type) in spinal cord neurons in vitro. This selective targeting has not been consistently confirmed, however, and no effect was observed on the same type of Ca^{2+} channel in hippocampal neurons in vitro. One possible reason for these inconsistencies is that neurological and psychiatric diseases are mediated by neuronal networks rather than single CNS neurons. Networks of neurons have additional emergent properties at which drugs can potentially act – properties that may not be present on the same neurons in vitro.

Network Perspective on Brain Function

The complex functions of the CNS involve distributed neuronal networks. For example, a network has been identified that mediates normal locomotion. New neuronal network connections can be formed to support increased brain function, as seen in learning. Many brain malfunctions involve disrupted neuronal network functions, as in Parkinson's disease. Other brain malfunctions are due to the formation of abnormal neuronal networks, for example mossy-fiber sprouting that occurs in some forms of epilepsy.

Neuroanatomy provides knowledge about the connections between brain neurons, but a neuronal network differs from the neuroanatomy of a pathway. Functional studies indicate that many anatomical connections, when activated physiologically, only result in subthreshold responses. These subthreshold responses can reach threshold under pathophysiological conditions, resulting in the creation of a specific disease network. Such findings have led to the idea that CNS diseases are largely governed by what could be termed the 'Law of Conservation of Networks.' That is, pathophysiology of CNS diseases may involve abnormal interactions between existing networks. For example, audiogenic seizures (AGS), as described later, involve the normal auditory network interacting abnormally with the normal network for locomotion to produce the motor (convulsive) seizure. Thus, the convulsion is in response to over-stimulation of the auditory pathway, which extensively activates the locomotor network that is only minimally activated under normal conditions (such as the acoustic startle response). Many mechanisms are known to control network function (**Table 1**).

Networks in Epilepsy

As noted earlier, all forms of epilepsy involve a distributed epileptic network rather than abnormal neurons in one brain location. This view is based on studies of small networks in vitro, animal models of temporal lobe epilepsy, human intracranial EEG, and human and animal brain imaging. Imaging is one of the currently most useful approaches to networks. However, the minimum time scale that is achievable with imaging is too great to observe brain changes that occur during a seizure, which is itself relatively brief. Imaging does not indicate the nature (inhibition or excitation) of neuronal firing changes, which are the critical events underlying elaboration of the seizure. Although imaging is very useful to indicate brain changes associated with neurological or psychiatric disease, imaging data do not tell us if the

Table 1 Mechanisms controlling network function

Gap junctions
Burst firing
Neuroactive substances
a. excitatory *or* inhibitory
b. endogenous *or* exogenous
c. synaptic *or* volume transmission
d. tonic *or* phasic
Multiplicity of synaptic inputs
Extracellular ions and gases (O_2)
Temperature
Interneuron activity
Astrocytic integration
External stimuli
Development and age
Synaptic plasticity
a. strengthening
b. weakening
c. synapse remodeling
d. synaptogenesis
Neurogenesis

observed changes are essential, ancillary, or compensatory to the disease process, nor can it be determined if the observed changes are excitatory or inhibitory. Direct observation of neuronal firing changes, recorded with extracellular microelectrodes in behaving animals, that occur before and during the seizure provides a millisecond time scale of events, and provides details about the presence of excitation or inhibition during the seizure – thus allowing observation of the dynamic function of a network. The effects of drug treatments have also elucidated mechanisms of network function in epilepsy.

Current Classification of Anticonvulsant Drug Mechanisms

The current mechanisms of anticonvulsant drug action, which were largely determined in vitro, include effects on neurotransmitter receptors or ion channels primarily. Enhancement of the inhibitory action of γ-aminobutyric acid (GABA) at $GABA_A$ receptors is thought to contribute in a major way to the anticonvulsant effects of barbiturates, benzodiazepines and tiagabine, and this action may contribute to the anticonvulsant effects of valproic acid and gabapentin. Block of sodium channels is another important anticonvulsant drug mechanism for phenytoin, carbamazepine, oxcarbazepine, and lamotrigine. Antagonism of specific types of calcium channels is another mechanism of anticonvulsant drug action, including ethosuximide and gabapentin. Blockade of glutamate receptors has also been observed with barbiturates, felbamate, and lamotrigine. Many anticonvulsant agents exert multiple actions at the single-cell level. However, the actions of these anticonvulsants have had minimal evaluation in seizure networks, and the limited seizure network research that has been done has shown differential effects on neurons within specific nuclei of the associated networks. Such differential effects may be particularly relevant to therapeutic mechanisms when the doses given to the intact animal do not greatly exceed those required to produce anticonvulsant effects (in the same animal). By limiting the doses, the nontherapeutic (adverse) effects that are exerted by all anticonvulsants in excessive doses are reduced or eliminated, as discussed later.

Audiogenic Seizures

Seizure models in animals have played a key role in the discovery of all antiepileptic drugs. In vitro testing *cannot* replace animal models, because it cannot model the entire spectrum of pharmacodynamic actions required for seizure blockade, does not provide information about the pharmacokinetics that occur in intact organisms, and avoids toxicological effects that may be induced in the intact animal.

A few animal models have well-established neuronal networks. These include an induced model of epilepsy – electrical kindling, which is a model of temporal lobe epilepsy produced in normal animals by repeated electrical stimulation in sites such as the amygdala. Genetics plays a major role in a number of human epilepsies, and in several animal models – e.g., genetically absence-prone rats (GAERs), which model absence epilepsy, and genetically epilepsy-prone rats (GEPRs) and Wistar audiogenic rats (WARs), which model generalized convulsive seizures. (GEPRs discussed below are the severe seizure strain (GEPR-9s), and work done in the moderate seizure strain (GEPR-3) is not included here.) GEPRs are abnormally sensitive to many seizure induction methods, including those evoked by loud sounds; such seizures are called AGS. There are many different rodent forms of naturally-occurring AGS, and several gene knockout mouse strains also exhibit AGS susceptibility. AGS susceptibility can also be induced in previously nonsusceptible rodents with a variety of treatments, including withdrawal from ethanol in ethanol-dependent rats. In all forms of AGS, high-intensity acoustic stimulation results in ictal behaviors that include wild running, clonus, and/or tonus, followed by postictal depression of behavior (much like that seen in generalized tonic–clonic seizures in humans). In human epileptic patients, generalized clonus and tonus occur, but these convulsions are rarely evoked by acoustic stimuli, although they may be evoked by visual stimuli. This sensory difference between humans and rodents may be due to the fact that rodents, unlike humans, are nocturnal and rely less than humans do on the visual system. Many anticonvulsant drugs have been developed using AGS models, and the anticonvulsant effect of levetiracetam was initially observed in an AGS model but not in the standard models.

Neuronal Network for AGS

AGS models provide very specific experimental advantages for network determination and functional studies. There is exact stimulus control – especially of intensity, which allows external control of seizure induction and the ability to evaluate responses to stimuli that are below and above the seizure threshold. Research on the neuroanatomy and neurophysiology of the auditory system is well developed. This established background can be readily applied to AGS, including potential candidate sites within the auditory pathway to evaluate for involvement in AGS as well as computer analysis methods for evaluating neuronal responses. A list of methods that are being used to evaluate neuronal networks is found in Table 2. The methods primarily emphasized in the author's lab are discussed here.

Methods

Chronic Surgical Implantation

An anesthetized rat is implanted stereotaxically with a cannula guide tube or microwire implant in the nuclei of interest. An antibiotic is given to minimize postoperative infection.

Focal Microinjection

One week after surgery, the rats are tested for AGS susceptibility, using an electric bell ($\geq$100 dB sound pressure level (SPL)) in a sound-attenuating chamber. One day later, the cannulae are inserted, and vehicle or active drug (in a volume $\leq$ 1 µl) is infused, using a microsyringe and infusion pump. AGS susceptibility is tested at appropriate times based on the drug's pharmacokinetics.

Extracellular Neuronal Responses

The microwire electrode assembly consists of a connector attached to several 13-µm diameter insulated tungsten wires. One week after implantation, neuronal firing in a nucleus is recorded from the rat, maintained in a clear plastic cylinder inside a sound-attenuating chamber. Tone burst stimuli (12 kHz, 100-ms duration, 5-ms rise–fall, at 0.5- or 1-Hz stimulation repetition rates) are presented. Neuronal firing in response to acoustic stimuli is recorded initially at intensities below that which will initiate AGS and then at seizure-inducing parameters. Neuronal firing and behavior are videotaped (split-screen) simultaneously during AGS. The data prior to AGS are analyzed using computer-based single unit discrimination methods to isolate recordings with a ratio of $\geq$3 to 1 to the background activity. The data are analyzed using post stimulus–time histograms, and quantified neuronal firing changes are statistically compared.

Chronic neuronal recordings are instituted after a network site is putatively identified using imaging techniques and/or focal microinjection to determine whether blockade of the site affects AGS. This assessment involves microinjecting an inhibitory substance, such as a $GABA_A$ receptor agonist or a NMDA receptor antagonist. If the results of this treatment indicate that the seizure can be reduced or blocked reversibly with such drugs, this site will be evaluated in other ways, including electrical stimulation and/or microinjection of an excitatory substance (such as a $GABA_A$ receptor antagonist or an excitatory amino acid receptor agonist) to mimic network activation. If the latter approaches render a normal animal susceptible to the seizure, this result is interpreted to confirm the importance of the structure as a requisite part of the seizure network.

The AGS is triggered using pure tone stimuli at 12 kHz, which is the most sensitive frequency for seizure induction in rats. The use of acoustic attenuators precisely controls stimulus intensity, which allows the neuronal responses to acoustic stimuli below the threshold for seizure induction to be evaluated (since only high intensities trigger seizures). The animals are not anesthetized, which is important since anesthetics significantly alter network properties and can prevent seizures. The neuronal response changes and simultaneous behavior changes are observed before, during, and after seizure. The convulsive seizures in GEPRs exhibit a stereotypical pattern of wild running followed by tonus, and culminate in tonic hind-limb extension (TE); the convulsive behaviors are rated using the seizure severity scale of Jobe. Extracellular neuronal recording in awake, behaving rats indicates if the neurons in a given site, which was previously implicated in the AGS network, change their activity associated with convulsive behaviors. If the results of this experiment suggest an important role of this structure, in vitro experiments on this site are evaluated.

Table 2 Methods of seizure network identification

Anatomical studies (e.g., c-fos, 2-deoxyglucose imaging)
Focal inactivation (reversible blockade* or lesion)
*a. excitatory neurotransmission block
*b. inhibitory neurotransmission increase
Stimulation mimicry (electrical/chemical*)
Acute neuronal electrophysiolgy in vivo
Chronic neuron electrophysiology & behavior in vivo
Combined stimulation and neuronal recording in network sites in vivo
In vitro patch or sharp intracellular recordings to determine cellular mechanisms

Recent Results

Establishing Inferior Colliculus as the Consensus AGS Initiation Site

An example of the earlier-mentioned evaluation of a network site is the experimentation done on the inferior

colliculus (IC) in AGS. A consensus has been reached, through the work of a number of laboratories, that the IC plays a critical role as the site for seizure initiation of AGS. The consensus is based on the application of a variety of techniques (described earlier) in the most thoroughly studied AGS models, GEPRs, and WARs, and also in normal rats made susceptible to AGS during ethanol withdrawal. The major defect that leads to AGS susceptibility in GEPRs is the reduced effectiveness of GABA-mediated acoustically-evoked inhibition in IC neurons. This reduced inhibition allows high-intensity acoustic stimuli to overdrive IC neurons, which in turn results in excess activation of neurons in sensorimotor brainstem sites to which the IC projects (deep layers of superior colliculus (DLSC), periaqueductal gray (PAG), and pontine reticular formation (PRF)) (**Figure 1**). Thus, excessive activity in the normal auditory network abnormally activates the locomotor network, which projects to the spinal cord and produces the convulsive behaviors.

Neuronal responses to acoustic stimuli in each of the AGS network sites in GEPRs are significantly greater than in normal animals, even in response to stimuli below the seizure threshold (**Figure 2**). The firing patterns of neurons in the AGS network during the seizure change dramatically in each site. However, the patterns are consistently different in each site, as the seizure progresses, depending on the type of convulsive behavior that is occurring.

AGS Network Hierarchy

Neuronal recording studies indicate that the seizure network operates hierarchically during AGS; that is, intense activation of each network nucleus occurs in a consistent and specific order preceding each convulsive behavior (**Figure 3**). IC neurons fire most intensely just prior to and during AGS initiation, while neurons in DLSC fire most intensely as the wild running is beginning. PAG and PRF neurons fire most intensely just prior to and during tonic–clonic behavior, and PRF neurons fire most intensely just prior to and during TE. During postictal depression, only neurons in the PRF remain active until recovery of the righting reflex, when neurons in all sites become active again. These findings suggest that the network hierarchy begins with IC dominance (greatest change from preseizural firing), but the DLSC becomes dominant during wild running. Then the PAG and PRF become dominant during tonic behavior, while the PRF dominates during TE.

Anticonvulsant Drug Action on the AGS Network

As noted earlier, abnormal acoustically-evoked neuronal firing prior to seizure occurs in all AGS network nuclei of the GEPR, and the effects of several anticonvulsant drugs on these elevated neuronal responses were evaluated in

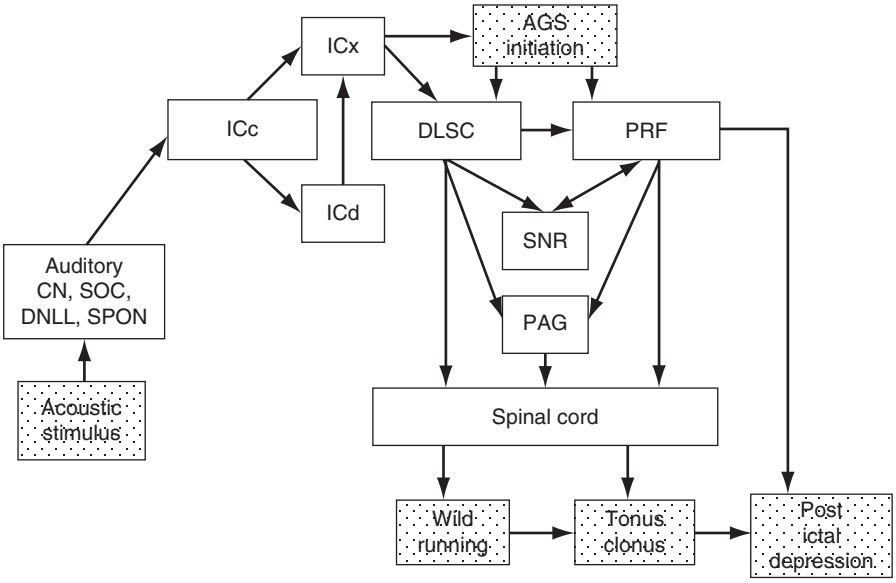


Figure 1 Diagrammatic representation of the major nuclei of the neuronal network that subserves audiogenic seizures (AGS) in GEPRs. The inferior colliculus (IC) central nucleus (ICc) in the midbrain receives input from the lower nuclei in the auditory pathway and initiates AGS via projections to the IC dorsal nucleus (ICd) and particularly to the IC external nucleus (ICx). The ICx projects the sensory input to the motor output nuclei. These include the deep layers of superior colliculus (DLSC), which initiate wild running, and the periaqueductal gray (PAG) and pontine reticular formation (PRF), which are dominant in triggering the clonic and/or tonic phase via their projections to the spinal cord. The PRF is the only region active during postictal depression. The substantia nigra reticulata (SNR) may exert a gating role for elaboration of the convulsive behavior. CN, cochlear nucleus; DNLL, dorsal nucleus of lateral lemniscus; SOC, superior olivary complex; SPON, superior periolivary nucleus. Modified from Faingold, CL **Figure 1** of Jasper's Basic Mechanisms of the Epilepsies, 3rd edn., *Advances in Neurology* 79, Chapter 18: Neuronal networks in the genetically epilepsy-prone rat, pp. 311–321, 1999, with permission from Lippincott.

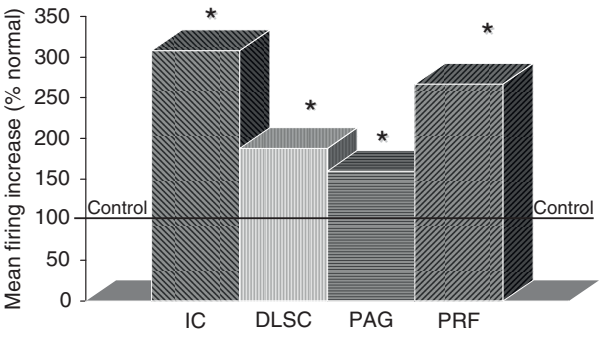


Figure 2 Mean neuronal firing increases in GEPRs (as percentage of normal rat neurons) in nuclei in the AGS network. Neuronal firing was evoked by stimuli (12 kHz, 90–110 dB SPL) that are below the seizure threshold in inferior colliculus (IC), deep layers of superior colliculus (DLSC), periaqueductal gray (PAG), and pontine reticular formation (PRF). All responses were significantly greater than control at $^*p < 0.05$ level (student's *t* test).

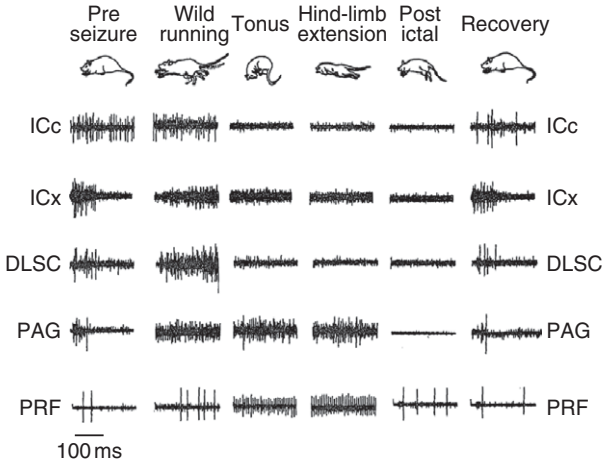


Figure 3 Composite behavioral activity in genetically epilepsy-prone rats (GEPRs) and typical examples of sequential neuronal firing patterns in the audiogenic seizure (AGS) network nuclei. These include the inferior colliculus central nucleus (ICc), inferior colliculus external nucleus (ICx), deep layers of superior colliculus (DLSC), ventrolateral periaqueductal gray (PAG), and pontine reticular formation (PRF). The *preseizure* normal behavior and neuronal responses to the acoustic stimulus in GEPRs just before AGS initiation are shown on the left with burst firing in ICc and ICx as the initial events. Neurons in the DLSC begin to fire at a high tonic rate just prior to the onset of *wild running*. DLSC firing continues at the rapid tonic rate during AGS, and ceases abruptly at the onset of postictal depression. PRF and PAG activity show slow but tonic rates of firing. The PRF and PAG exhibit rapid tonic firing just prior to the loss of righting reflex and entry into *tonus* and tonic *hind-limb* extension. The tonic extension is followed by postictal depression and the neuronal firing in all areas but the PRF ceases, whereas activity in the PRF slows its firing rate. All areas but PRF remain silent until the end of the postictal depression when *recovery* of the righting reflex occurs and neuronal responses begin to revert toward pre-AGS firing patterns in all areas. (Stimulus: 12 kHz, 100-ms duration, 5-ms rise–fall, 95–100 dB SPL). Reprinted from Faingold, CL **Figure 3** of Jasper's Basic Mechanisms of the Epilepsies, 3rd edn., *Advances in Neurology* 79, Chapter 18: Neuronal networks in the genetically epilepsy-prone rat, pp. 311–321, 1999, with permission from Lippincott.

these seizure network nuclei in awake, behaving GEPRs. The anticonvulsant drugs examined thus far (at doses that effectively reduce AGS) exerted differential effects on acoustically-evoked neuronal firing of neurons in each network nucleus (see **Table 3**). As noted earlier, the network appears to operate hierarchically. This hierarchy of network sites suggests that the site in the network at, or closest to, the seizure initiation site in IC is likely to be a critical site for drug action when it is administered at the lowest effective dose (to determine the most sensitive therapeutic target). This view is based on the previous findings that inhibitory substances ($GABA_A$ receptor agonists or NMDA receptor antagonists) will completely block AGS when focally microinjected into each network site. Competitive NMDA receptor antagonists are effective anticonvulsants against many forms of experimental seizures, including AGS in GEPRs. Systemic administration of these agents at anticonvulsant doses, prior to seizures, results in significant reduction of IC neuronal responses to acoustic stimuli at all stimulus intensities, suggesting that competitive NMDA receptor antagonists act in the AGS network at or afferent to the IC. However, an uncompetitive NMDA receptor antagonist, MK-801 (dizocilpine), did not alter the responses of IC neurons, despite the fact that it blocked AGS in very low doses.

Tiagabine blocks cellular uptake of GABA, prolonging its action. Systemically administered tiagabine blocked AGS in GEPRs, but unlike the competitive NMDA receptor antagonists, significant IC neuronal firing reduction prior to seizure was seen only at high acoustic intensities. The time course of the reduced IC neuronal firing paralleled that of AGS suppression with tiagabine, suggesting that tiagabine enhanced that form of $GABA_A$ receptor-mediated inhibition in IC neurons that is prominent at high acoustic intensities. However, other anticonvulsant drugs, including phenytoin, do not affect IC neuronal responses to acoustic stimuli at doses that block AGS, indicating that their actions are exerted at network sites efferent to the IC.

The PAG and PRF are implicated in the generation of tonic convulsive behaviors. AGS in GEPRs that culminate in TE, and elevated acoustically-evoked rapid tonic and/or burst neuronal firing, immediately preceding TE, have been observed in these brain sites. Phenytoin can block AGS completely, but lower doses of this agent can block the TE phase of AGS selectively. Anticonvulsant doses of phenytoin induce consistent changes in PAG and PRF neuronal firing and behavior in GEPRs. Phenytoin in doses that selectively suppressed TE did not consistently

Table 3 Drug Action on Neurons in Audiogenic Seizure Network

Brain Site	*Drug*	*Drug action*	*Effect in vivo*
IC	AP7	competitive NMDAR antagonist	firing down (all intensities)
	CPP	competitive NMDAR antagonist	firing down (all intensities)
	tiagabine	GABA uptake inhibitor	firing down (high intensities)
	ethanol	$GABA_A$ enhancement, NMDA antagonist	firing down (high intensities)
	MK-801	uncompetitive NMDA antagonist	no effect
	phenytoin	Na^+ channel blocker	no effect
PAG	phenytoin	Na^+ channel blocker	firing down *
	MK-801	uncompetitive NMDA blocker	no effect
	lamotrigine	Na^+ channel blocker	firing down
PRF	phenytoin	Na^+ channel blocker	firing down
	MK-801	NMDA channel blocker	no effect

Abbreviations: IC = inferior colliculus, PAG = periaqueductal gray, PRF = pontine reticular formation; * depending on dose
(Adapted from Faingold CL, Progress in Neurobiology, 72:55–85, 2004; Table 1, page 70, Reprinted with kind permission from Elsevier)

alter PAG neuronal responses to acoustic stimuli prior to seizure, but the same doses of phenytoin resulted in significant suppression of PRF preseizural acoustically-evoked neuronal firing and suppressed firing during seizure as well. Doses of phenytoin, which completely blocked AGS, significantly reduced PAG acoustically-evoked neuronal firing and more greatly decreased PRF firing. These results suggest a critical role for PRF, but not for PAG neurons, in the generation of tonic convulsive behaviors of AGS. The suppression of PAG and PRF neuronal firing induced by phenytoin, associated with complete seizure blockade, is consistent with vital roles for both structures in the seizure network. The differential suppressive effect of phenytoin on both preseizural and seizural neuronal firing in PRF as compared with the lack of effect on PAG firing indicate that this experimental approach can identify the most sensitive therapeutic target for anticonvulsant drug action. For an anticonvulsant drug to completely block seizures, however, actions on multiple network sites or on critical circuits within the network are needed, to significantly affect the emergent properties of the seizure network. MK-801 administration did not consistently affect preseizural PRF, PAG, or DLSC acoustically-evoked neuronal firing, despite blocking AGS in GEPRs. The substantia nigra reticulata (SNR) is implicated in the seizure network for several types of seizures, including AGS, and is also implicated in networks subserving normal locomotion. MK-801 induced a significant increase of neuronal firing in SNR neurons concomitant with suppression of AGS. However, MK-801 exerted little effect on action potential firing rates in response to electrical stimuli over a broad range of stimulus intensities when perfused directly onto SNR neurons in slice studies in vitro, despite using high concentrations (up to 200 μM) in vitro. These findings suggest that the significantly increased firing induced by systemically administered MK-801 is an effect on the emergent properties of the AGS network, critically involving SNR neurons. How can a drug produce a major effect on specific neurons in vivo, but not produce this effect on the same neurons in vitro? As noted earlier, there are a number of mechanisms that control network function. In the intact unanesthetized brain in vivo, all of these mechanisms are present. However, in vitro, many of these mechanisms are absent.

If we examine the influences exerted on neurons in a specific brain site, such as the IC, we can see that there are numerous influences, some of which are shown in **Figure 4**. When IC neurons are studied in in vitro brain slices, many of these influences are eliminated or greatly modified (**Figure 4**). Also, achieving an appropriate concentration of the drug in vitro is highly problematic. An 'amplification' phenomenon occurs in vivo, so that much smaller concentrations of drugs affecting seizures are needed in vivo as compared with in vitro to obtain the same effect, as demonstrated by the lab of Narahashi.

The possibility that functioning brain networks possess emergent properties has led to the hypothesis that these properties themselves are potentially critical targets for the action of CNS drugs. This hypothesis, in turn, has led to the idea that '*the intact brain is greater than the sum of its cells*.' When neurons are isolated (in vitro) from their network, regional selectivity of drug action can be lost. In this situation, a neuron in vivo may be affected by a given drug, but the same drug may not affect the same neuron in vitro, as seen with MK-801. Conversely, the emergent property theory also suggests that a neuron may not be affected by a given drug in vivo, even if the same drug has clear effects on the same neuron in vitro – which is observed with ethanol. Thus, the network can actually alter the sensitivity of neurons to a drug, as seen in data comparing the regional effects of ethanol in vivo and in vitro from the lab of Breese.

Neuronal networks may be responsible for conferring the selectivity of drug action. Most CNS drugs at therapeutic concentrations involve selective actions on neurons in a specific site within the brain. However, this selectivity is not due solely to the intrinsic properties of those neurons, and this selectivity may change when the neuron is isolated from the network. The human brain is estimated

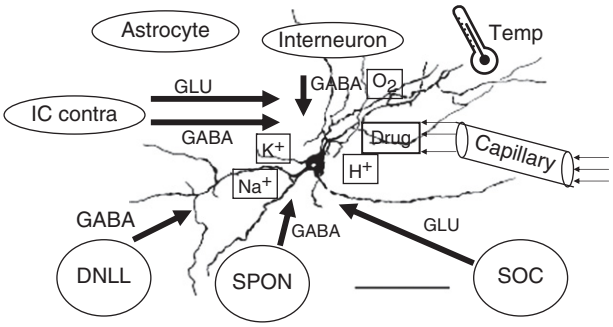


Figure 4 A diagram showing many of the important network-related influences exerted on an inferior colliculus (IC) neuron in vivo. The synaptic inputs to IC neurons include those from the pons via the superior olivary complex (SOC), which projects glutamate (GLU)-mediated excitation onto IC. Inhibitory γ-aminobutyric acid (GABA)-mediated inputs project onto IC neurons from the dorsal nucleus of the lateral lemniscus (DNLL) and the superior periolivary nucleus (SPON). There are also extensive descending inputs to IC (not shown). A number of these inputs, including those from DNLL, SPON, and SOC are eliminated, and many others are significantly modified when the IC undergoes preparation of the slice. Thus, the drug is delivered to the neuron via a capillary in the intact animal, but is directly perfused onto the neuron in vitro. The elements in the extracellular milieu, including the levels of Na^+, K^+, H^+, and O_2, are subject to physiologically-mediated changes in the intact animal but are largely controlled by the experimenter in the slice preparation, as is the temperature. Certain of the influences on IC neurons remain at least partially intact, including the excitatory and inhibitory projections from the contralateral (contra) IC, inhibitory GABAergic input from interneurons and input from local astrocytes contained within the slice. Elimination and alteration of the network-related influences on IC neurons can result in altered effects of any drug in vivo as compared with those exerted by the same agent in vitro, as seen in our studies. The anatomical part of the figure is reprinted from **Figure 2B** of Li Y, Evans MS, and Faingold CL (1999) Synaptic response patterns of neurons in the cortex of rat inferior colliculus. *Hearing Research* 137: 15–28.

to contain 50–100 billion neurons and over a trillion synapses. Several nuclei have been shown to become organized into complex interconnected systems or networks that perform complex functions in health and disease. Network function is subject to 'complexity theory' – i.e., when the multitude of elements in a complex system interact, new and unexpected properties can emerge due, in part, to self-organization. Based on complexity theory, a network can confer emergent properties on a neuron or set of neurons, and those properties can be a selective target of CNS drug action.

Networks and CNS Pathophysiology

As noted earlier, CNS neuronal networks can exhibit emergent properties. Specific elements within these networks may be a major therapeutic target on which CNS drugs exert their pharmacological effect. These network properties are in addition to any specific intrinsic properties of a particular type of neuron in a specific brain site. The net drug effect in an intact organism is the sum of all the actions exerted on various levels of these networks by the drug, and may vary by dose, particularly toxic doses. It is well-known that many neurological and psychiatric diseases are mediated by networks of brain nuclei. A drug's effect may be exerted on multiple networks or common elements of these networks.

Anticonvulsant drugs successfully treat seizure disorders, but many of these same drugs are effective in treating chronic pain disorders and psychiatric diseases – e.g., anxiety disorders and manic-depression (bipolar disorder). Each of these disorders is thought to be subserved by specific brain networks. The gate theory of pain involves an example of an early-described neuronal network in the spinal cord, and brain imaging has identified a neuromatrix that is activated in chronic pain syndromes. Imaging differences are seen between patients in pain and pain-free individuals, and many of these differences are reversible with effective drug therapy. A number of anticonvulsant drugs (e.g., carbamazepine, phenytoin, pregabalin, and gabapentin) effectively treat chronic pain syndromes. These agents may act by altering the properties of the neuronal network involved in the pathophysiology of pain.

Brain imaging techniques have also identified pathophysiological networks in psychiatric disorders, such as anxiety, that express major differences between anxious patients and normals – and many of these differences are also reversed with successful therapy. Evidence suggests that anticonvulsant drugs (e.g., valproate, lamotrigine, pregabalin, and gabapentin) may be effective in treating anxiety disorders.

Future Directions

This neuronal network approach adds another dimension to the understanding of how anticonvulsants produce their antiseizure effects. Additional experimentation is required to develop the usefulness of this approach fully. A catalog of the variety of emergent properties that exist in epileptic neuronal networks, in a number of seizure models, is needed to establish the general applicability of the mechanisms. There is a hierarchy of levels within the nervous system where this concept may apply. Whether the network-modifying mechanism is always involved in drug actions on epilepsy needs to be evaluated. This emergent property approach was developed in the GEPR, and additional anticonvulsants need to be evaluated in this model. More comparisons between in vitro and in vivo effects of the same agent are needed. The generality of this approach needs to be tested in closely related seizure models, such as the moderate seizure strain of the GEPR (GEPR-3) and ethanol withdrawal

models that exhibit similar AGS. Then the mechanisms need to be examined in other seizure models to identify whether differences and similarities occur. This methodology can generate a compendium of emergent targets for drug action. This approach is potentially important for the development of improved drug treatment for epilepsy – and a similar approach may be applicable to other CNS diseases, such as anxiety and pain, to determine if there are common actions.

Seizure networks are subject to plasticity, which may result in the expression of additional emergent properties. For example, a partial seizure model initially involves a relatively circumscribed neuronal network, but if the seizure secondarily generalizes, the seizure network expands and additional properties emerge. These properties may be additive with the original emergent properties. Blockade of these separable emergent properties may require drugs with different actions. Alternatively, effective anticonvulsants may act at multiple levels. Phenytoin, for example, acts on isolated sodium channels, which can be considered the 'ground level' in the hierarchy of emergent properties. However, phenytoin also acts on emergent properties at higher organizational levels in vivo, including specific network nuclei, as seen in the studies cited earlier where it selectively targets PRF neurons within the AGS network.

Identification of how anticonvulsants affect the emergent properties subserving seizure pathophysiology is vital in order to identify drugs that more selectively target these properties. This study applies this approach by investigating network mechanisms for seizure control during AGS, and evaluating anticonvulsant drug actions on neurons in the nuclei of the network. These studies are yielding a detailed understanding of the way this seizure network functions, and how anticonvulsant drugs modify these mechanisms. The results of initial studies indicate that specific sites within the intact network are most sensitive to therapeutic doses of a given drug; this site sensitivity may be different with each specific anticonvulsant examined, since even drugs with similar mechanisms of action can act at different brain sites.

Acknowledgements

The author thanks Dr. Ronald Browning, Dr. M. Steve Evans, and Sri Tupal for their comments on the manuscript and Diana Smith for editorial assistance. This work was supported by NIAAA AA 11628 and Southern Illinois University School of Medicine, Excellence in Academic Medicine Internal Grant.

See also: **Antiepileptic Drugs**: Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models; **Models**: Models of Generalized Seizures in Freely Moving Animals; Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; The Substantia Nigra in the Control of Seizures.

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Antiepileptic Drug Mechanisms

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Introduction

A seizure is defined as a single episode of neurological dysfunction arising from abnormal neuronal discharge, and can lead to clinically overt changes in motor control, sensory perception, behavior, or autonomic function. Epilepsy is a condition of recurrent spontaneous seizures arising from such aberrant electrical activity within the brain. Epileptic seizures are divided into two broad groups, depending on their site of origin and pattern of spread. Partial or focal seizures arise from a localized region of the brain, and the associated clinical manifestations relate to the function ordinarily subserved by that area. Focal seizures can remain localized, but can also spread to adjacent cortical areas and potentially involve the entire cortex. In contrast, primary generalized seizures begin with abnormal electrical discharges in both hemispheres simultaneously, and critically involve reciprocal connections between the thalamus and neocortex.

Epileptic seizures reflect enduring alterations in structure and function required to support abnormal neuronal firing. At a cellular level, two hallmark features of epileptiform activity are neuronal hyperexcitability and neuronal hypersynchrony. It is important to understand that a single abnormally discharging neuron is insufficient to produce a clinical seizure. For a seizure to manifest electroclinically, there must be synchronous activity of large populations or networks of neurons. Notwithstanding this important context, determination of the mechanisms of action of antiepileptic drugs (AEDs) usually begins with, or is reduced to, modulation of membrane-bound ion channels.

The neuronal cell membrane is populated by a variety of ion channels that convert chemical signals into electrical activity. As such, ion channels have traditionally been regarded as the 'currency' of neuronal excitability. A variety of chemical signals act through ligand-gated and voltage-gated ion channels to generate excitatory or inhibitory signals, which may be subthreshold or suprathreshold for subsequent action potential initiation – which in turn give rise 'downstream' to action potential-propagated neurochemical signals. Traditionally, there are three major classes of molecular targets believed to be relevant for limiting epileptic activity. These include: (1) voltage-gated sodium and calcium channels; (2) γ-aminobutyric acid ($GABA_A$) receptors; and (3) ionotropic glutamate receptors. Clinically useful antiepileptic AEDs exert their effects principally on one or more of these targets.

Background

Voltage-Gated Ion Channels

Sodium channels

Each sodium channel exists as a complex of polypeptide subunits. There is a major α-subunit and one or more β-subunits, which influence the kinetic properties of the α-subunit. Voltage-dependent sodium channels are activated upon membrane depolarization and underlie the fast upstroke of the action potential. The sodium current is a self-limiting process in that the channel inactivates within milliseconds, thus terminating the action potential. Repolarization of the neuronal membrane primes the sodium channel for subsequent activation. The kinetics of the onset and recovery from inactivation allow sustained, high-frequency action potential firing during normal and epileptic activity. Under normal conditions, there is an additional mechanism in the form of 'slow' inactivation that prevents excessive action potential firing. Slow inactivation occurs during prolonged depolarizations, in which full onset and recovery occur over several seconds. Consequently, these lengthy depolarizations can participate in the termination of repetitive action potential bursts.

Upon the decay of the transient sodium current, a remaining or 'persistent' sodium current (I_{NaP}) is observable and constitutes less than 4% of the total sodium current. It has been proposed that the non-inactivating I_{NaP} is either produced by the overlap between activation and inactivation voltage ranges of sodium channels (resulting in a 'window current') or generated by sodium channels entering an infrequently visited open state that lacks fast inactivation. In any case, the voltage range of I_{NaP} activation is immediately subthreshold for action potential initiation, and is thus repeatedly crossed during an action potential train. Accordingly, I_{NaP} occupies a unique position, and its presence or absence can regulate repetitive firing and the spread of hyperexcitability.

Calcium channels

Similar to other voltage-dependent ion channels, calcium channels are macromolecular protein complexes composed of multiple subunits that form a calcium-permeable pore activated by depolarization. There are two broad families of calcium channels, high voltage-activated (HVA) and low voltage-activated (LVA). As the name suggests, HVA calcium channels (L-, R-, P/Q-, and N-types) require strong membrane depolarization to induce opening, and are

predominantly responsible for the regulation of calcium entry and neurotransmitter release from presynaptic axon terminals in response to the arrival of an action potential. LVA calcium channels (T-type) are named as such because they can be activated by small depolarizations of the plasma membrane. T-type calcium channels regulate neuronal firing by participating in bursting and intrinsic oscillations. Thus, given their roles, both HVA and LVA calcium channels represent important targets in the modulation of neuronal excitability.

Synaptic Excitation

Neuronal excitation in the central nervous system is mediated by ionotropic glutamate receptors (i.e., α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid or AMPA, kainate, and *N*-methyl-D-aspartate or NMDA) and metabotropic glutamate receptors (see **Figure 1**). Inhibition of excitatory glutamatergic neurotransmission would appear the most obvious way to limit excessive excitatory activity, and this conceptual notion has been supported by many in vivo and in vitro preclinical studies. However, in clinical trials, selective glutamate receptor antagonists – while efficacious – have been hampered by unacceptable side-effects. Of the currently available AEDs, only two are known to exert activity, in part, on ionotropic glutamate receptors – and none interact principally with metabotropic glutamate receptors.

Synaptic Inhibition

Neuronal inhibition in the central nervous system is primarily achieved by the presynaptic release of the neurotransmitter GABA, which binds to ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ and $GABA_B$ receptors mediate fast and slow inhibition in the brain, respectively. During normal neuronal activity, communication between inhibitory GABAergic interneurons and excitatory glutamatergic neurons provides the critical balance necessary to prevent the development of synchronized epileptiform discharges – discharges that would naturally arise in an unchecked, recurrently connected

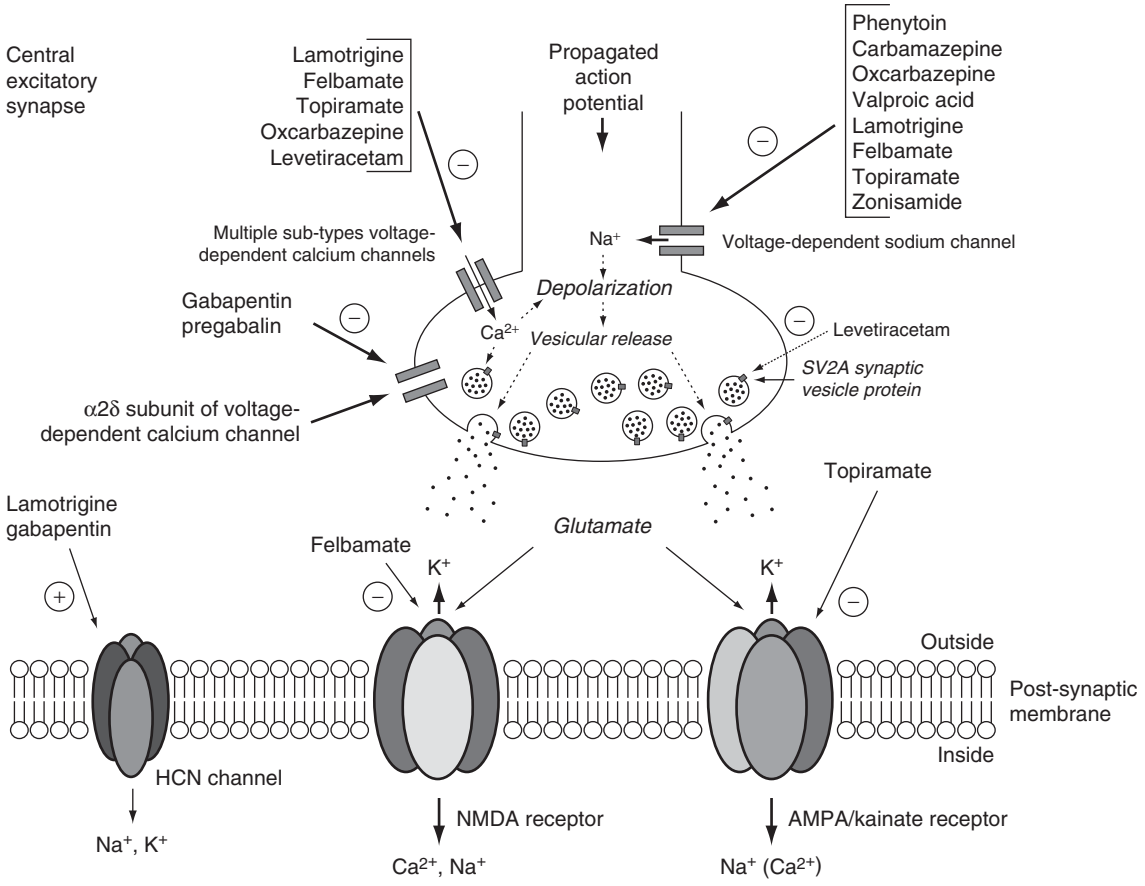


Figure 1 Schematic representation of an excitatory synapse in the central nervous system, and the putative major sites of action of various antiepileptic drugs (AEDs). Plus or minus signs denote activation/potentiation or inhibition, respectively. NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid; HCN, hyperpolarization-activated cyclic nucleotide-regulated cation channels. Modified with permission from Rho JM, Sankar R (1999) The pharmacologic basis of antiepileptic drug action. *Epilepsia* 40(11): 1471–1483.

excitatory network. However, the role of inhibition with respect to epileptiform activity is not simple. For example, it appears that inhibitory neurotransmission may actually participate in the synchronization of large populations of neurons and the subsequent propagation of seizures to adjacent brain regions. Thus, AEDs that act principally to augment GABAergic neurotransmission may prevent the initiation of epileptiform activity; however, such agents may also – in some cases – exacerbate the synchronization and spread of seizure activity, notably in thalamocortical circuits. There are several distinct ways that GABAergic inhibition can be augmented: (1) increased $GABA_A$ and $GABA_B$ receptor function; (2) enhanced GABA synthesis and decreased degradation; and (3) inhibition of GABA re-uptake into neuronal and glial cells (see **Figure 2**).

Methods – AED Screening

Traditionally, the antiepileptic activity of compounds is first identified in animal models of seizures. Screening by the Anticonvulsant Drug Development (ADD) Program at the University of Utah has contributed to the successful development of nine second-generation AEDs: felbamate (1993), gabapentin (1994), lamotrigine (1994), fosphenytoin (1996), topiramate (1996), tiagabine (1997), levetiracetam (1999), zonisamide (2000), and oxcarbazepine (2000). The ADD program employs a testing paradigm of a battery of animal models to determine efficacy against different seizure types. The 'opening-gate' screens are maximal electroshock (generalized tonic-clonic seizures), subcutaneous pentylenetrazol administration (generalized absence epilepsy), and the 6-Hz psychomotor seizure test (potential therapy-resistance). After a compound proves efficacious in one of these models, furthers tests are performed to characterize its anticonvulsant potential against audiogenic seizures, limbic seizures, and acute clonic seizures. These additional tests reveal whether a compound has a narrow or broad spectrum of anticonvulsant activity. Although the current process for AED discovery has been largely successful, the anticonvulsant screening paradigm is a dynamic flow chart – so that the ADD Program continually evaluates new animal models for possible incorporation into the screening process.

Once a new AED is identified, a wide array of techniques are available to determine its possible mechanisms of action, including (but not limited to) binding studies, imaging, electrophysiology, molecular biology, and molecular

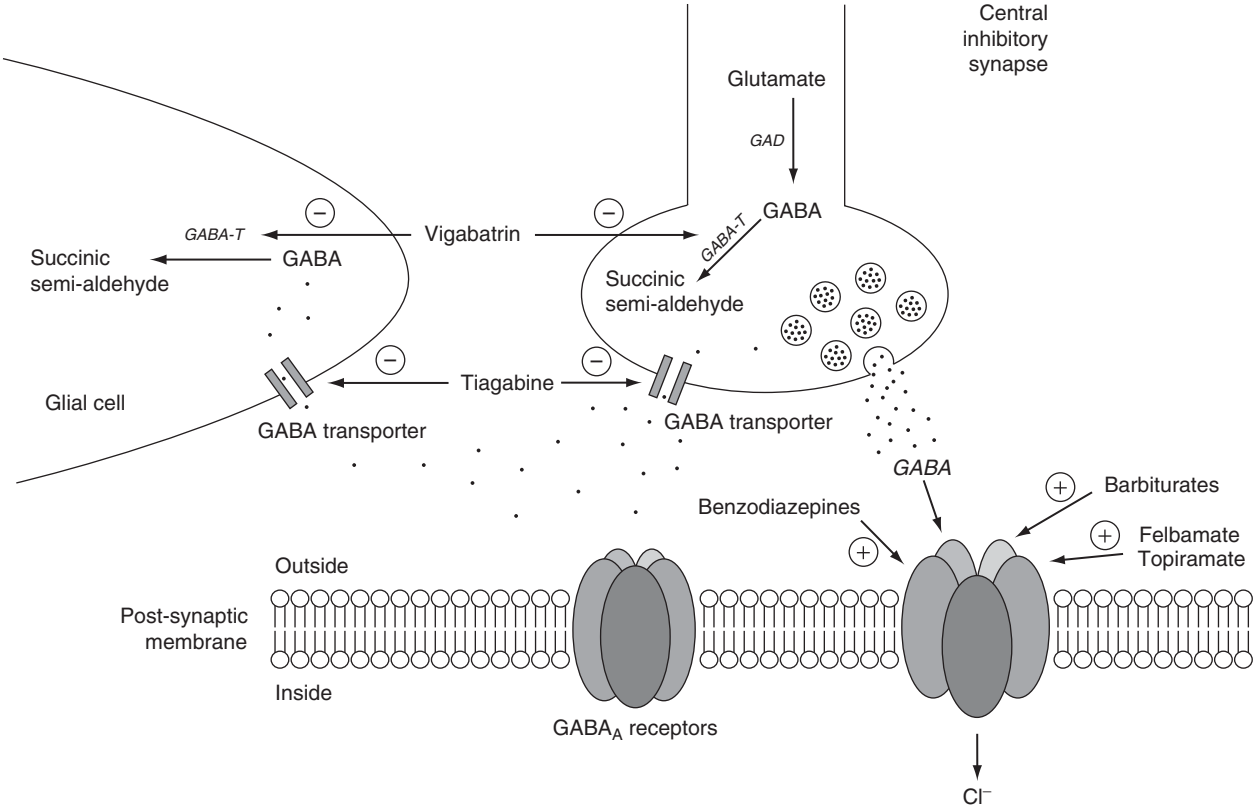


Figure 2 Schematic representation of an inhibitory synapse in the central nervous system, and the putative major sites of action of various antiepileptic drugs (AEDs). GABA, γ-aminobutyric acid; GABA-T, GABA transaminase; GAD, glutamic acid decarboxylase; SV2A, synaptic vesicle protein 2A. Plus or minus signs denote activation/potentiation or inhibition, respectively. Modified with permission from Rho JM, Sankar R (1999) The pharmacologic basis of antiepileptic drug action. *Epilepsia* 40(11): 1471–1483.

genetics. Competitive binding studies determine the specificity of the interaction between a radiolabeled AED and its binding site(s) by examining the ability of various neurotransmitters, hormones, or even other drugs to compete for the binding site(s). If no radiolabeled AED is available, then the inverse experiment can be performed using radiolabeled neurotransmitters, hormones, or drugs with known binding sites. However, this technique has limited or no sensitivity if the AED binding site is completely novel.

In recent years, high-throughput fluorescent imaging techniques (e.g., FLIPR-fluorometric-imaging plate reader, Molecular Devices, Union City, CA) have been used to ascertain test drug effects on cellular calcium concentration or membrane potential. Typically, these experiments are performed by adding a receptor agonist, such as the neurotransmitter glutamate, or depolarizing agent, such as potassium, to induce a change in fluorescence; then the effectiveness of varying concentrations of a drug of interest, in either reducing or preventing the change in fluorescence, is quantified. Understandably, these experiments, when performed using isolated cultured primary neurons, have low specificity and are unable to reveal a putative binding site for the AED, thus providing limited information about mechanism of action. Specificity is increased if the study uses a cell line expressing individual recombinant ion channels or receptors.

Electrophysiological evidence remains the 'gold standard' for discerning AED mechanism(s) of action, even though this approach is time-consuming and labor intensive. Fundamentally, electrophysiological techniques record changes in voltage or current in a group of neurons, within individual cells, or associated with single ion channels. These experiments are generally performed in vitro with acutely dissected brain slices or cultured neurons. Extracellular recordings provide information about AED effects on population events and provoked epileptiform activity. Whole-cell patch clamp techniques allow the investigator to determine AED effects on the single-cell resting membrane potential, input resistance, action potential characteristics (i.e., threshold, duration, and peak), and action potential firing characteristics (i.e., frequency, adaptation, and repetitive firing susceptibility). Furthermore, AED effects on specific voltage- and ligand-gated ion channels are resolved by isolating ionic currents, by changing the membrane potential or applying a receptor/channel agonist. Once a particular channel is identified as an AED target, single channel recording techniques establish AED effects on channel opening and closing kinetics and detail the exact manner in which the AED modulates ion channel function.

The disciplines of molecular biology and molecular genetics have become essential in establishing and determining AED binding sites on specific molecular targets. Molecular biology techniques have provided the technology to overexpress a known ion channel in a cell line that is essentially devoid of endogenous voltage- and ligand-gated channels. Combined with electrophysiology, such molecular approaches provide unequivocal evidence of AED action. Site-directed mutagenesis of ion channel proteins permits a detailed map of the molecular binding site for the AED. Molecular genetics has allowed investigators to identify specific mutations in particular ion channels that are associated with various epilepsies (i.e., GEFS+ type 1 and 2, childhood absence, and many others). Studies of the functional consequences of such natural mutations have been possible using site-directed mutagenesis and gene knockout and knock-in technology. AED modulation of these mutated ion channels can be assessed, and possibly correlated to refractoriness to individual AEDs. The growing structural and functional characterization of AED molecular targets, and increasing understanding of how AEDs modulate these targets, provide a framework for creating improved epilepsy therapies, and have encouraged many investigators in their attempts to develop drugs with specific molecular targets.

Recent Results

Voltage-Gated Ion Channels

Sodium channels

Several AEDs effective in the treatment of partial-onset epilepsy (i.e., phenytoin, carbamazepine, oxcarbazepine, lamotrigine, zonisamide, felbamate, topiramate, and valproate) inhibit voltage-gated sodium channels and block sustained, high-frequency, repetitive firing of neurons. Most of these AEDs appear to inhibit sodium channels in a voltage- and use-dependent manner (i.e., the block is more effective at depolarized rather than hyperpolarized potentials, and the potency of the block increases with increasing action potential frequency or prolonged depolarizations). These observations suggest that these AEDs act in a manner similar to, or modulate the kinetics of, slow inactivation – thus accounting for the selective block of high-frequency firing without affecting normal neuronal action potential discharge.

Phenytoin, carbamazepine, topiramate, ethosuximide, and valproate may additionally inhibit the non-inactivating, persistent sodium current that results from the overlap between activation and inactivation voltage ranges. As mentioned already, the persistent sodium current constitutes less than 4% of the total sodium current, but modulation of this current by AEDs may regulate epileptiform activity due to current activation in the voltage range immediately sub-threshold for spike initiation.

Calcium channels

HVA calcium channels consist of one α_1 subunit (with 7 known subtypes) that forms the channel pore, and various auxiliary subunits (including the $\alpha_2\delta$ subunit).

Two structural analogues of GABA (gabapentin and pregabalin) are effective in the treatment of partial seizures, and have been shown to interact with $\alpha_2\delta$-1 and $\alpha_2\delta$-2 auxiliary subunits. Interestingly, despite strong evidence for this unique site of action, neither gabapentin nor pregabalin has been consistently shown to exert an effect on calcium currents. This result is perhaps not surprising since $\alpha_2\delta$-1 and $\alpha_2\delta$-2 auxiliary subunits can combine with many of the 7 subtypes of HVA α_1 subunits, yielding tremendous heterogeneity in molecular composition – and hence, pharmacosensitivity. Even so, gabapentin and pregabalin can decrease presynaptic neurotransmitter release, at both inhibitory GABAergic and excitatory glutamatergic synapses. In contrast, various subtypes of HVA channels are inhibited by phenobarbital, levetiracetam (N-type), lamotrigine (N- and P/Q-types), felbamate (L-type), oxcarbazepine (N-type), and topiramate (L- and R-type), reducing neurotransmitter release in most cases.

Absence seizures are a unique seizure type associated with brief lapses of consciousness that begin and terminate abruptly, and are a result of generalized spike-wave discharges seen on the scalp electroencephalogram (EEG). Absence seizures reflect widespread and heightened phase-locked oscillations between excitation (i.e., spike) and inhibition (i.e., slow wave) in mutually connected thalamocortical networks. T-type calcium channels generate low-threshold calcium currents that depolarize the membrane and trigger bursts of action potentials. Such excitatory bursts are believed to underlie the 'spike' portion of a generalized spike-wave oscillation. Inhibition of T-type calcium channels by the AEDs ethosuximide, valproate, and zonisamide terminates the 3-Hz spike-wave discharges and correlates with efficacy in the treatment of generalized absence seizures. However, whether augmentation of T-type calcium currents is essential for the genesis of absence seizures remains somewhat controversial.

Synaptic Excitation

NMDA receptors are macromolecules formed by co-assembly of an obligatory NR1 subunit and at least one type of NR2 subunit. Felbamate, at therapeutically relevant concentrations, inhibits NMDA-evoked currents in hippocampal neurons and appears to be a low-affinity open channel blocker. Studies conducted in in vitro expression systems have demonstrated that felbamate is a more potent antagonist of NMDA receptors containing NR2B subunits. The NR2B subunit is abundantly expressed in the immature brain and largely restricted to the forebrain in the adult. These expression patterns may explain the effectiveness of felbamate in pediatric epilepsies and low neurobehavioral side-effects in adults.

As the primary mediators of fast excitatory neurotransmission, AMPA and kainate receptors have differential but important roles in seizure genesis and spread. The AED topiramate has been demonstrated to inhibit both AMPA- and kainate-receptor mediated currents. Kainate receptors are particularly interesting as AED targets because they are not restricted to postsynaptic membranes; they can also be found on presynaptic axon terminals. Presynaptically, activation of kainate-receptors modulates glutamate release and inhibits GABA release. As such, kainate-receptor mediated inhibition represents a very potent mechanism of seizure termination. Like other ligand-gated ion channels, AMPA- and kainate-receptors are composed of various subunits, and topiramate is selective for GluR5-containing kainate-receptors.

Synaptic Inhibition

$GABA_A$ receptors are oligopentameric, macromolecular protein complexes consisting of a variety of subunits that form a pore selectively permeable to chloride and bicarbonate ions. Biophysical and pharmacological properties of $GABA_A$ receptors are determined by their inherent subunit composition. In general, pharmacological blockade and enhancement of $GABA_A$ receptors causes seizures and attenuates seizures, respectively. The AEDs phenobarbital, diazepam, felbamate, and topiramate have been experimentally shown to modulate $GABA_A$ receptors in differential ways to augment inhibitory neurotransmission.

$GABA_A$ receptor function can be enhanced by increasing the channel conductance and/or open state of the channel (either channel 'burst frequency' and/or open state duration). Accordingly, the barbiturate phenobarbital increases mean open duration, the benzodiazepine diazepam increases open state frequency without affecting duration, and topiramate increases both open state and burst frequency and duration. Felbamate has been demonstrated to enhance GABA-mediated currents in hippocampal and cortical neurons, and although the precise effects on single channel kinetics remain unclear, there is evidence to suggest a barbiturate-like action.

Studies of $GABA_A$ receptors expressed in oocytes or mammalian cells, and comprising different subunit combinations, have revealed subunit-specific modulation of $GABA_A$ receptors by AEDs. Diazepam binds to a recognition site juxtaposed between the α and γ subunits, and enhances GABA activation of most receptors comprised of an $\alpha\beta\gamma$ subunit combination with varying affinities; however, it is ineffective for $\alpha_{4\text{ or }6}\beta\gamma$ combinations. Very high concentrations of diazepam are able to directly activate and enhance receptors comprised of only $\alpha\beta$ subunits. Therapeutic concentrations of topiramate enhance $\alpha\beta_{2\text{ or }3}\gamma_2$ combinations and directly activate $\alpha_4\beta_3\gamma_2$, whereas higher concentrations of this AED directly activate $\alpha\beta_{2\text{ or }3}\gamma_2$ combinations and inhibit $\alpha\beta_1\gamma_2$ combinations. Studies utilizing the *Xenopus* oocyte expression system indicate that a therapeutic concentration of felbamate (300 μM) exclusively enhances $GABA_A$ receptors

comprised of $\alpha_{1 \text{ or } 2}\beta_{2 \text{ or } 3}\gamma_2$, and is either ineffective or inhibits every other subunit combination tested. In contrast, phenobarbital positively modulates every $GABA_A$ receptor subunit combination, although potency does depend on α and β subunit subtypes. It is safe to speculate that such subunit specificity, along with possible variations in local AED concentrations, may contribute to the unique anticonvulsant profile of each AED and its effectiveness against particular seizure types.

$GABA_B$ receptors are coupled via a guanosine 5′-triphosphate-binding protein to calcium or potassium channels. The GABA structural analogue, gabapentin, has been reported to modulate inwardly rectifying potassium channels and N-type calcium channels via preferential interaction with $GABA_B$ receptors containing $GABA_{B1a}$ subunits. Gabapentin's subunit specificity was initially thought to contribute to its anticonvulsant activity by selectively reducing excitatory neurotransmitter release with a simultaneous sparing of inhibitory neurotransmission. However, subsequent studies have been unable to replicate these findings-with failure to identify binding at radioligand sites related to $GABA_B$ receptors, failure to demonstrate an inhibitory effect on gabapentin actions by $GABA_B$ receptor antagonists, and failure to prevent gabapentin action by co-application of the $GABA_B$ agonist, baclofen.

Ambient levels of extracellular GABA are controlled by enzymes involved in the GABA life cycle and GABA uptake by transporters. Two pyridoxal-5′-phosphate-dependent enzymes, glutamate decarboxylase (GAD) and GABA transaminase (GABA-T) are involved in GABA synthesis and degradation, respectively. The AED vigabatrin (γ-vinyl GABA) is a GABA analogue that has no activity on GABA receptors, but acts by inhibiting GABA-T, which results in significant elevations in brain GABA levels. Vigabatrin increases ambient extracellular GABA either by reversal of GABA transporters, or by shifting the concentration equilibrium to a value that supports increased extracellular GABA as a result of the marked elevation in intracellular GABA, or by effects on GABA uptake that are unrelated to GABA-T. Other GABA analogues, such as gabapentin and pregabalin, were once thought to enhance GAD and inhibit GABA transferase, resulting in increased GABA concentrations – but subsequent studies have disproved these interactions at therapeutically relevant drug concentrations. The GABA transporter, GAT1, is inhibited by the AED tiagabine, resulting in prolonged postsynaptic inhibitory currents that are enhanced during repetitive interneuron activation, activity that is expected to occur during epileptiform discharge. The increase of ambient GABA through any of these mechanisms would be expected to act on $GABA_A$ and $GABA_B$ receptors. However, the anticonvulsant consequences are more likely due to effects on extrasynaptic rather than synaptic GABA receptors, given the relatively higher GABA affinity and lower desensitization properties of extrasynaptic GABA receptors.

Additional Molecular Targets

HCN channels

Another important ion channel involved in the regulation of thalamocortical rhythmicity is the hyperpolarization-activated cation channel (HCN channel), responsible for the so-called I_h or h-current. HCN channels are highly expressed in neuronal dendrites (and to a lesser extent in the soma) in both thalamus and hippocampus, are activated by hyperpolarization, and produce a depolarizing current carried by a flux of Na^+ and K^+ ions. This depolarization helps to bring the resting membrane potential toward the threshold for T-type calcium channel activation.

Importantly, unlike other voltage-gated conductances that are classified as either inhibitory or excitatory, h-currents are both inhibitory *and* excitatory. HCN channels possess an inherent negative-feedback property. That is, hyperpolarization produces an activation of HCN channels, which then leads to depolarization and immediate de-activation of these channels. The net effect of HCN channel activation is a decrease in the input resistance of the membrane, which is a measure of the voltage change produced by a given synaptic current. H-currents tend to stabilize the neuronal membrane potential toward the resting potential against both hyperpolarizing and depolarizing inputs.

The relevance of HCN channels in the pathogenesis of absence seizures was recently underscored by the experimental finding that deletion of a specific HCN isoform results in absence epilepsy and sinus dysrhythmia in mice. Furthermore, lamotrigine, an AED that is highly effective against absence seizures, enhances activation of dendritic h-currents in hippocampal pyramidal neurons by shifting the voltage-dependent activation towards depolarized potentials. Increasing I_h reduces input resistance, length constant, and temporal summation, and suppresses action potential initiation by dendritic inputs. Gabapentin has also been shown to enhance I_h amplitude by increasing the channel conductance, which results in a slight membrane depolarization and reduced input resistance.

Potassium channels

Potassium channels represent a large and heterogenous family of ion channels, and generally function to dampen neuronal excitation by inducing membrane hyperpolarization. Hence, they represent a natural target for AED development. Previously, it was thought that none of the AEDs available for clinical use today act directly on voltage-gated potassium channels. However, recently

published studies implicate potassium channels as part of the mechanism of action of several AEDs. For example, topiramate induces a steady membrane hyperpolarization mediated by a potassium conductance, whereas levetiracetam blocks sustained repetitive firing by paradoxically decreasing delayed rectifier voltage-gated potassium currents. Phenytoin inhibits A-type potassium channels by increasing the probability of the inactivated state. Delayed rectifier and A-type potassium channels participate in membrane repolarization after an action potential and play a role in determining the frequency of repetitive firing. Presumably, inhibition of either potassium channel slows membrane repolarization, thereby decreasing sodium channel de-inactivation, and effectively preventing responses to fast depolarization by limiting the frequency of repetitive firing. Lamotrigine and zonisamide also inhibit A-type channels, but at supra-therapeutic concentrations.

Carbamazepine, at therapeutically relevant concentrations, blocks recombinant rat brain small conductance, calcium-activated potassium channels which underlie the slow after-hyperpolarization of action potentials that mediate the phenomenon of spike frequency adaptation. In contrast, large conductance, calcium-activated potassium channels participate in spike repolarization and fast hyperpolarization, and are activated by zonisamide in a manner that shifts voltage sensitivity to less positive potentials and decreases mean closed durations, thereby increasing open probability. Interestingly, polymorphisms and mutations in small and large conductance, calcium-activated potassium channels, respectively, are associated with generalized epilepsies.

As described above, the multitude of different types of potassium channels predicts diverse actions of AEDs on potassium conductance. This diversity of actions is illustrated by the AED retigabine, an investigational compound with broad efficacy in animal seizure models, which enhances activation of KCNQ2 and KCNQ3 potassium channels. This mechanism of action is particularly intriguing given that mutations in genes encoding these potassium channel subunits have been linked to a rare form of inherited epilepsy, benign familial neonatal convulsions.

Carbonic anhydrase inhibition

Several AEDs, including acetazolamide, topiramate and zonisamide, act either principally or in part by inhibiting certain carbonic anhydrase isoforms. The clinical use of carbonic anhydrase inhibitors covers the last half century, but it is unclear how anticonvulsant effects are achieved with these agents. Interestingly, the demonstration that carbonic anhydrase II knockout mice have decreased susceptibility to pentylenetretrazole (PTZ)- and fluorothyl-induced seizures emphasizes the relevance of this family of enzymes in modulation of neuronal excitability. Inhibition of brain carbonic anhydrase results in an increase in total CO_2 concentrations in neurons, and increased pH and a decreased HCO_3^- concentration in neuroglia. One downstream effect of intracellular alkalinization by acetazolamide is an up-regulation of I_h in thalamocortical neurons due to a shift in voltage-dependent activation of HCN channels to more positive potentials. This pH-mediated up-regulation of I_h results in decreased membrane responses to hypepolarizing stimuli and a reduced ability to generate rebound calcium-mediated spikes. These changes also lower interstitial K^+ concentration, as well as pH. It is well known that a reduction in extracellular K^+ leads to diminished neuronal excitability, but the impact of decreased extracellular pH is less clear, despite the fact that – depending on subunit composition – extracellular protons can block NMDA receptor-mediated excitation and enhance $GABA_A$ receptor function.

Synaptic vesicle protein

One of the newest AEDs has challenged our conceptual understanding of relevant mechanisms of action. Unlike traditional agents, levetiracetam failed two standard screening tests in animals – the maximal electroshock (MES) and PTZ seizure threshold tests – yet had a profound effect in retarding amygdala kindling in rats. Levetiracetam's principal molecular target, originally identified as a specific high-affinity neuronal binding site, was recently shown to be a specific synaptic vesicle protein, SV2A, which is involved in neurotransmitter release. The functional consequences of an interaction between SV2A and levetiracetam remains to be elucidated. However, a putative augmentation by levetiracetam can be tentatively hypothesized based on the observation that mice lacking SV2A have severe seizures.

Future Directions and Challenges

This last example cearly illustrates that we have yet much to learn about mechanisms underlying seizure genesis and epileptogenesis. Certain molecular targets, such as $GABA_A$ receptors, are widely regarded as relevant to the clinical effects of AEDs such as barbiturates and benzodiazepines. And there is an emerging consensus that the clinical activity of many AEDs across a broad spectrum of seizure types may be a consequence of the multiplicity of molecular actions for any given AED. For example, topiramate's actions on voltage-gated sodium and calcium channels, combined with its effects on both $GABA_A$ and glutamate receptors, are consistent with efficacy against multiple seizure types.

Yet despite their diverse molecular actions, current AEDs fail to 'cure' or modify the disease process. Rather, they are effective in most patients in eliminating the major symptom – seizures – by dampening neuronal excitation, synchronization, and spread of seizure activity. Little experimental evidence exists to support the notion that

these drugs are truly 'antiepileptogenic' or 'antiepileptic' (i.e., prevent the development or maintenance of the epileptic state). As epileptogenic mechanisms become better understood, an important goal will be to design more specific and rational interventions to treat the disease as well as the symptoms.

See also: **Antiepileptic Drugs**: Anticonvulsant Drugs as Neuronal Network-Modifying Agents; Clinical Drug Development in Epilepsy; Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models; Preclinical Drug Development in Epilepsy; **Ketogenic Diet**: Dietary Therapies for Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; Seizure Models in Acute and Organotypic Slices; **Pediatric Epilepsy**: Quantitative and Qualitative Changes of Action of Antiepileptic Drugs in Developing Experimental Animals; **Plasticity**: Seizure-Related Changes in Dendritic Spine Structure.

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GABA Transporters as Targets of Antiepileptic Drugs

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Introduction

Seizures and epilepsy are a consequence of neuronal hyperexcitability resulting from a variety of mechanisms. These include genetic or acquired alterations in ion channel function, aberrant network connectivity resulting from neurogenesis and axonal sprouting, enhancement of excitatory glutamatergic synaptic transmission, and reductions in inhibitory (GABAergic) synaptic transmission. The predominant mechanism(s) of hyperexcitability are likely to vary between the different types of epilepsy syndromes (e.g., idiopathic generalized epilepsy, temporal lobe epilepsy (TLE), posttraumatic, or poststroke epilepsy, postinfectious epilepsy, and tumor- or mass-associated epilepsy). However, drugs that augment $GABA_A$ receptor function (phenobarbital, benzodiazepines) are among the most potent and reliable agents used to prevent or acutely treat seizures in almost every type of epilepsy syndrome. Their effectiveness and versatility make the GABAergic system an attractive target for pharmacologic modulations that would be effective for chronic treatment of a variety of types of epilepsy.

GABA is the primary inhibitory neurotransmitter in the brain. GABAergic inhibition in the brain occurs as a result of presynaptic release of GABA that acts on postsynaptic $GABA_A$ receptors to transiently open Cl^- channels, resulting in a hyperpolarizing inhibitory postsynaptic potential (IPSP). GABA released from presynaptic terminals also acts on pre- and postsynaptic $GABA_B$ receptors – G-protein-coupled receptors that exert several effects,

these drugs are truly 'antiepileptogenic' or 'antiepileptic' (i.e., prevent the development or maintenance of the epileptic state). As epileptogenic mechanisms become better understood, an important goal will be to design more specific and rational interventions to treat the disease as well as the symptoms.

See also: **Antiepileptic Drugs**: Anticonvulsant Drugs as Neuronal Network-Modifying Agents; Clinical Drug Development in Epilepsy; Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models; Preclinical Drug Development in Epilepsy; **Ketogenic Diet**: Dietary Therapies for Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; Seizure Models in Acute and Organotypic Slices; **Pediatric Epilepsy**: Quantitative and Qualitative Changes of Action of Antiepileptic Drugs in Developing Experimental Animals; **Plasticity**: Seizure-Related Changes in Dendritic Spine Structure.

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Introduction

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including opening potassium channels to produce slow hyperpolarization. These forms of GABA signaling are referred to as phasic inhibition. In contrast, ambient extracellular GABA concentrations ($[GABA]_0$) act in a tonic manner on extrasynaptic $GABA_A$ receptors to cause persistent Cl^- currents, termed tonic inhibition. GABA transporters on neurons and glia serve to terminate synaptic events, limit crosstalk between neurons from GABA molecules diffusing away from synapses, maintain ambient [GABA] at appropriately low concentrations, and provide a means for neurons to reclaim synaptically released GABA for repackaging into synaptic vesicles. Thus, GABA transporters are important pharmacologic targets that modulate both phasic and tonic GABAergic inhibition in the brain. GABA transporters can also operate in the reverse direction, and thus can become an important source of GABA release. In this article, we review the types of GABA transporters, their relevance to epilepsy, and the anticonvulsant drugs that target them.

Background

GABA Transporter Function, Subtypes, and Cellular Localization

GABA transporters are Na^+- and Cl^--dependent symporters that translocate one GABA molecule, two Na^+ ions, and one Cl^- ion per thermodynamic reaction cycle. GABA may be moved into cells against its concentration gradient by this coupled translocation due to the energy stored in the steep transmembrane concentration gradient for sodium established by the Na^+/K^+-ATPase. GABA transporters are electrogenic, moving one net charge into cells during an uptake cycle, and therefore are voltage-dependent. Although GABA transporters may primarily work in the "forward" direction (i.e., moving GABA into cells), they may also work in the reverse direction, releasing GABA into the extracellular space. The direction and magnitude of GABA transport is dependent on the transmembrane concentration gradients of transported substrate and membrane potential. GABA transporters under control conditions have a reversal potential (the membrane voltage at which transport reverses direction) near typical resting membrane potentials of neurons, allowing GABA transporters to easily operate in reverse and release GABA with relatively minor membrane potential changes. During synaptic events, when extracellular GABA may transiently reach as high as 1 mM, GABA transporters will work exclusively in the forward direction. In contrast, elevations in intracellular sodium or membrane depolarization lead to favorable thermodynamic conditions for reverse transport and release of GABA into the extracellular space. As there are continuous fluctuations in membrane potential, ion concentrations, and GABA concentrations during normal brain function, it is predicted that the rate and direction of GABA transport would constantly change as the conditions vary. GABA transporters also play an important role in regulating the level of ambient $[GABA]_0$, and under normal conditions attempt to clamp this level at around 90–250 nM – which is in the range of concentrations necessary – to activate high-affinity extrasynaptic $GABA_A$ receptors that cause tonic inhibition.

GABA transporters belong to the superfamily of Na^+- and Cl^--dependent co-transporters. There are four cloned isoforms: GAT1–3 and the betaine/GABA transporter-1 (BGT-1). BGT-1 differs from the other GABA transporters in that it translocates 3 Na^+ ions during each thermodynamic reaction cycle. GAT-1 is the primary neuronal GABA transporter, but can also be expressed in astrocytes. GAT-1 is concentrated in presynaptic terminals, where its surface expression is dynamically regulated by $[GABA]_0$ and neural activity. GAT-2 expression in brain is greatest in leptomeningeal cells, although there is also some expression of GAT-2 in astrocytes. GAT-3 is the primary GABA transporter expressed in glial cells, and exists in perisynaptic and extrasynaptic glial membranes. BGT-1 expression is seen in glia, to a lesser degree than GAT-3, and in extraneural tissue including liver and kidney.

GABA Transporter Pharmacology

Pharmacologic agents that inhibit GABA transport have been indispensable tools in the study of GABA transporter function. Early antagonists of GABA uptake were the GABA analogs β-alanine, guvacine, and nipecotic acid (NPA). NPA is a nonselective GAT inhibitor that also induces GABA release through a process termed 'heteroexchange.' Many of the GABA transporter antagonists subsequently identified have been the result of chemical modifications to NPA or guvacine. These chemical modifications include addition of lipophilic groups to allow transfer of putative antieleptics across the blood–brain barrier; however, these modifications have also affected the pharmacologic action of these molecules, yielding highly selective inhibitors for specific GABA transporter subtypes. Commonly used research tools to selectively inhibit the neuronal GABA transporter GAT1 include the compounds SKF-899761 and NO-711 (also known as NNC-711). Tiagabine is also a relatively selective GAT1 antagonist. The compound SNAP-5114 is selective for GAT2 and GAT3. Antiepileptic drug development is an active field that has identified additional compounds with varying specificities for GABA transporters that promise to extend the list of research tools and potential therapeutic agents.

Methods

Electrophysiology

The study of GABA transporters and the effects of antiepileptic drugs on these molecules often employ

electrophysiologic measurements of membrane voltage or current from neurons in tissue culture or acute brain slices. These recordings are carried out most frequently with patch clamp methods. Patch clamp recordings allow the identification of both phasic and tonic forms of GABAergic inhibition, and allow the assessment of the effects of GABA transporter antagonists on these forms of inhibition. The effects of drugs that stimulate GABA release via GABA transporters may also be detected with patch clamp recordings often as increased tonic inhibition.

Measurements of GABA

Measurements of human brain GABA concentrations in vivo have been made with two different approaches. One approach is invasive and involves sampling of extracellular fluid with microdialysis catheters placed in or near an epileptic focus of a patient undergoing intracranial EEG investigations (as part of a presurgical evaluation for medically-refractory epilepsy). These samples can then be analyzed by high-performance liquid phase chromatography (HPLC) to determine GABA concentrations. This technique also provides the ability to perfuse the area of tissue under study with test solutions, including solutions with elevated K^+ to stimulate neurotransmitter release and those with zero Ca^{2+} to reduce vesicular release of neurotransmitter. A noninvasive approach that has been applied specifically to the study of antiepileptic drug effects on brain GABA concentrations is magnetic resonance spectroscopy (MRS). This method allows quantification of whole brain GABA concentrations (intracellular vs. extracellular GABA cannot be distinguished). Within study protocols, MRS has allowed investigators to determine the effects of some antiepileptic drugs (gabapentin, vigabatrin, valproic acid) on brain GABA concentration, both in control and in epileptic subjects, and at various time points following a single test dose of drug.

Results

GABA Transporters in Epilepsy

GABA transporters are active participants in the regulation of phasic and tonic inhibition. By controlling the time course of synaptic events and ambient [GABA], GABA transporters influence inhibitory tone and the excitation-inhibition balance in the brain. This balance is disrupted in epilepsy, leading to seizures. The roles of GABA transporters in epilepsy are complex. There is support for altered GABA transporter expression and function in both human and experimental epilepsy. These reported alterations include both downregulation and upregulation of GABA transporter number, altered patterns of expression, and diminished uptake capacity.

Surgery is an effective treatment for epilepsy that is refractory to medications, and tissue obtained during these therapeutic procedures has provided an opportunity to study the cellular and molecular properties of human epileptogenic tissue. In human TLE with hippocampal sclerosis (HS), GAT1 expression is reduced in the granule cell layer of dentate gyrus and the pyramidal cell layer (see **Figure 1(a)**). If this downregulation of GAT1 occurred in isolation, it might be expected to prolong IPSPs and promote elevations in ambient [GABA], thereby providing a compensatory, anticonvulsant increase in GABAergic inhibition in the setting of a hyperexcitable, epileptic neural network. However, in TLE with HS, the decrease in GAT1 may be due to cell death and loss of GABAergic terminals, so the anticonvulsant effect of GAT1 loss could be overshadowed by the proconvulsant effect of a reduction in GABA synapse number. Complicating conclusions about how a reduction in GAT1 expression might affect excitability, GABA transporters readily operate in the reverse direction in response to the membrane depolarization and elevation of intracellular sodium that occurs in neurons during high-frequency firing. Consistent with this reversal, measurements of extracellular GABA in human hippocampus (with microdialysis catheters) have shown that GABA is released during seizures and this GABA release is due, in part, to Ca^{2+}-independent, nonvesicular release of GABA by reversal of transporters. In this role, as a source of GABA release, GABA transporters exert an anticonvulsant effect which would be diminished by GABA transporter downregulation. Indeed, the reduction of GAT1 immunoreactivity seen in TLE with HS is associated with less GABA release during seizures (and in response to exogenous glutamate) compared to the contralateral, nonepileptogenic hippocampus (see **Figure 1(b)** and **(c)**). Electrophysiological measurements from human hippocampal tissue with HS have confirmed GABA transporter downregulation. During whole-cell recordings, the responses to exogenous GABA are prolonged in tissue with HS and the responses to NPA, a nonselective GAT inhibitor that induces GABA release, are attenuated (see **Figure 1(d)**). These results demonstrate reduced GABA transporter function (including GABA release) in at least one form of human epilepsy (i.e., TLE with HS). GABA transporter expression is reduced in genetically epilepsy prone rats as well.

Although the importance of GABA transporter downregulation in epileptogenesis and seizures is not firmly established, our consideration of GAT1 downregulation in TLE with HS illustrates the duplicitous nature of GABA transporters in regulating excitability and the conflicting effects produced by pharmacologic inhibition of GABA transporters. The inhibition of GABA transporters delays the removal of synaptically released GABA thereby prolonging IPSPs – an anticonvulsant effect – while at the

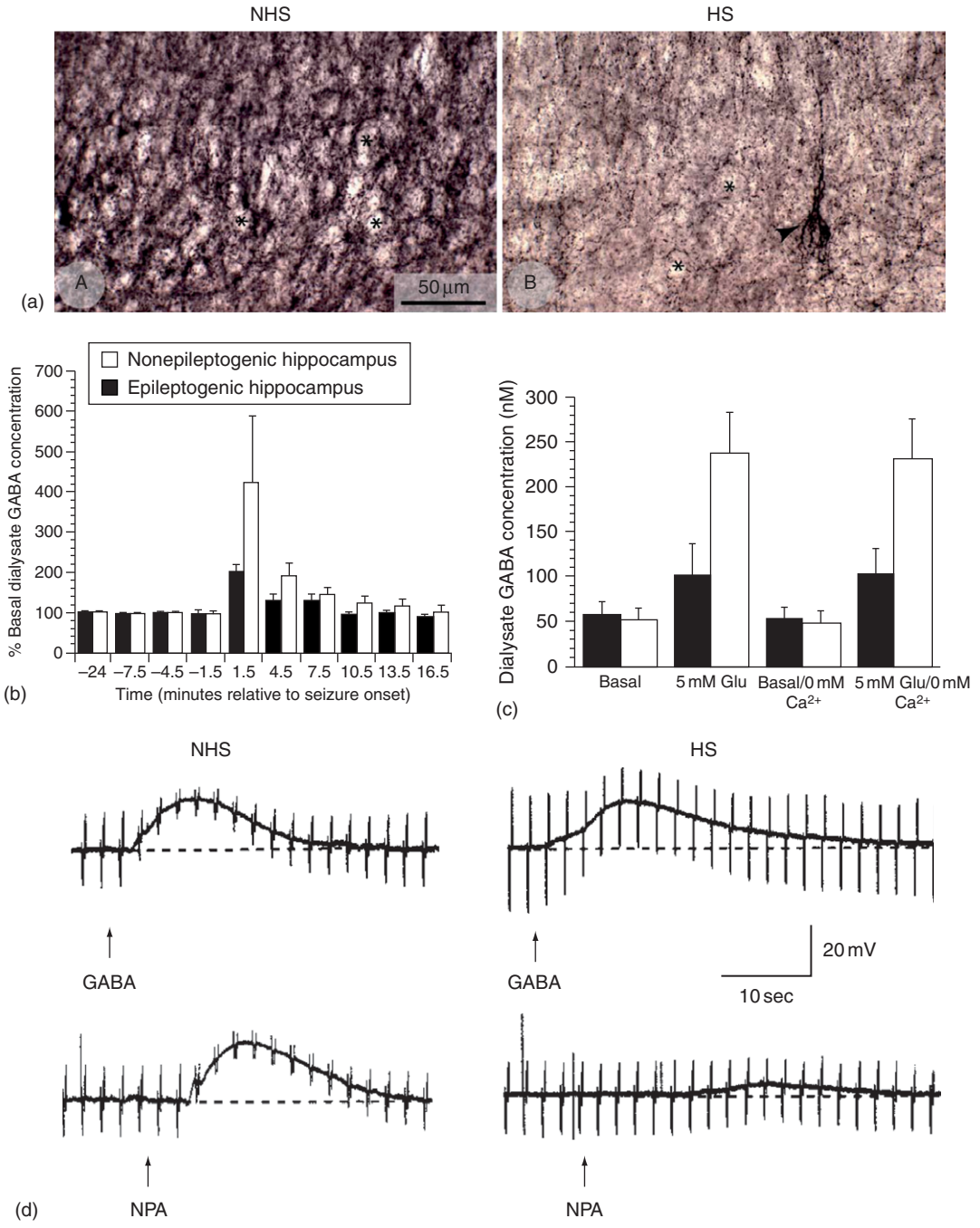


Figure 1 GABA transporter expression and function in human temporal lobe epilepsy with hippocampal sclerosis (HS). (a) Micrographs of immunohistochemical labeling for the GABA transporter GAT-1 in granule cell layer of human dentate gyrus. Tissue from a patient with a temporal lobe tumor but no hippocampal sclerosis (left, ''NHS'') showed diffuse labeling surrounding granule cell somata (asterisks). In contrast, in TLE with HS (right, ''HS'') immunostaining is reduced and only rare granule cells displayed immunostaining (arrowhead). Reproduced from Lee TS, Bjornsen LP, Paz C, Kim JH, *et al*. (2006) GAT1 and GAT3 expression are differently localized in the human epileptogenic hippocampus. *Acta Neuropathologica* 111: 351–363, with permission from Springer-Verlag. (b) Microdialysis catheter measurements of extracellular GABA during spontaneous seizures from human temporal lobe in vivo. GABA release during seizures is reduced in the hippocampus in which secondarily-generalized seizures originated (epileptogenic hippocampus). Reproduced from During MJ and Spencer DD (1993) Extracellular hippocampal glutamate and spontaneous seizure in the conscious brain. *The Lancet* 341: 1607–1610, with permission from Elsevier. (c) Microdialysis catheter measurements of extracellular GABA under basal conditions and during glutamate stimulation with or without extracellular calcium. GABA release in response to glutamate is primarily due to Ca^{2+}-independent, nonvesicular release and is reduced in epileptogenic hippocampus. Same legend as for part (b). Reproduced from During MJ, Ryder KM, and Spencer DD (1995) Hippocampal GABA transporter function in temporal-lobe epilepsy. *Nature* 376: 174–177, with permission from Nature Publishing. (d) The effects of exogenous GABA and NPA on

same time reducing nonvesicular GABA release – a proconvulsant effect. The situation is made even more complex by the presence of $GABA_A$ receptors on both principal cells and inhibitory interneurons, the variety of GABA transporter subtypes with different cellular localizations, the regulation of GABA transporter surface expression by extracellular GABA itself, and the fact that $GABA_A$ receptor activation can sometimes be depolarizing. Which action of GABA transporter inhibition (anticonvulsant or proconvulsant) dominates? Clinical and experimental studies have shown that drugs that selectively inhibit GAT1 are effective in reducing seizure occurrence, supporting the utility of this approach to treat epilepsy. Additionally, several effective antiepileptic drugs work indirectly through GABA transporters by modifying intracellular GABA metabolism thereby promoting GABA release and increasing ambient extracellular GABA concentrations. Moreover, some antiepileptic drugs exert a variety of anticonvulsant effects and have GABA uptake inhibition as part of their mechanistic profile.

GABA Transporters and Antiepileptic Drug Action

Tiagabine

Tiagabine is a structural analog of NPA, with an additional hydrophobic chain that allows for delivery across the blood brain barrier and alters the pharmacologic specificity of tiagabine. Tiagabine is the only direct GABA transporter inhibitor approved for clinical use, with an indication as adjunct therapy for partial epilepsy. It is effective in preventing seizures in several animal models of focal and generalized epilepsy (e.g., amygdaloid kindling, pentylene–tetrazol, audiogenic seizures). Tiagabine preferentially inhibits uptake by GAT1, but is active at other GABA transporters at higher concentrations (10^{-4} g mol^{-1}). GAT1 inhibition with tiagabine prolongs the time course of evoked IPSPs in hippocampal pyramidal cells without influencing IPSP amplitude (see **Figure 2(a)**). Tiagabine and other GAT1-selective inhibitors have little or no effect on the amplitude or time course of miniature synaptic potentials; presumably diffusion is sufficient to 'terminate' miniature IPSPs (due to smaller numbers of GABA molecules) without engaging GAT1. In hippocampus, selective GAT1 inhibition can also sometimes result in elevation of ambient [GABA] and increased tonic inhibition. These observations suggest that, tiagabine exerts its anticonvulsant actions through modulation of both phasic and tonic inhibition. In addition to its efficacy in patients and in animal models, tiagabine inhibits evoked epileptiform activity of single hippocampal neurons in situ (see **Figure 2(b)**).

Vigabatrin

Vigabatrin is an example of an antiepileptic drug that works indirectly through GABA transporters. GABA transporters are controlled by the transmembrane gradients of transported substrate and membrane potential. Elevation of cytosolic [GABA] is one perturbation that alters GABA transporter equilibrium, which in turn potentiates nonvesicular GABA release and leads to higher values of ambient [GABA]. Cytosolic GABA is normally degraded by the enzymatic action of GABA transaminase. Vigabatrin acts by irreversibly inhibiting GABA transaminase, causing elevation of GABA concentrations in vivo and in vitro. Vigabatrin, paradoxically, reduces the frequency and/or amplitude of IPSCs, demonstrating that enhancement of phasic inhibition does not underlie the anticonvulsant action of vigabatrin. Rather, the anticonvulsant action is due to enhancement of tonic inhibition. During perforated patch clamp recordings from hippocampal neurons, in the absence of extracellular calcium to block vesicular GABA release, tonic GABA receptor-mediated currents are enhanced in a time- and concentration-dependent manner by vigabatrin (see **Figure 3(a)** and **(b)**). Most importantly, pretreatment with the GAT-1 blocker NO-711 prevents the enhancement of tonic currents and IPSC amplitude reduction that are induced by vigabatrin, indicating that GABA is released by reverse transport. The elevation of ambient [GABA] caused by vigabatrin can desensitize some classes of $GABA_A$ receptors on neurons, and this desensitization may be the basis of the reduced amplitude and frequency of phasic synaptic events observed following vigabatrin treatment.

Gabapentin and pregabalin

Gabapentin and pregabalin are alkylated GABA derivatives that are effective in the treatment of seizures and neuropathic pain. Although these drugs are structurally similar and have overlapping clinical utility, it is not established that they share all the same mechanisms of

membrane potential of human hippocampal neurons recorded *in situ* after excision. Neurons in epileptic tissue with HS (right, ''HS'') have prolonged responses to exogenous GABA (intracellular chloride concentration is high in these experiments so GABA is depolarizing) and heteroexchange GABA release due to NPA is reduced compared to neurons from tissue with no HS (left, ''NHS''). These results demonstrate a decrease in function of the GABA transporter in HS, with less transporter-mediated GABA uptake and GABA release. Reproduced from Partrylo PR, Spencer DD, and Williamson A (2001) GABA uptake and heterotransport are impaired in the dentate gyrus of epileptic rats and humans with temporal lobe sclerosis. *Journal of Neurophysiology* 85: 1533–1542, with permission from American Physiological Society.

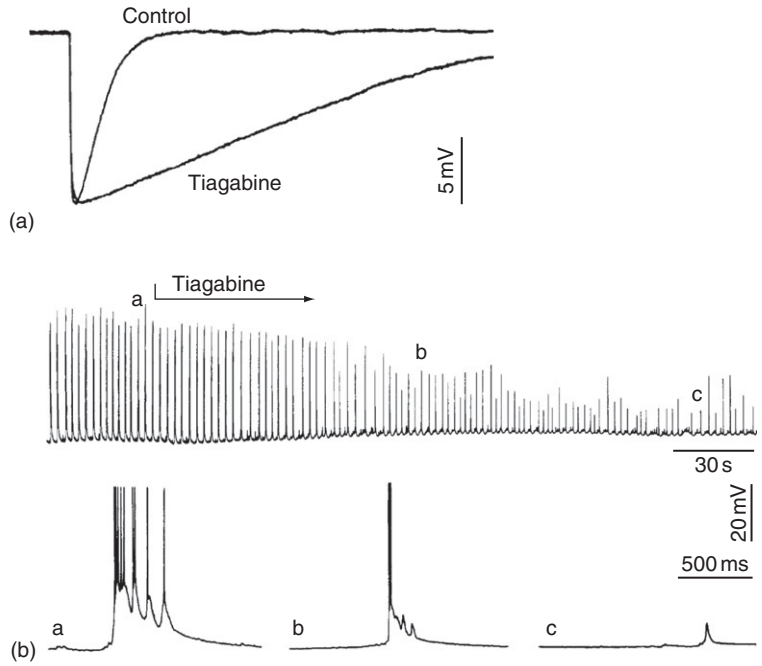


Figure 2 The GAT-1 inhibitor tiagabine prolongs inhibitory postsynaptic potentials (IPSPs) and inhibits epileptiform discharges. (a) Whole-cell recordings of IPSPs in a hippocampal neuron. Tiagabine prolonged IPSPs without affecting amplitude. (b) Whole-cell recording of epileptiform bursting induced by elevated K^+ (8.5 mM) from a hippocampal neuron in organotypic slice culture. This form of hyperexcitability is inhibited by tiagabine (25 μM). The bottom panel illustrates portions of the record from the top panel on an expanded time base, indicated by the letters. Reproduced from Thompson SM and Gahwiler BH (1992) Effects of the GABA uptake inhibitor tiaganine on inhibitory synaptic potentials in rat hippocampal slice cultures. *Journal of Neurophysiology* 67: 1698–1701, with permission from American Physiological Society.

action. Gabapentin, being the older of the two drugs, has been more extensively studied. The precise mechanism of action underlying the anticonvulsant effect of gabapentin (and pregabalin) is not known. The finding that gabapentin can antagonize presynaptic, voltage-sensitive calcium channels containing the α2δ subunit, thereby limiting neurotransmitter release, has focused attention on this mechanism as the action underlying the therapeutic effects of both gabapentin and pregabalin in epilepsy and pain. However, considerable data exist in support of gabapentin also causing effects on the GABAergic system. Gabapentin stimulates glutamic acid decarboxylase in vitro (GAD, the GABA synthetic enzyme) and gabapentin administered to patients in vivo rapidly (within 1 h) increases mean brain GABA concentration to 283% of control values (as determined with MRS). Gabapentin has no acute effects on GABA receptors themselves, but potentiates heteroexchange (transporter-mediated) release of GABA induced by NPA from axons and from hippocampal neurons in slices (see **Figure 4(a)**). This result could be explained by gabapentin's stimulation of GAD and increased cytosolic [GABA], a direct modulatory action on GABA transporters, or an increase in surface expression of GABA transporters. An increase in cytosolic [GABA], with a resultant change in GABA transporter equilibrium and elevated ambient [GABA], is a plausible hypothesis, because gabapentin also increases tonic inhibition of hippocampal neurons 2–3-fold following prolonged treatment (see **Figure 4(b)**). Interestingly, the effects of gabapentin and vigabatrin on tonic inhibition are synergistic. GABA transporters undergo regulated redistribution between the surface membrane and the intracellular compartments in response to a variety of stimuli (e.g., elevated extracellular GABA and depolarization). Gabapentin and pregabalin both induce a similar redistribution of GABA transporters to plasma membrane. Incubation of hippocampal neurons with gabapentin or pregabalin for two hours doubles the uptake of [^{3}H] GABA (see **Figure 4(c)**). These effects are not observed with acute application of drug during the assay period. Biotinylation experiments to define the intracellular and membrane fractions of GAT1 during western blotting demonstrated that the enhanced GABA uptake is due to mobilization of GAT1 to the plasma membrane (see **Figure 4(d)**). Independent of gabapentin's actions on calcium channels, its effects on the GABAergic system would be expected to contribute to its antiepileptic action via elevated GABA concentrations, enhanced transporter reversal, and increased tonic inhibition. The increase in plasma membrane GABA transporter number could

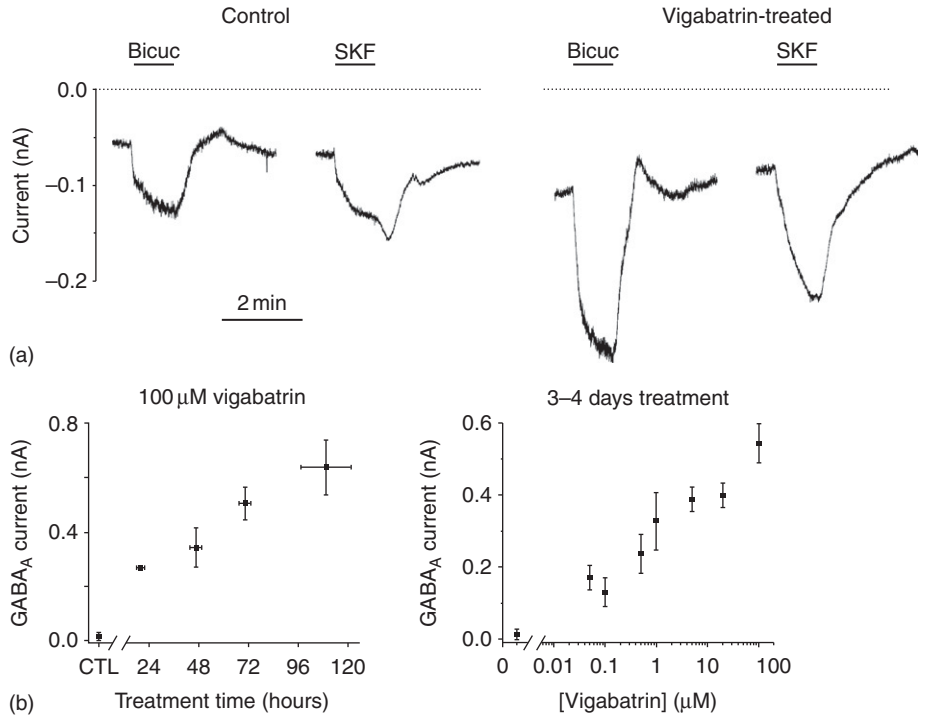


Figure 3 Vigabatrin increases ambient [GABA] and tonic inhibition through GABA transporter reversal. (a) whole-cell currents of hippocampal neurons recorded at −60 mV using gramicidin-perforated patch clamp techniques. Bicuculline, a $GABA_A$ receptor antagonist, blocked a tonic, outward current. The tonic currents were 2–3-fold larger after treatment with vigabatrin (100 μM) for 7 days. Tonic currents under control conditions and following vigabatrin treatment were blocked by the GAT1 inhibitor SKF-89976A indicating that GABA efflux was mediated by reversal of the GABA transporter. (b) Time and concentration dependence for vigabatrin potentiation of tonic inhibition in hippocampal neurons. Note that the effect of vigabatrin is very slow to develop (days), but occurs with as little as 50 nM. Reproduced from Wu Y, Wang W, and Richerson GB (2001) GABA transaminase inhibition induces spontaneous and enhances depolarization-evoked efflux via reversal of the GABA transporter. *Journal of Neuroscience* 21: 2630–2639, with permission from Society of Neuroscience.

shorten the duration of IPSCs (a proconvulsant effect), but perhaps more importantly would enhance nonvesicular GABA release during intense neural activity such as occurs during seizures (an anticonvulsant effect).

Valproic acid

Valproic acid is an anticonvulsant drug effective against focal and generalized epilepsy syndromes, with several well-characterized antiepileptic mechanisms of action that include Na^+ and Ca^{2+} channel inhibition, as well as modulation of GABA metabolism. As is the case with gabapentin and vigabatrin, alterations of GABA metabolism are expected to work indirectly through GABA transporters (potentiation of nonvesicular release, elevation of ambient [GABA]). Elevations of whole brain GABA levels are seen with valproic acid treatment, and valproic acid has in vitro activity against GABA metabolizing enzymes. These in vitro activities include stimulation of GAD (GABA synthetic enzyme) and inhibition of GABA transaminase (GABA degradative enzyme). Additionally, valproic acid has been reported to directly inhibit GABA transport of GAT1-transfected oocytes.

Future Directions

GABA transporter isoforms in the brain have unique cellular localizations and identifying pharmacology. The expression and function of GABA transporters are altered in some forms of epilepsy. GABA transporters serve to terminate synaptic events; their pharmacologic inhibition prolongs IPSPs and can increase tonic inhibition. Drugs such as tiagabine that directly inhibit the neuronal GABA transporter GAT1 exert their antiepileptic effect through this mechanism. GABA transporters exist near equilibrium at typical resting membrane potentials of neurons and operate in the reverse direction to release GABA under appropriate conditions including during spontaneous seizures. Several antiepileptic drugs (gabapentin, pregabalin, vigabatrin, and valproic acid) work indirectly through GABA transporters by increasing intracellular GABA, thereby altering GABA transporter equilibrium and leading to increased ambient [GABA] through facilitation of GABA release.

Future research promises to expand our knowledge of the changes in the expression and function of the different GABA transporter isoforms in epilepsy. This information

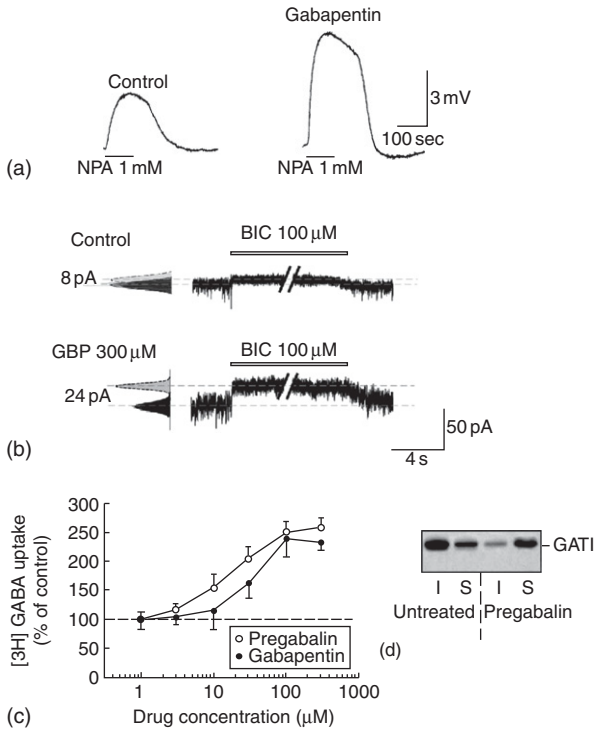


Figure 4 Gabapentin and pregabalin, alkylated GABA derivatives, potentiate nonvesicular release of GABA by GABA transporters, increase tonic inhibition, enhance GABA uptake, and increase membrane surface expression of GAT1. (a) Membrane potential of rat optic nerve axons during nipecotic acid (NPA, 1 mM) application. Pretreatment of nerves with gabapentin (100 μM) for 1 h robustly potentiated the release of GABA induced by NPA. Gabapentin had no effect on depolarization produced with exogenous GABA. Axons maintain high intracellular Cl^- concentrations so GABA responses are depolarizing. A similar response occurs in neurons in hippocampal slices. Reproduced from Kocsis JD and Honmou O (1994) Gabapentin increases GABA-induced depolarization in rat neonatal optic nerve. *Neuroscience Letters* 169: 181–184, with permission from Elsevier. (b) Tonic membrane currents of hippocampal neurons. Pretreatment of neurons with gabapentin (300 μM) for 36–48 h (bottom panel) produced a threefold increase in tonic inhibition compared to control neurons (top panel). Insets on left are all point histograms of current recordings before and during bicuculline application used to define the tonic, $GABA_A$ receptor mediated current. Reproduced from Cheng VY, Bonin RP, Chiu MW, Newell JG, MacDonald JF, and Orser BA (2006) Gabapentin increases a tonic inhibitory conductance in hippocampal pyramidal neurons. *Anesthesiology* 105: 325–333, with permission from American Society of Anesthesiologists. (c) Gabapentin and pregabalin both produce an approximately twofold increase in GABA uptake. Shown are dose–response curves for GABA uptake by hippocampal neurons. Neurons were incubated in drug for 2 h. (d) Immunoblot of biotinylated, surface (S) and nonbiotinylated, intracellular (I) GAT1 protein. Pretreatment with pregabalin for 2 h produced a shift of GAT1 protein from the intracellular to the surface fraction. Reproduced from Whitworth TL and Quick MW (2001) Upregulation of g-aminobutyric acid transporter expression: Role of alkylated γ-aminobutyric acid derivatives. *Biochemical Society Transactions* 29: 736–741, with permission from Biochemical Society.

will advance our understanding of the roles and function that GABA transporters play in health and disease. Antiepileptic drug development has focused on identifying new specific inhibitors of GABA transporter isoforms that may be employed, not only as research tools but also as medical therapy. The development of specific inhibitors for GABA transporter isoforms has the potential to allow recognition of previously underappreciated mechanisms in epilepsy, such as the betaine–GABA transporter (BGT-1). Better defining the pro-convulsant versus the anticonvulsant roles of GABA transporters may lead to more efficacious pharmacological treatments that block the former and enhance the latter.

See also: **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability; Neuronal Cl Transport: Setting the Stage for Successful Inhibition; Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

Further Reading

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Local Delivery of Antiepileptic Drugs

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Introduction

Medical therapy for epilepsy is unsatisfactory in about one-third of treated patients. Increasing dosage of medication increases efficacy, but also leads to toxicity. One strategic approach to this conundrum is development of local drug delivery to regions of brain involved in generation of seizures. Local delivery would allow relatively high and effective concentrations, but with minimization of side effects. Side effects intrinsic to the action of the drug on local circuits – for example, impairment of memory with delivery of drug into the hippocampus – would theoretically remain. However, side effects that are incidental to the antiepileptic mechanism – such as hepatotoxicity, suppression of blood counts, kidney stones, and possibly some neurological side effects – would be attenuated by local delivery.

Background

Local delivery of drug to CNS has been employed in clinical practice for pain (epidural or subdural morphine) and spasticity (epidural baclofen). Wafers (which slowly release agent into a resection bed of a tumor), and by catheter delivery into brain, enhanced by convection/pressure have been used in the chemotherapy of brain tumors. Delivery of antiepileptic drugs to the neuraxis has not yet been employed for seizure patients. For this procedure to become a reality in epilepsy therapy, methods to deliver a compound to a seizure focus – or, alternatively, to a part of brain that influences the excitability of a seizure focus – will be needed. Treatment must be shown to be both safe and effective. In the case of devices that deliver drug to brain, safety and efficacy must be demonstrated both for the drug and for the device.

Two general strategies could be used for local drug delivery. One would supply drug on a regular dosing schedule. The other would deliver drug in response to a seizure, or perhaps on command of a supplementary device (not yet available) that is able to predict the imminent onset of a seizure.

Several routes of direct administration of drugs to the brain have been considered, with different degrees of invasivity, ranging from intravenous infusion of liposomes or circulating prodrugs to devices or catheters implanted in brain tissue or cerebrospinal fluid (CSF). **Table 1** shows a few promising approaches to drug delivery to the CNS.

Methods/Results

Seizure-Activated Prodrugs

A prodrug is in an active compound that circulates harmlessly until activated by some metabolic process or clinically relevant event. The commonly used AED oxcarbazepine is, in fact, a prodrug for its monohydroxy derivative, which is the active form. Fosphenytoin is a prodrug of phenytoin, less irritating for intravenous injection. However, metabolic conversion of these two existing AEDs confers no special advantage in terms of penetration or concentration in the brain. One drug that does confer such a potential advantage for epilepsy is under testing, in the form of phosphatidycholine-valproic acid (DP-VPA). The phosphatidylcholine moiety attached to the valproic acid molecule renders the drug inactive until a phospholipase, released at a seizure focus, cleaves the drug to active valproic acid. In animal models, this drug did not block the first seizure, but impeded development of subsequent seizures, presumably because of accumulation of valproic acid at the site of seizure initiation. Clinical trials are underway. A 28-day double-blind crossover trial documented a 30% reduction in seizure frequency compared with placebo, an improvement that is comparable with the results of adding oral valproate to a drug

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regimen. Such trials may or may not lead to release of this particular agent, but they do serve as an interesting proof-in-principle for prodrugs acting at the seizure focus. Further understanding of metabolic activities that are relatively specific to a seizure focus would be useful, since such an understanding might provide other targets for local therapy at the seizure focus (e.g., in the form of agents blocking a focus-specific enzyme).

Carrier Vehicles

Carrier vehicles encapsulate drugs in an inert shell in order to enhance pharmaceutical potency and reduce toxicity in nontarget tissue. These vehicles include conventional liposomes, chemically modified liposomes, microsomes, and polymeric nanoparticles. A liposome is a spherical vesicle composed of a phospholipid and cholesterol bilayer membrane, encapsulated in an aqueous solution. Liposomes are able to carry both hydrophilic solutes as well as hydrophobic chemicals. The lipid bilayer fuses with the cellular membranes to release the contents of the liposome into the tissue, typically over a period of days to weeks, with permeability of the drug depending upon factors such as particle size, pH, osmolality, and other adjustable factors. Liposomes become particularly interesting when coupled to a targeting mechanism – e.g., based on immune affinity or receptor binding (**Figure 1**). The liposomes then can be injected systemically, home to a target of interest, and slowly deliver high concentrations of medications in the region of interest. One example of such use is liposomal doxorubicin for treating hepatic tumors; this delivery method provides significantly reduced systemic and cardiac toxicity.

Table 1 Methods for antiepileptic drug delivery directly to brain

Seizure-activated prodrugs
Nanoparticles: Liposomes and microsomes
CSF delivery
Implanted slow-release AEDs
Epidural drug
Local perfusion by catheter

Mori and associates studied the efficacy of liposomal phenytoin in an amygdala seizure focus in the rat, induced by injecting dibutyryl-cAMP and ethylenediaminetetraacetic acid into amygdala and bilateral sensorimotor cortex. Two doses of liposomal phenytoin, given intravenously at 40 mg kg^{-1} at a time 30 and 60 min after injection of cAMP, were able to suppress interictal and ictal amygdalar discharges for up to 170 min. Labeling liposomes with horseradish peroxidase demonstrated preferential accumulation of liposomes in the amygdalar seizure focus. Amiloride is a sodium ion exchange inhibitor with efficacy against animal models of seizures. Its use, however, is limited, since it produces systemic hyperkalemia when conventionally injected. However, Ali and associates showed that liposomal amiloride, delivered intravenously in mice, can inhibit chemically induced seizures, without producing significant hyperkalemia.

Microsomes are conceptually similar to liposomes, but are fabricated from colloidal matrices of polymers. Investigators have recommended that nanoparticles (liposomes or microsomes) should have particle diameter less than 100 nm, and should participate in receptor-mediated transcytosis across brain capillary endothelial cells in order to penetrate into brain. A better technique to enhance carrier-mediated transport across the blood–brain barrier (BBB) is to link a liposome to an antibody, such as OX26, which piggybacks on a transport mechanism for iron into the brain. Several other antibodies and transporter processes are candidates for assisting penetration of nanoparticles into brain. The antigenic targets that would be most useful for putative AEDs presently are unknown, and fabrication of liposomal antiseizure preparations can be technically challenging.

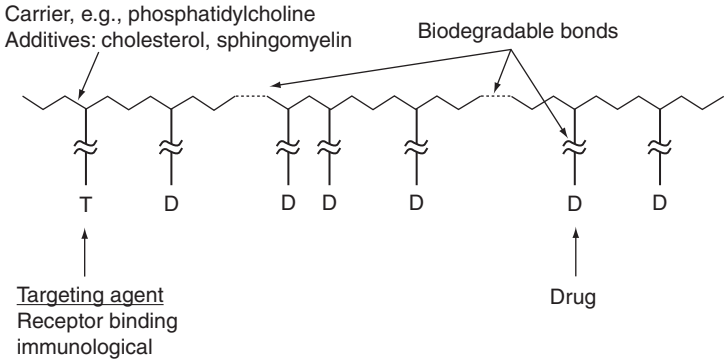


Figure 1 A general structure of a polysome or microsome. Liposomes are colloidal particles composed of phospholipid molecules assembled in a cell membrane-like bilayer or a multilayer sheet-disk configuration. Microsomes use artificial polymers, such as polysorbate to package the drug (D). A targeting agent (T) can be an affiliated receptor binding compound or antibody that will adhere to the desired target tissue.

CSF Delivery Methods

The BBB protects brain from the systemic microenvironment. Most of the AEDs in current use are lipophilic, so they can pass the lipid component of the BBB membrane. Drugs that do not cross the BBB are not suitable candidates for systemically administered epilepsy therapy. In the special case of status epilepticus, consideration might be given to using mannitol to transiently open the BBB for nonlipophilic medications. One example in the clinical literature entails use of mannitol to deliver higher doses to brain of systemically administered chemotherapy in patients with brain tumors. This approach is so far a theoretical possibility; no such candidate drug for treatment of status epilepticus is in clinical use.

The most direct way to reach the CSF is to place a catheter into ventricles or the subarachnoid space, partially bypassing the BBB. Medication can be administered directly through the catheter or by an implanted programmable infusion pump. This approach has been used in clinical practice for antibiotics, chemotherapy, spasticity, and pain. Laboratory investigations have also looked at intrathecal or intraventricular administration of AEDs. For example, intraventricularly administered phenobarbital and carbamazepine were shown to attenuate amygdala-induced seizures in rats; phenytoin inhibited seizures, but only for the first 15 min after administration. A subsequent study showed that intraventricular valproic acid inhibited both partial and generalized seizures in the amygdala-kindled rat. We have shown that fluorothyl-induced seizures in rats were delayed by a prior 5-day infusion of gabapentin into ventricle by an implanted osmotic pump.

Medication administered into ventricles does not quickly penetrate brain parenchyma. A study of ^{111}In diethylenetriamine pentaacetic acid placed in human ventricles showed that the drug reaches the cisterna magna in 5 min, the lumbar cord in 30 min, and the Sylvian cisterns in 50 min. Penetration to brain deep nuclei by passive diffusion requires several hours or longer. This observation suggests that intraventricular administration of AEDs would not be suitable for responsive therapy to a detected seizure, unless the focus were to be located very near the ventricle. Medications can be administered to the CSF space via a lumbar spine catheter, thereby eliminating risk of direct brain trauma from the procedure. However, the degree to which thecally administered drug penetrates and mixes with ventricular CSF is uncertain, since CSF flow is ventriculo-pedal. Intrathecal administration of pain medications may obviate this problem by having effects directly on spinal cord neuronal systems.

Implanted Slow-Release AEDs

The concept of implanting slow-release forms of medications into the brain was pioneered in the treatment of glioblastoma, with polymer matrix wafers containing BCNU (carmustine). Implantable biodegradable microspheres also have been studied in brain tumor patients as another method to provide sustained drug delivery; for example, 5-fluorouracil-loaded microspheres have been surgically implanted into the tumor bed of patients with an unresectable glioma. The CSF and the blood concentration of the drug were found to be negligible. Phenytoin can be conjoined to a poly-ε-caprolactone microcarrier to make stable dissolvable particles, which might be of use for treating seizures.

The wafer polymer concept has been adapted, experimentally, to treat seizures in a rat model of epilepsy produced by focal application of cobalt to neocortex. Phenytoin was prepared in an ethylene–vinyl acetate (EVAc) controlled-release polymer and surgically implanted over the seizure focus. Phenytoin was released for over a year, with a calculated total release time of 3.5 years. Implantation of polymer loaded with phenytoin reduced both EEG epileptiform activity and behavioral seizures compared with inert polymer controls. Implantation of slow-release AEDs provides potential benefits in terms of high concentrations of drug at a relevant brain site, with low concentrations – and therefore low side effects – elsewhere. The technique also has disadvantages. Drug release from the wafer is nonlinear over time. With narrow therapeutic/toxic ratios for many AEDs, this factor could pose a problem in dosing. Many of the AEDs have low potency and poor solubility in water, making it necessary to design large volume wafers. Finally, the need to replace the polymer via a craniotomy every few years is a serious practical limitation. Trials in epilepsy patients may await solutions to these concerns.

Epidural Drug

Epidural placement of a drug reservoir may be less traumatic than is subdural or intraparenchymal placement of drug. An additional advantage of epidural delivery is use of a defined space surrounded by bone and dura to assist with localization of the drug over the intended target. Disadvantages include inability to approach sites remote from the convexities of brain, and limited penetration through dura. Proof-in-principle of epidural drug administration was shown by our laboratory, and developed by others in studies of a neocortical seizure focus in rats. A cup was surgically implanted over intact parietal lobe dura, and later filled with convulsant acetylcholine or anticonvulsant phenobarbital. Starting with phenobarbital prevented subsequent acetylcholine-induced seizures. Phenobarbital administration following acetylcholine terminated seizures within 2.5 min. In another study, these investigators showed that epidural GABA could prevent acetylcholine-induced seizures, but could not terminate them once they were developed.

Human dura mater is about one-third of a millimeter thick, which would be expected to present much more of a barrier to drug penetration than does the thinner rodent dura. This barrier would obviously be a disadvantage for acute administration of antiseizure medications, but it might be less of a problem for chronic administration – where, for example, epidural medication is known to work for pain control.

Local Perfusion by Catheter

Catheters can deliver AEDs from a reservoir associated with a programmable infusion pump to the region of a seizure focus in brain. Such a strategy has, to date, been used in animal models of epilepsy but not in the clinical setting. Injection of the excitatory amino acid antagonist 2-amino-7-phosphono-heptanoate into substantia nigra, entopeduncular nucleus, or piriform cortex blocks seizures produced by pilocarpine injection in rats. Infusion of 2% lidocaine into deep prepiriform cortex of rat blocks seizures induced by bicuculline.

We have further examined laboratory methods to deliver putative antiseizure medications directly to a seizure focus. Interictal spikes in rat neocortex, produced by the GABA-antagonist bicuculline methiodide, can be reduced to about 2% of baseline levels by applying diazepam to the seizure focus via an epidural cannula (**Figure 2**). Ictal events in rats, produced by cobalt and pilocarpine, could be eliminated or delayed by diazepam applied to the seizure focus. Systemic levels of diazepam were insufficient to account for the antiseizure action. Diazepam infusion also was able to prevent epileptiform activity when administered to the region of the seizure focus in advance. Left hippocampus was implanted with an injection catheter in 15 rats, along with hippocampal and surface EEG recording electrodes. Diazepam was then injected into hippocampus, followed 5 min later by bicuculline. Compared with control vehicle, diazepam reduced numbers of interictal spikes and numbers of electrographic and behavioral seizures, and increased latency to those seizures that did occur. The naturally occurring neuromodulator adenosine, which suppresses synaptic activity via the A1 receptor, can produce a similar prophylactic action when infused via a hippocampal cannula prior to administration of bicuculline methiodide into the same cannula.

The process of focal antiseizure drug application can be coupled to seizure detection. In an experiment in our laboratory, neocortical interictal spikes and behavioral-

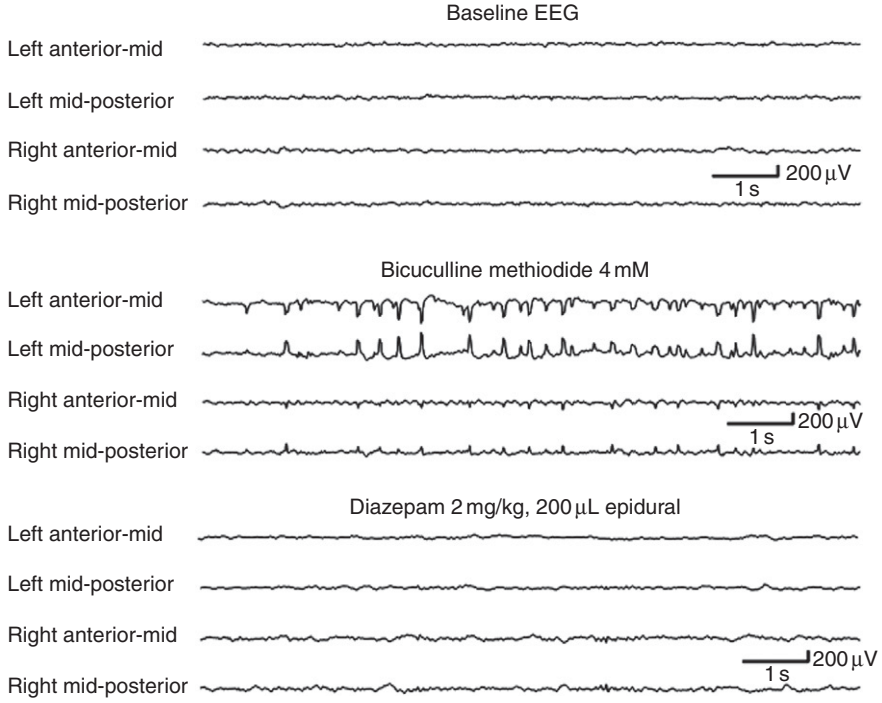


Figure 2 Each panel shows four channels of EEG recorded from bone screws implanted into rat skull, two from the left and two from the right. Anterior, mid and posterior refer to screw placement in the anterior–posterior dimension. The convulsant GABA antagonist, bicuculline methiodide (BMI) 4 mM is placed in the epidural space adjacent to the 'left mid' electrode. Compared with baseline in the top four EEG channels, administration of BMI can be seen to produce frequent left epileptiform spikes. In the bottom panel, administration of diazepam, a GABA-A receptor allosteric modulator and clinical AED, eliminates the spiking within a few minutes.

electroencephalographic seizures were created by epidural bicuculline methiodide in a rat. Bone screws placed near the seizure focus detected the ictal EEG activity by a digital seizure detection algorithm, and then triggered an external programmable infusion pump to deliver diazepam to the same epidural space. Diazepam administration occasionally shortened the detected seizure, but more often depressed regional EEG activity and eliminated emergence of other seizures (within the 40-min duration of the experiment). Mean numbers of seizures were reduced from 2.9 for vehicle-treated control animals to 0.2 for diazepam-treated animals. This paradigm might be expected to work even better if coupled with seizure-prediction algorithms that are now under development.

Reduction of epileptiform activity by local infusion of antiseizure medicines is established in principle, but several important issues need resolution before the technique is adopted clinically. First, what is the best drug, one that is both effective and safe, even if it spreads beyond the expected infusion area? Diazepam would fail these criteria, because its pH in solution is very alkaline, and because it can depress brainstem respiratory centers. A suitable drug for direct brain infusion has two advantages over usual candidate AEDs: it need not be orally absorbed and it need not be able to cross the BBB. Peptides might therefore make good candidates for focal infusion. Second, will the drug lead to tolerance or even a rebound effect, with more seizures at times of non-infusion? Continuous infusion with GABA for a few days, followed by cessation, leads to a withdrawal model of epilepsy. Muscimol, in contrast, seems not to produce such a rebound. Third, can the drug be distributed to relevant tissue? Passive diffusion is a slow process, requiring hours or days. If drug is carried away from the site of interest by bulk CSF flow or vascular uptake, then adequate concentrations may never be achieved.

Convection-enhanced delivery (CED) is a method of delivering drug by bulk flow driven by pressure, usually provided by a continuous infusion pump. Penetration into brain substantially exceeds that of simple diffusion. Chemotherapeutic agents have been administered to patients by CED. Infusion of the GABA agonist, muscimol, into the globus pallidus interna has been used without complication in a patient with Parkinson disease, to transiently relieve contralateral rigidity and bradykinesia. Distribution of muscimol was studied in monkey hippocampus after administration by an infusion pump and implanted hippocampal catheter. Autoradiography and MRI imaging of brain water demonstrated a 'teardrop' of muscimol distribution, with backflow along a space around the catheter pathway through hippocampus. Drug distribution was substantially greater than could be accounted for by passive diffusion. This backflow can be useful for drug distribution through an elongated brain structure, but if greater localization to the catheter tip is desired, then a reflux-free cannula may be of use. CED has not yet been used for treatment of clinical epilepsy.

Other Techniques

Two additional techniques, brain cell transplants and gene therapy, have potential for focal chemical delivery to a region of seizure production in brain, but are beyond the scope of this review. Those interested can see references in Further Reading.

Directions for Future Research

Several problems must be solved before local delivery of AEDs to brain will become a practical clinical approach. The main problem is one of drug distribution. Passive diffusion of a drug is very slow, and even slower if drug is metabolized in brain or taken up into venous blood. Recent experiments on convection-enhanced drug delivery may partially solve this problem. Penetration through the BBB for large liposomes, microsome, or virosomes remains a limiting factor. Recent research linking these delivery vehicles to proteins that are actively transported into brain is promising, but amount of drug so delivered remains small.

A second problem to be solved is managing possible compensatory changes in brain receptors that are exposed chronically to high concentrations of drugs. Research will be required to characterize long-term effects of drug exposure on brain excitability. Future research must show that local drug perfusion or carrier delivery does not result in a 'rebound' of increased excitability when drug delivery is interrupted.

The third major issue is selection of the best drug or drugs for local delivery. A useful drug needs to act where it is delivered and stay away from brain regions where it might have negative effects (e.g., on brainstem cardiorespiratory reflexes). Currently used AEDs are brought to market, in part, because of availability with oral administration and absence of deleterious peripheral metabolism. These concerns are not obvious limitations with optimal design of drugs for direct delivery to brain tissue. Therefore, a useful antiepileptic drug for local delivery to brain is likely to be a novel agent or an existing agent used in a new way.

Acknowledgements

The senior author (RSF) is supported by the Maslah Saul MD Chair and the Anderson Research Fund and the Susan Horngren Research Fund for Epilepsy. Additional support was contributed by DeFreyne and Littlefield.

See also: **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Resculpturing Synaptic Transmission with Neuropeptides; Focal Cooling as a Potential Treatment for Neocortical Epilepsies; Gene Therapy in Epilepsy; Immunoliposome Gene Therapy and its Potential use in Treatment of the Epilepsies; Viral Vector Approaches for Antiepileptic Gene Therapy.

Further Reading

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Pathways of Drug Diffusion into the Epileptic Brain

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Introduction

The central nervous system (CNS) is protected by physical and vascular barriers – namely, the skull, the blood-to-CSF, and the blood–brain barriers (BBB). While the skull protects the brain from mechanic insults, the BBB protects the brain from potentially harmful substances present in the bloodstream, while at the same time supplying the brain with the nutrients required for proper functions (**Figure 1(a)**). The BBB strictly regulates the brain penetration of drugs and controls the trafficking of cells of the immune system from the blood into the brain. Failure of these barriers is often associated with CNS pathologies (e.g., malformations of cortical development (MCD), brain tumors, epilepsy, and multiple sclerosis). Due to the presence of such complex, yet delicate mechanisms of protection, pharmacological targeting of CNS pathologies is challenging. Effective treatment often requires new approaches to drug delivery in order to outwit vascular barriers. Several neurological diseases are refractory to drug treatment. For example, while long-term antiepileptic drug (AED) treatment is the standard therapeutic approach to block seizure occurrence in epileptic patients, a significant number of patients consistently fail to respond to AEDs. The probability of seizure freedom becomes very low if the administration of three (or more) AEDs, given alone or in combination, fails to control seizure activity – hence the term 'multiple drug

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Pathways of Drug Diffusion into the Epileptic Brain

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Introduction

The central nervous system (CNS) is protected by physical and vascular barriers – namely, the skull, the blood-to-CSF, and the blood–brain barriers (BBB). While the skull protects the brain from mechanic insults, the BBB protects the brain from potentially harmful substances present in the bloodstream, while at the same time supplying the brain with the nutrients required for proper functions (**Figure 1(a)**). The BBB strictly regulates the brain penetration of drugs and controls the trafficking of cells of the immune system from the blood into the brain. Failure of these barriers is often associated with CNS pathologies (e.g., malformations of cortical development (MCD), brain tumors, epilepsy, and multiple sclerosis). Due to the presence of such complex, yet delicate mechanisms of protection, pharmacological targeting of CNS pathologies is challenging. Effective treatment often requires new approaches to drug delivery in order to outwit vascular barriers. Several neurological diseases are refractory to drug treatment. For example, while long-term antiepileptic drug (AED) treatment is the standard therapeutic approach to block seizure occurrence in epileptic patients, a significant number of patients consistently fail to respond to AEDs. The probability of seizure freedom becomes very low if the administration of three (or more) AEDs, given alone or in combination, fails to control seizure activity – hence the term 'multiple drug

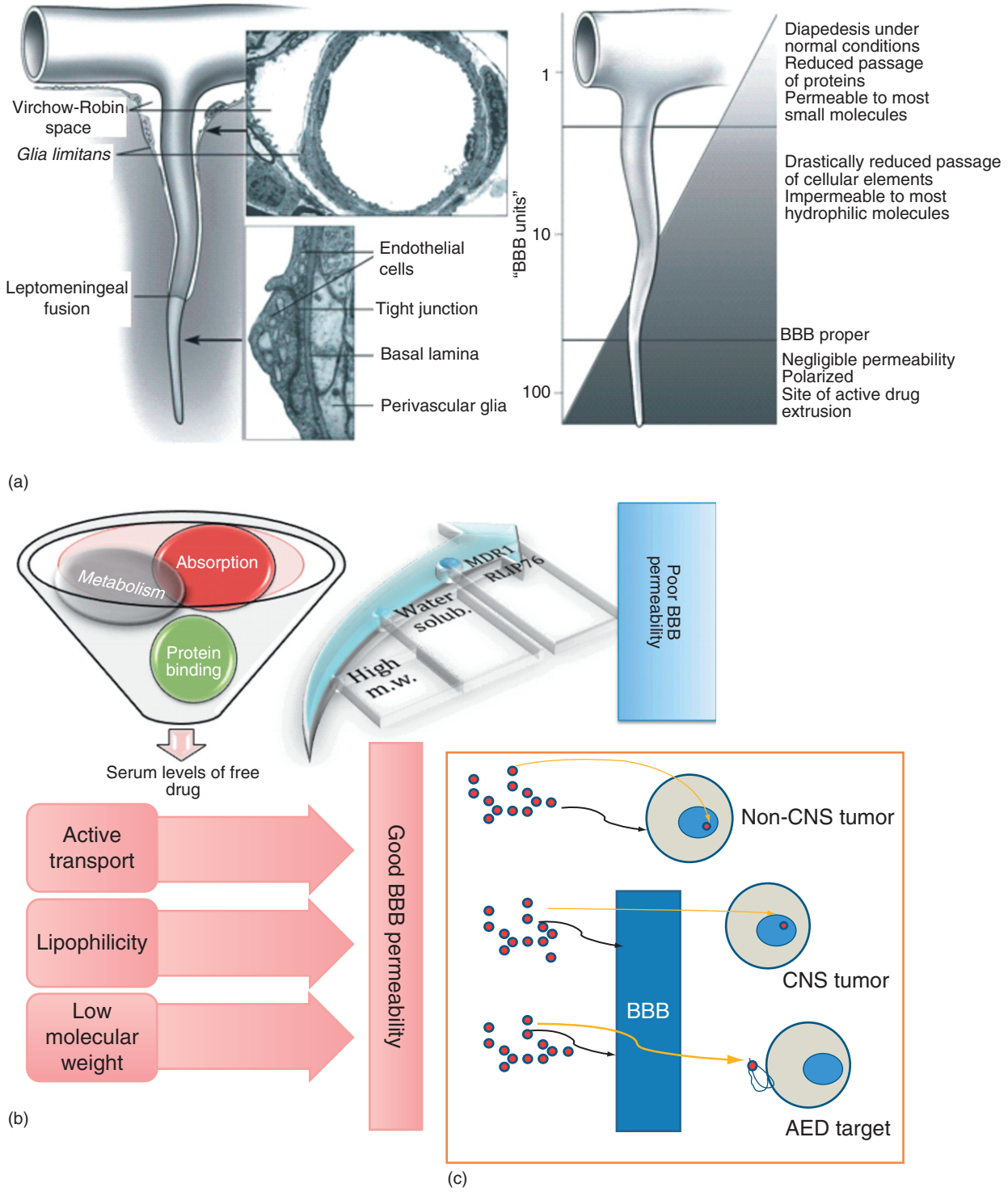


Figure 1 (a) Endothelial cells constitute the BBB. These cells are oriented perpendicular to the direction of blood flow and are exquisitely sensitive to shear stress. Several known contributory factors leading to CNS disorders are believed to act, at least in part, by direct damage to the endothelial cell layer. While the morphological appearance of brain microvascular endothelial cells at the light microscopy level is not dissimilar from their systemic counterpart, it is known today that several distinct submicroscopic and physiological features endow unique properties to these cells. Since both cerebral and systemic microvascular endothelial cells are exposed to comparable levels of shear stress, it is evident that mechanisms other than exposure to blood flow alone must be involved in the process of inducing peculiar BBB properties in undifferentiated endothelial cells. As shown in the *inset*, perivascular glial cells are ideally situated for abluminally influencing the endothelium and it is now accepted that the 'BBB organ' is constituted not only by endothelial cells, but by glial end feet as well. Current understanding of the mechanisms of neuroimmunological interactions of specialized cells with the BBB has also suggested involvement of both perivascular pericytes and microglia as active components

Table 1 Mechanisms supporting the pharmacokinetic hypothesis of multiple drug resistance

	Previous data available?	
	MDR1	*RLIP76*
Phenytoin	Substrate	Substrate
Carbamazepine	Unclear (Substrate?)	Substrate
Oxcarbazepine	Substrate	Data not yet available
10-OH carbazepine	Substrate	Data not yet available
Valproic acid	Data not yet available	Data not yet available
Levatiracetam	Data not yet available	Data not yet available
Topiramate	Data not yet available	Data not yet available
Lamotrigine	Substrate	Data not yet available
Felbamate	Substrate	Data not yet available

resistance.' In these cases, neurosurgical resection of the epileptic tissue often represents the last alternative to seizure control. In the past ten years, several new AEDs have been released, but no major reduction in the overall percentage of drug-resistant patients has been achieved. This situation underscores the nonspecificity of the drug refractory process, suggesting that a broad-range/general mechanism can affect the efficacy of all the available AEDs. In this article, we attempt to summarize the possible impediments and determinants of successful drug delivery into the epileptic brain.

Background – Drug-Resistance in Epilepsy

In the normal brain, several mechanisms regulate, directly or indirectly, penetration of drugs to their site of action in the brain (**Figure 1**). These mechanisms include metabolic transformation by specialized enzymes, binding of drugs to serum protein, drug transporters at the BBB, and transformation of 'prodrugs' into active forms. Drug resistance in epilepsy is further complicated by the variable nature of the pathophysiological process, the possible evolution of resistance over time, and the individual sensitivity to drugs (congenital or acquired). Two general hypotheses – pharmacokinetic and pharmacodynamic – have been proposed to explain refractoriness to AEDs. This article focuses particularly on the pharmacokinetic hypothesis.

Altered AED Brain Distribution – The Pharmacokinetic Hypothesis

Drug resistance in epilepsy may occur if AEDs are extruded into the vascular bed before entry into the brain. In this respect, molecular and functional studies have implicated several drug transport proteins at the BBB and blood–cerebrospinal fluid barrier as potentially important factors in the development of drug-resistant epilepsy. The pathways of drug brain penetration are dictated by a number of factors involving the intrinsic physical properties of the drugs and their interactions with 'drug resistance proteins' expressed at the BBB (**Figure 1(a)** and **1(b)**). Moreover, the BBB itself can undergo anatomical and functional modification as a result of an underlying pathology and development of disease-related symptoms. Indeed, seizures themselves may influence drug distribution in the brain parenchyma. In human epilepsy, a plethora of pathological changes have been observed in the epileptic zone and surrounding brain. These changes involve the cerebrovasculature, neurons, and glial cells. Many investigators now ascribe altered AED penetration in the brains of drug resistant patients to the over-expression of drug resistance transporters at the BBB (**Table 1**). However, due to the complexity of the epileptic pathology, other phenomena may play an important role in AED resistance.

ATP-Binding Cassette ABC Proteins

ABC-drug transporters identified in the brain are cell membrane proteins that play an important role in regulating entry into the CNS of a large variety of clinically relevant drugs (**Figure 2**). These proteins were initially discovered and characterized for their ability to confer resistance of cancer cells to chemotherapeutics. Indeed, the best-studied phenomenon of CNS drug resistance relates to brain tumor pathology. In brain tumors, mechanisms of drug extrusion similar to those observed in epileptic brain have been described. Specifically, over-expression of multidrug transporters protein (ABC-binding cassette) has been demonstrated for both tumoral cells resistant to chemotherapy and epileptic brain tissues resistant to AED treatment. In particular, over expression of *p*-glycoprotein (MDR1) was observed at the endothelium of brain

of the BBB. Of relevance is also the fact that not all brain endothelial cells form a 'BBB proper' and that a gradient of tightness exists, with passage of blood-borne cells and substances being minimal in the distal parenchymal portion of the vessels and maximal in pial vessels. Also relevant for drug diffusion studies is the fact that capillaries and presumably venules interface lumen with brain cells, while pial vessels and in general larger vessels are surrounded by CSF. (b) Pathways and determinants of drug diffusion into brain. See text for details. (c) Schematic representation of intrinsic and extrinsic (pharmacodynamic) drug resistance.

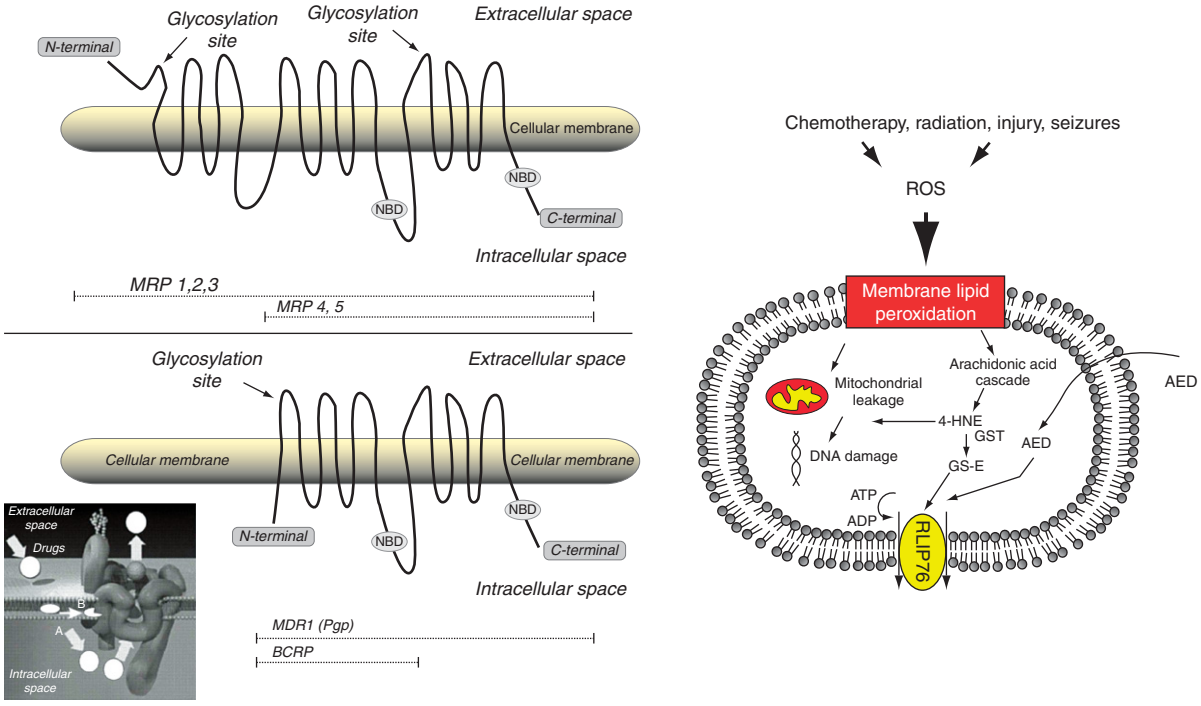


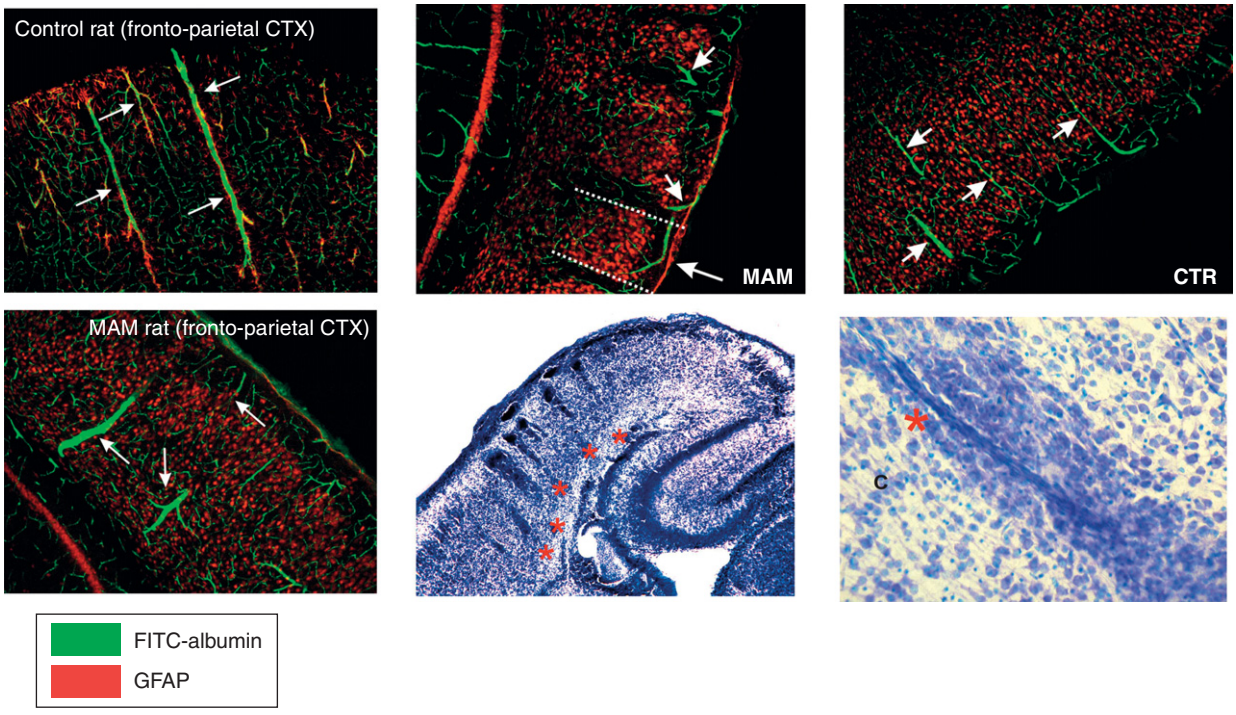
Figure 2 ABC and non-ABC transporters contribute to active drug extrusion from the epileptic brain. *Left panel:* Molecular structure of MRPs, BCRP, and MDR1 transporters. Note the variable length of transmembrane spanning elements and the 'flippase' mechanism of drug binding and extrusion (*inset*). The *right panel* depicts the hypothesis linking metabolic activity and toxic byproducts to RLIP76 (or ABC) transporters. Note that drug transport is fueled by ATP hydrolysis and is driven by intracellular events independent from the presence of the transported drug.

specimens obtained from drug resistant epileptic patients or animal models of epilepsy (Figure 2). There is, however, a significant functional difference between expression of drug transporters in tumoral cells and in refractory epileptic tissue (Figure 1(c)). In particular, while cancer cells may express 'intrinsic drug resistance' that prevents the cellular drug uptake and the subsequent interaction with the nuclear target, resistance to AEDs is more commonly ascribed to abnormally low AED accumulation in the brain extracellular space. The functionally relevant overexpression of drug transporters (limiting the AED brain penetration) occurs at the BBB level, not at the targeted cells (neurons). Further, in contrast to chemotherapic agents for the treatment of cancer cells, AED act on targets present on the membrane surface of cellular targets – so a hypothetical overexpression of drug-transporter on the neuronal cell membrane is unlikely to influence the pharmacologic effect of AED (Figure 1(c)).

RLIP76: A New Model of Multiple Drug Resistance?

Transport mechanisms for the exclusion of toxic xenobiotics and their metabolites from the cellular environment are crucial for living organisms. This trafficking of toxins and their metabolites is an energy dependent, primary active process, involving the hydrolysis of nucleotide triphosphates (ATP or GTP), while transferring substrate molecules across the cell membrane, against a concentration gradient of the substrate. While these transport mechanisms constitute an important part of the cellular defense machinery, they also pose a formidable threat to the efficacy of chemotherapy, including AED efficacy.

RLIP76 is a protein cloned previously as a Ral-effector and Ral-GAP that bridged the Ras and Ral pathways. RLIP76 also displayed GAP activity towards Rho/Rac G-proteins (Figure 2). In independent studies seeking the genetic identity of DNP-SG ATPase, a multispecific transporter protein, investigators established the identity of DNP-SG ATPase as RLIP76. The catalytic activity of RLIP76 and its function in transporting a number of xenobiotic compounds have also been demonstrated. The relevance of these findings to drug-resistance is based on the relatively promiscuous nature of RLIP76 with respect to substrate; numerous amphiphilic chemotherapy drugs can function as transported substrates. Recently published evidence supports a role for RLIP76 in drug resistant epilepsy. RLIP76 is expressed in brain tissue, preferentially in the luminal surface of the endothelial cell. RLIP76 has been shown to be capable of transporting phenytoin and carbamazepine, thus indicating a possible role of this transporter in drug resistant epilepsy (Figure 2).



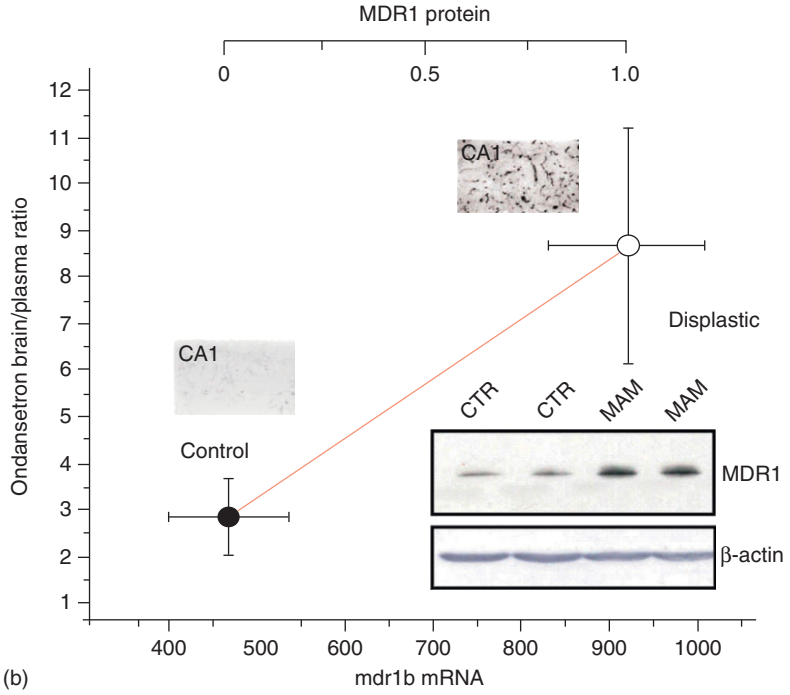


Figure 3 (a) Morphological comparison of a normal and dysplastic BBB in control and MAM rodents. The truncated vessels present in the frontal portion of the cortex are suggestive of inadequate perfusion and therefore of uneven delivery of xenobiotics present in the blood stream. Similar pattern is observed in the temporal cortex. Note how the vessels are interspersed to malformed regions, resulting in morphologically distinct regions of neurovasculature. Loss of glial, neuronal and vascular radiality is also observed in dysplastic brain. The Cresyl violet stained sections depict the appearance of early postnatal MAM brain sections emphasizing the abnormal relationship between neuronal and vascular (*asterisks*) elements. (b) Direct relationship between MDR1 expression levels and drug levels in CNS cast a doubt on MDR1's role in drug extrusion in dysplastic brain. MDR1 levels were measured by RT-PCR and western blotting in dysplastic regions of MAM-treated and control brain. Drug levels were measured by HPLC. Note that increased expression of MDR1 was associated with *elevated* drug levels, suggesting that MDR1 does not participate significantly to drug extrusions in dysplastic regions.

Methods

Prenatal Exposure to Methylaxozymethanoic Acid and Immunohistochemistry

Prenatal exposure to methylaxozymethanoic acid (MAM) at E15 interferes with proper neuronal migration and cerebral vessel formation. These structural alterations assume a characteristic phenotype (neurovascular ectopias and BBB leakage) in the adult animals. We assessed the pattern of drug brain penetration in these animals compared to controls. Interestingly, overexpression of the multidrug resistance protein, MDR-1, at the BBB was discovered in these animals. The MDR-1 substrate, ondansetron, was used to evaluate the relevance of BBB damage and MDR-1 overexpression to drug brain penetration.

For prenatal exposure to MAM, pregnant Sprague–Dawley rats received two injections of MAM (15 mg kg^{-1} of maternal body weight, i.p., in sterile saline, 12 h apart) on day E15. Prenatally exposed adult rats were anesthetized with isofluorane. After surgical exposure of the ribcage, the midline thoracic cutis was incised and the sternum opened. The apex of the left ventricle was cannulated with a 24-gauge polyethylene catheter (BDInsyte, BDVialon, Becton Dickinson, Mountain View, CA, USA), and perfused (1 ml/min) with a FITC–albumin (1% in 0.1 M phosphate-buffered saline). The right atrium was incised to prevent elevation in systemic blood pressure. Immediately following perfusion, animals were decapitated and the brains removed. Brains were fixed for 24 h in 10% formalin solution, then cryoprotected in 30% sucrose solution for 2 days. Frozen brain samples were mounted on a cryostat and cut in the coronal plane at 35 μm thickness. Sections were collected and floated in 0.1 M phosphate buffer solution (PBS) and mounted on positive-charged, glass slides (Fisher Scientific, Pittsburgh, PA). Sections were dried and processed with cresyl violet and/or fluorescence to visualize blood vessels and cells. Immunostaining was performed as described earlier (see Further Reading). The primary antibodies used were: mouse anti-NeuN monoclonal antibody (1:500); mouse anti-GFAP monoclonal antibody (1:200); the secondary antibody was Texas-Red dye conjugated Affinipure donkey anti-mouse IgG (1:100).

MDR1 Western Blot, RT-PCR and HPLC Quantification of Ondansetron (Figure 3(b))

Briefly, MDR1 mRNA levels were evaluated by RT-PCR, while protein expression was quantified by western blot and anatomically localized by immunohistochemistry. The MDR-1 substrate, ondansetron, was injected intravenously at the dose of 1 mg kg^{-1} in MAM-treated and control rats. Animals were sacrificed 30 after and blood and brain collected. Sample preparation and high performance liquid chromatography (HPLC) analysis are described in the Further Reading section. We have correlated the levels of expression of MDR-1 with the brain-to-plasma ondansetron ratio measured in MAM-treated and control rats.

Intracarotid Injection of Hyperosmolar Mannitol

A hyperosmotic bolus of mannitol is injected directly in the carotid artery, provoking a rapid opening of the BBB (within few seconds). This technique allows the study of the effect of acute BBB damage on physiological parameters such as brain electrical activity, as recorded by EEG. Adult male Sprague–Dawley rats were used for this procedure. After anesthesia, a 2–3 cm vertical incision was made from the suprasternal notch to below the chin. After exposure of the sternothyroid muscle, the common carotid artery was exposed and was separated from the posteriorly placed vagal nerve. After exposure of the bifurcation of internal and external carotid arteries, a silk tie (3-0, Ethicon, 24″) was looped around the external branch of the carotid artery (ECA), while a microclamp was positioned ready at the common artery (CCA). In temporal sequences the following steps were then performed within 2–3 min: (1) clamp the CCA; (2) ligate the distal ECA, leaving approximately 5 mm stump proximally; (3) insert the polyethylene catheter (27 gauge) into the proximal stump of the ECA towards the bifurcation; (4) tighten the silk tie around the catheter; (5) inject a hyperosmolar mannitol solution (1.4 M bolus in saline, 0.1 ml s^{-1}, 2 ml); (6) remove the catheter and ligate the proximal stump of the ECA; and (7) release the clamp at the CCA. Sham procedures were conducted using identical protocol and injecting 2 ml of sterile PBS instead of the mannitol hyperosmolar solution.

Radiolabeled Chemical Injection and Quantification

^{3}H-sucrose, ^{3}H-deoxyglucose, ^{14}C-phenytoin, and C^{14}-diazepam (1 μ Cu, Sigma Aldrich) dissolved in 500 μl of saline were injected after sham procedures (with isoosmolar PBS) or after BBB disruption (BBBD) through the intracarotid catheter. These drugs were chosen based on their dissimilar lipophilicity (sucrose and glucose being very idrophylic, diazepam and phenytoin liphophylic); therefore, they have dissimilar tendencies to distribute between plasma and brain. We have examined how this intrinsic physical property would affect drug brain distribution in the presence (or not) of a damaged BBB. Three minutes after BBBD, peripheral blood was drawn from the left heart ventricle, and the brain dissected out (including frontal, parietal, and occipital cortex and cerebellum). Radioactivity was determined by a β-counter Beckman-Coulter. Brain samples were weighted and homogenized with ice-cold solubilizing buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 150 mM sodium chloride, 60 mM octylglycoside) using a glass potter (150 μl of solution per 10 mg of tissue). Radioactivity in brain was normalized

by tissue weight. Radioactivity counted in serum was normalized by volume. Brain-to-plasma ratio for each drug was then calculated from these values.

FITC–Albumin and Fluorescent Doxorubicin Injection

A microangiographic technique based on the injection of fluorescent probes enables confocal visualization of the entire brain micro- and macrovasculature. Simultaneous observation of functional changes in BBB permeability was also possible with utilization of appropriate microangiographic molecules. To this end, fluorescent albumin was used, since albumin does not extravasate abluminally if the BBB is intact. The fluorescent albumin solution was prepared by reconstituting 500 mg of bovine desiccate albumin–fluorescein isothiocyanate (FITC–albumin, MW 69 KDa; 12 mol mol^{-1} albumin; Sigma, St. Louis, MO) in 50 ml of phosphate-buffered saline (0.1 M PBS) lacking magnesium and calcium ions. Presence of vascular leakage was evaluated by visual inspection and by confocal microscopy. A solution containing 10 mg ml^{-1} of FITC–albumin and red fluorescent doxorubicin (1 μM) was injected as described above. Doxorubicin is used as a small red fluorescent tracer (MW 540 Da).

Recent Results – BBB in Epilepsy and its Role in Mediating Drug Resistance

In addition to the overexpression of multidrug transporter proteins, other morphological and functional modifications can occur at the BBB, all of which perhaps contributing to the phenomenon of drug resistance. In the mammalian cortex, the cortical vasculature is characterized by organized large radial vessels that branch out into a homogeneous network of capillaries; in the hippocampus, a region-specific vascular density (CA1 > CA3) has been reported. Medically intractable epilepsy is often associated with malformations of cortical development (MCD). Even though the term MCD includes a number of brain pathologies, each characterized by a peculiar morphological neuronal appearance and architecture, a propensity to generate seizures is often observed in dysplastic regions. One common model of MCD is obtained by prenatal exposure of rats to the antimitotic agent methylazoxymetanoic acid (MAM). The resulting structural abnormality in MAM-exposed animals mimics the periventricular nodular heterotopias observed in human dysplastic brain. Importantly, vascular dysplasia is also observed in this model. Altered angioarchitecture in brain heterotopic areas (**Figure 3(a)**, GFAP, NeuN, and FITC–albumin staining) includes the loss of the cortical radial vessels in the frontal and temporal cortex and the presence of tortuous side-branched vessels. Regions of leaky BBB are also commonly observed in these models.

Overexpression of MDR1 in Dysplastic BBB: Overall Relevance to Drug Brain Penetration

Recently, overexpression of MDR1 was demonstrated in dysplastic regions of MAM-treated rats, leading to the hypothesis that MDR1 overexpression in heterotopic brain areas may represent a response to aberrant brain development. Overall, analysis of vasculature within heterotopic brain regions in MAM-exposed rats showed significant changes in the intrinsic expression of MDR1, vascular malformation, and the presence of concomitant BBB damage. When evaluating the functional consequences of these observations for drug brain penetration, investigators also observed a general increase in the brain-to-plasma ratio of the MDR1 substrate, ondansetron, compared to control (**Figure 3(b)**) (ondansetron is an antiemetic drug which is known to be transported by MDR1). This observation led to the apparent paradox of increased brain expression of MDR1 in the vasculature *and* increased drug levels of an MDR1 substrate in the brain. These findings suggest that in addition to MDR1, there must be other powerful mechanisms that control drug uptake in dysplastic brain.

BBB Leakage and Epilepsy: MRI Findings

Is there a link between leakage of the BBB and seizure/epilepsy pathology? Several studies have shown BBB damage during or immediately after seizures. A profound remodeling of the cerebrovasculature associated with leakage and extravasation of serum proteins, in consequence of spontaneous seizures, are observed in rodent models of temporal lobe epilepsy. Moreover, it was recently demonstrated that angiogenesis occurs in human TLE (as well as in rodent models of TLE) in consequence of seizures. In particular upregulation of VEGF in neurons, accompanied by an increase in vascular density, has been described after acute, short or long-lasting seizures. Once initiated, the angiogenic processes increase progressively, even in the absence of seizure activity, as observed during the latent period (e.g., in pilocarpine-treated rats), or after single short seizures induced by electroconvulsive shock. Conversely, it has also been repeatedly shown that BBB leakage promotes seizures or epileptogenesis. Whatever the temporal relationship between BBB leakage and seizures, it is clear that epileptic brain is characterized by an abnormal blood–brain interface.

Integrity of the BBB and presence of edema can be evaluated with MRI techniques. Specifically, structural changes at a cellular level can be assessed by diffusion-weighted imaging (DWI), which calculates the extent of passive water motion or diffusivity (apparent diffusion coefficient, ADC). Curiously, contradictory data have

been obtained when evaluating the changes in brain water perfusion in rodent models and in epileptic patients. DWI analysis in animal studies has demonstrated an early and transient decrease of water diffusivity during provoked status epilepticus or sustained seizures. Pre- and postictal human studies, using DWI or diffusion tensor imaging (DTI), have also shown (in some cases) transiently decreased local diffusivity. However, diffusion studies have demonstrated areas of significantly *increased* diffusivity, during the interictal period, in patients with partial epilepsies.

BBB Leakage, Edema, Protein Binding, and Drug Diffusion

Substantial progress has been made in the understanding of the pathophysiology and mechanisms involved in the changes of BBB permeability. In many diseases that affect the brain, the cerebral endothelium plays an active part in the disease process, with the BBB becoming disrupted, or modified, in such a way that there is a dramatic increase in vascular permeability. Theoretically, there are several ways by which various molecules can pass the endothelium, including intercellular routes, vesicular transport, or direct transcellular penetration through damaged endothelium. BBB dysfunction may be a cause or consequence of a particular disease process. Diseases in which increased BBB permeability has been reported include neoplasia, ischemia, hypertension, dementia, epilepsy, infection, multiple sclerosis, and trauma.

The effect of a disease on BBB function will secondarily affect cerebral blood flow and vascular tone in the brain, which then may further influence transport of substances across the BBB. A question of great significance is whether, in certain neuropathological conditions, the BBB disturbance constitutes the *primary* pathogenic factor, which then triggers a sequence of events molding the final pathological state. A notable consequence of BBB damage is cerebral edema, which may be classified into two major types: vasogenic or 'wet' edema, and cellular cytotoxic or 'dry' edema.

The extravasation of plasma proteins resulting from a weakened BBB causes vasogenic edema. When considering the formation of vasogenic edema, it is not clear how water extravasates through a leaky blood-brain barrier under conditions where total brain osmolarity is not substantially different from the osmolarity of serum (**Table 2**). By pure diffusion motion, protein will escape across a leaky blood brain barrier from the blood into the brain according to their chemical gradient (see **Figure 5**). However, it is very unlikely that additional extravasation of other ionic solutes will drive sufficient water to account for brain swelling, since total ionic concentrations (and therefore osmolarity) in serum and brain are very similar (**Table 2**). Since vasogenic brain swelling is nevertheless commonly observed under conditions of impaired BBB function, mechanisms other than osmolarity differential between the two tissues may play an important role. In the case of epilepsy, it is well known that a significant shift of ions, from the intracellular (neurons) to the extracellular compartment, accompanies and follows seizures. For example, during seizures, extracellular potassium accumulation reaches levels that exceed 10-fold changes from baseline. This increased extracellular osmolarity may thus drive more water from blood to brain, initiating a self-sustaining process leading to brain edema.

Table 2 Ionic concentrations and osmolarity in serum and brain

	Brain	*Serum*	*Ratio*
Total osmolarity (mOsm l^{-1})	295	295	1
Glucose (mg dl^{-1})	60	90	0.66667
Na (meq l^{-1})	138	138	1
K (meq l^{-1})	2.8	4.5	0.62222
Ca (meq l^{-1})	2.1	4.5	0.4375
Mg (meq l^{-1})	0.3	1.7	0.17647
Cl (meq l^{-1})	119	102	1.16667
pH	7.33	7.41	0.98

The early process of vasogenic edema formation may also *initiate* seizures. In the presence of a leaky barrier (in nonepileptic brain), protein extravasation occurs very early after BBBD (**Figures 4** and **5**). For yet unknown reasons, albumin extravasation itself promotes pro-epileptogenic events. These events may be sufficient to trigger seizures, followed by water influx into the brain driven by increased osmolarity of the extracellular space.

Protein Binding and pH

From the earliest observations on AED accumulation in the brain, it became obvious that binding to serum protein is a crucial step regulating the passage of AED across the BBB. pH also represents an important determinant of drug pharmacokinetic properties, influencing the drug association/disassociation with serum proteins and affecting the penetration of the drug across the blood brain barrier. For example, **Figure 4(a)** depicts a cholinergic drug that is impermeant across the BBB because of a charge present on the molecule. This charge, however, is not present in the neutral form of the molecule, which exists at equilibrium with the ionic form (these two forms of the drug coexist according to the Henderson–Hesselbach equilibrium). Only the uncharged form of the drug will permeate the BBB and accumulate in the brain. As a result, the drug levels will constantly increase in the brain, in spite of steady levels in the serum. The importance of this fact, combined with protein binding (**Figure 4(b)**), has not been fully appreciated in studies of multiple drug resistance to AEDs. A consequence of seizures is a change in brain interstitial pH. Depending on the chemical properties of a given AED, changes in pH could determine a shift in the dissociation equilibrium of the drug, therefore affecting the

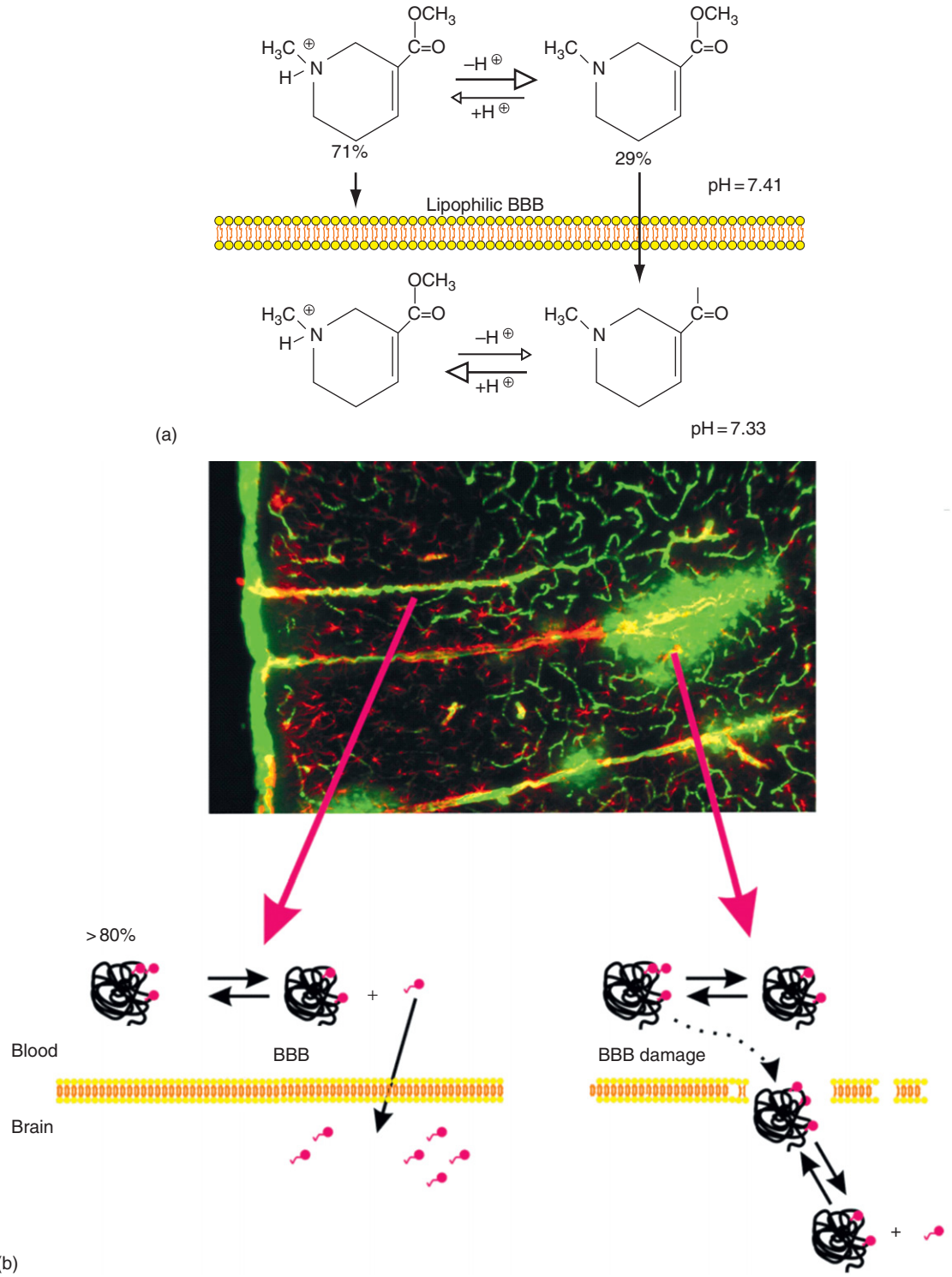


Figure 4 Effect of pH and protein binding on drug permeation across the BBB. (a) Protonation favors BBB passage of drugs by conversion into a nonpolar form. (b) BBB leakage and protein extravasation *decrease* the free drug levels if the drug is tightly bound to serum protein. In regions of intact BBB (green vessels filled with FITC–albumin; neurons are stained in *red*) the intravascular protein binding favors the unbound form since the latter is constantly removed from the equilibrium. This promotes CNS free drug levels higher than those in serum. Conversely, when protein leakage occurs, the CNS freed drug will drop to serum levels. The picture depicts BBB leakage observed in rat brain after exposure to pilocarpine.

overall binding to protein and partition across the lipophilic endothelial cell membrane.

Brain Edema and Drug Delivery in Epileptic Brain

It has been long suspected that seizures may induce cell edema. Routine MR techniques allow detection of focal cortical and hippocampal swelling after prolonged seizures in the area of the seizure focus. T_2-weighted imaging often shows focal increased signal intensity. The signal increase may involve cortical or limbic structures, particularly the hippocampus where posticta changes can be unilateral or bilateral. Similar results are obtained with diffusion weighted imaging, which usually overlaps with T_2-imaging

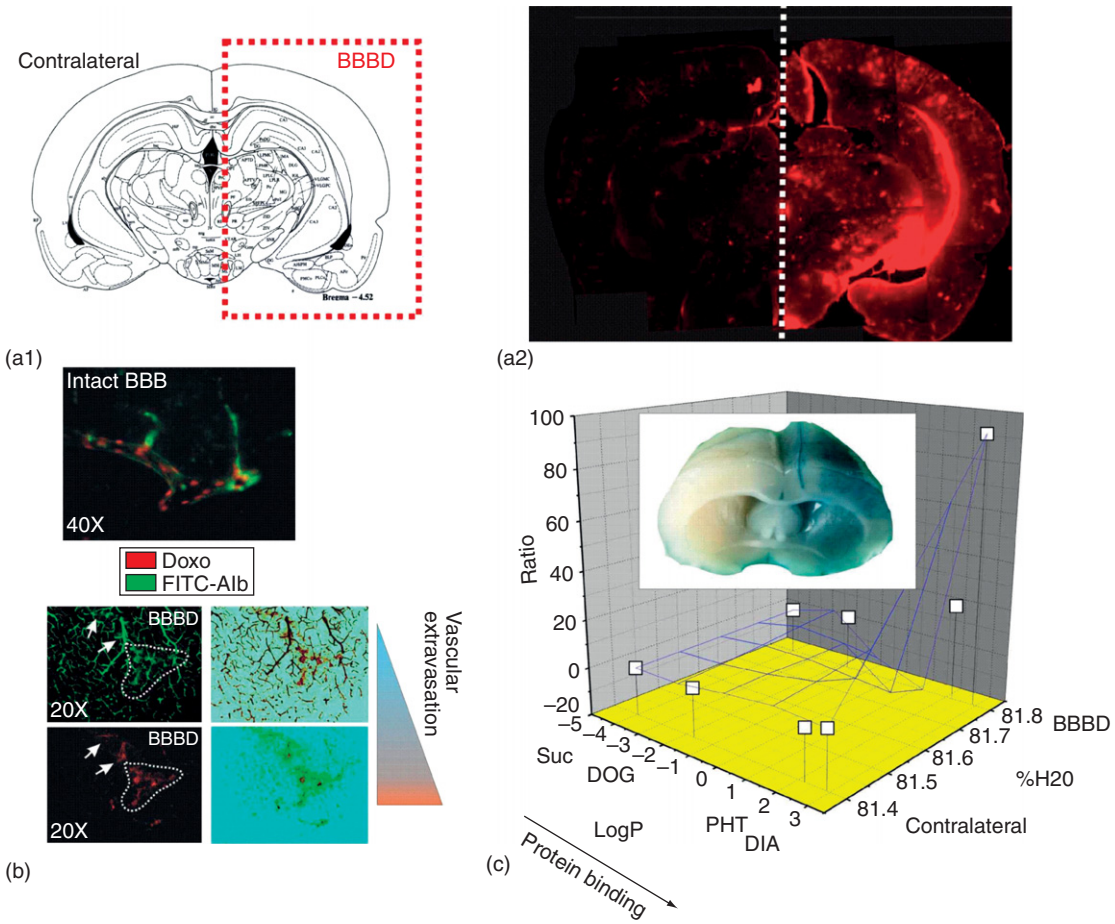


Figure 5 Effect of BBB osmotic disruption on protein and drug brain influxn. (a1–2) Hemispheric disruption promotes widespread leakage of serum albumin (in *red* due to binding to intravascular Evans Blue, which emits a red fluorescent signal). (b) Visualization of protein (FITC–albumin, in *green*) and the lipophilic drug doxorubicin (DOXO, in *red*) extravasation in regions of intact or leaky BBB. Note that intravascular protein and drug fail to migrate across an intact BBB, while protein and drug extravasate in the same spot when the BBB is breached. This is due to the tight binding of serum protein to drugs. At serum pH doxorubicin is 94% bound to plasma protein. Protein binding values (% fraction bound) are normally given as the percentage of the total plasma concentration of a drug that is bound to all plasma proteins. In most cases, binding to plasma proteins is reversible, and the concentration of the free and bound species of the drug at equilibrium may be expressed as: Free drug + Free Protein ↔ Drug protein complex. The total plasma concentration of the drug is expressed as the sum of the percent of free drug and the percent bound. Total plasma concentration = Free drug + Drug bound to protein. Depending upon whether the drug is a weak or strong acid or base, or is neutral, it can bind to a single blood protein, or to multiple proteins (e.g., serum albumin, acid–glycoprotein or lipoproteins). The most significant protein involved in the binding of drugs is albumin, which comprises more than half of all blood proteins. Albumin can interact with acidic or basic drugs in the plasma by van der Waals dispersion forces, hydrophobic bonding, hydrogen bonding, and ionic interactions. Under most conditions, the stability of the albumin-drug complex depends on pH. The drug/protein complex does not permeate phospholipid bilayers, including capillary the BBB. After much of the free drug has been cleared by the metabolic and excretory processes, the reversible drug/protein complex serves as a depot to replenish the concentration in vivo. For these reasons, drugs with high protein binding activity values tend to have a greater half-life compared to those with lower values. (c) Tridimensional graph showing the relationship between lipophilicity (log *P*), protein binding (arbitrary values indicated by the arrow), water content and drug uptake (ratio brain:plasma). Note the brain water content increased modestly after BBB disruption (BBBD) compared to the contralateral, intact hemisphere. However, under conditions of BBBD, the effect of protein extravasation (*e.g.*, part (b)) caused a massive influx of *bound* drug. Given the considerations given above, this will paradoxically result in *lower free drug levels.*

findings. Both types of imaging suggest cytotoxic edema, resulting from a metabolic mismatch between available energy and the demand to clear extra- and intracellular ion accumulation resulting from seizure activity.

MRI images have also been studied in animal models of temporal lobe epilepsy, where changes similar to epileptic human brain have been reported. Early after a seizure, changes may be detected by DWI in humans and animal models; T_2-weighted changes and swelling appear slightly later and may persist for a few days. The assumption that these changes reflect consequences of seizure activity was recently challenged by studies showing that changes seen in pilocarpine-treated animals may actually reflect brain cytotoxic or vasogenic edema occurring *prior* to epileptic seizures. It has also been shown that traumatic or chemical/iatrogenic disruption of the BBB may *lead* to seizures. **Figure 5** shows an example of brain drug permeation after osmotic opening of the BBB, induced by intracarotid injection of a hyperosmolar bolus of mannitol. Note that the disrupted BBB allows a rapid extravasation of Evans blue-proteins (in *red* in the fluorescent microscope image in A2). Also note that fluorescently labeled doxorubicin (DOXO, **Figure 5(b)**) is internalized by endothelial cells at the BBB when the BBB is intact, while extravasation occurs when the BBB is disrupted (BBBD). Panel (c) shows the effect of BBBD on the extravasation of other drugs and compounds of different lipophilicity (indicated by log *P*). These results show that:

1. Protein extravasation is an early consequence of BBBD.
2. No significant changes in water brain content take place immediately after BBBD.
3. Lipophilic drug *apparent* permeability is increased by BBBD, resulting from protein bound drugs accumulating into the extravascular compartment.

Does this situation apply to epileptic brain? This question was assessed in lithium–pilocarpine or pilocarpine models (**Figure 4(b)**). In animals injected with these agents, but *before* occurrence of status epilepticus, leakage of the BBB was observed. It is thus likely that the effects on drug accumulation in epileptic brain will follow patterns similar (i.e., apparent permeability increase) to those seen after intentional BBBD (**Figure 5(a)**). The results shown in **Figure 5** also suggest that penetration of lipophilic drugs (phenytoin and diazepam) is higher in regions of disrupted BBB integrity. Under these circumstances the extravasation of lipophilic drugs could be favored by the influx into the brain of serum proteins, the latter acting as carriers and favored by a positive serum-to-brain concentration gradient. However, CNS drugs can interact with neuronal targets only when present in the brain as free, therefore not bound to serum proteins. Such alteration of the equilibrium between free and bound drug in the CNS could affect the efficacy and the interaction with CNS targets.

Future Directions

This brief summary of the factors involved in drug permeation from the bloodstream into the brain focuses on several features that are believed to hamper the efficacy of drug treatments targeting the CNS, such as AED therapy. Multiple drug resistance to CNS drugs remains an important issue, particularly, while dealing with seizure disorders. Our data, and other considerations discussed above, shed light on the importance of brain environment and blood-brain barrier integrity in determining drug distribution to the brain. The data presented here were limited to nonepileptic animals; further studies are required to evaluate the role of BBB damage, serum protein extravasation, and changes in parenchymal water content in affecting the disposition of free AED in the epileptic brain.

Acknowledgements

This work was supported in part by the National Institutes of Health (NIH-NS43284, NIH-HL51614, NIH-NS46513, NIH-NS049514 and NIH-NS38195) to DJ.

See also: **Antiepileptic Drugs**: Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models; **Inflammation**: Cerebrovascular Diseases, Seizures and Epilepsy; **Pharmacoresistance**: Animal Models of Drug-Resistant Epilepsy; Brain Imaging of P-glycoprotein Function in Drug-Resistant Epilepsy; Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue; Mechanisms of Pharmacoresistance; Multidrug Resistance.

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Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models

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Introduction

Without a specific etiological understanding of the pathogenesis of seizures and epilepsy, approaches to drug therapy would be directed towards the control of symptoms – that is, the suppression of seizures. The treatment of choice in epilepsy is chronic administration of antiepileptic drugs (AEDs), and the treatment aim is to keep the patient seizure-free without interfering with normal brain function. A major goal in epilepsy research is to develop drugs with higher anticonvulsant efficacy and less toxicity than the existing drugs. The mechanisms of action of the currently marketed AEDs are still not completely understood. They finally involve alteration of the balance between neuronal excitation and inhibition. The immediate challenge is to establish the relative importance of individual mechanisms to the overall antiepileptic effect of each drug. During the past decades, a large number of new AEDs and improved formulations of older, 'first generation' drugs have been marketed worldwide. However, the proportion of intractable or difficult-to-treat patients has not changed substantially following the introduction of these new or improved drugs. The probability of intractability largely depends on the type of seizures and epilepsy, with complex partial seizures having the poorest prognosis of all seizure types in adults. A major obstacle in developing new strategies for treatment of pharmacoresistant epilepsy is that the biological basis of pharmacoresistance is only poorly understood. Most of the currently available AEDs and promising drugs in development have been identified by empirical screening in acute seizure models. However, it is increasingly recognized that drug targets in 'genuine' epilepsy models (i.e., models with chronic, spontaneous seizure occurrence) are modified or completely changed during epileptogenesis. Thus, there is a growing trend to test drugs in animal models that resemble specific types of epilepsy. This more focused approach allows development of new compounds with higher selectivity and efficacy for the altered targets in the epileptic brain. Furthermore, the study of the pathophysiological processes in epileptogenesis in proper animal models may yield new concepts for drug development. This article deals with current concepts of anticonvulsant drug action, new pharmacological targets and approaches (pharmacokinetic–pharmacodynamic (PK–PD) modeling), methodological aspects in determining the ultimate in vivo efficacy of drugs in specific animal models of epilepsy, and rational design of synergistic drug combinations.

Background

AEDs: Neurochemical Profiles and Mechanisms of Action

At the cellular level, three basic mechanisms of action of AEDs are now recognized: modulation of voltage-dependent ion channels (Na^+, Ca^{2+}, and K^+); enhancement of inhibitory neurotransmission (principally by GABA); and attenuation of excitatory (particularly glutamate-mediated) transmission. It is increasingly apparent that many of our AED compounds possess multiple mechanisms and cellular effects. Since epilepsies are, by definition, a group of disorders rather than a single disease entity, it is not surprising that the currently employed AEDs display such a range of pharmacological actions. In addition, many

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less well-characterized mechanisms may also contribute to the anticonvulsant effect of a given compound.

Excitation and inhibition are considered the prerogatives of glutamate and GABA. We, among others, correlated different types of chemically and electrically induced limbic seizures in rats with substantial increases in extracellular hippocampal glutamate and GABA levels caused by increased network activity during the initial stages of a seizure. These data are consistent with earlier human microdialysis studies in which seizure-associated increases in extracellular hippocampal glutamate and GABA were reported. These neurochemical changes presumably have a profound impact on the further course of a seizure. Increased glutamatergic excitation is considered a major cellular mechanism underlying seizure initiation, spread, and maintenance, while increased GABAergic inhibition is widely viewed as an anticonvulsant response in the epileptogenic tissue. We have used these neurochemical changes routinely as a biomarker for increased network activity, and have been able to demonstrate that a wide variety of AEDs (phenobarbital, carbamazepine, valproate, lamotrigine, and topiramate) and experimental compounds with anticonvulsant properties (adenosine-1 agonists, kainate antagonists, a group I metabotropic glutamate antagonist, angiotensin IV, somatostatin) suppress pilocarpine-induced increases in extracellular hippocampal glutamate and/or GABA. Moreover, we recently demonstrated that while increased hippocampal extracellular glutamate concentrations do not induce seizures per se, a pre-existing elevation in extracellular glutamate may aggravate seizures induced by subsequent intrahippocampal administration of a chemoconvulsant. However, whether glutamate and GABA increases can indeed effectively modulate seizure generation and spread – or are only an epiphenomenon – remains controversial. Studies in animal seizures models have often yielded conflicting data; sometimes no changes or even a decrease in glutamate and/or GABA were reported during seizures. Moreover, activation of different glutamate receptors subtypes differently affect seizure activity.

Monoaminergic receptors are widely distributed throughout the brain, and are expressed in almost all networks implicated in the regulation of seizures. Activation of dopamine, serotonin, and noradrenaline receptors is known to modulate seizure susceptibility in epilepsy models (both in vitro and in vivo). Despite convincing data, this class of neurotransmitters has not received much attention. Perhaps the lack of consensus in the field about which receptors and signalling pathways mediate the anticonvulsant effects may contribute to this relative lack of interest.

In recent years, more selective agonists and antagonists have become available, allowing a more reliable evaluation of the role of different monoaminergic receptor subtypes in seizure control. Recently, we demonstrated that increases in exogenous hippocampal dopamine or serotonin levels can have profound anticonvulsant effects in the focal pilocarpine model for limbic psychomotor seizures. These anticonvulsant effects were restricted to a well-defined anticonvulsant concentration range and were shown to be mediated by D_2 and 5-HT_{1A} receptor stimulation. Subsequently, we showed that GBR-12909, a highly selective dopamine reuptake blocker, and citalopram, a selective serotonin reuptake blocker, were anticonvulsant in the focal pilocarpine model. At the anticonvulsant threshold concentrations for both these compounds, significant increases in respectively endogenous hippocampal dopamine or serotonin levels within the previously determined anticonvulsant concentration ranges were observed. Moreover, our group was able to demonstrate that hippocampal dopamine and serotonin increases are an important constituent of the anticonvulsant mechanism of action of oxcarbazepine and its active metabolite 10,11-dihydro-10-hydroxycarbamazepine. These transmitter changes were defined as biomarkers for the anticonvulsant efficacy of these compounds. Our data are consistent with previous results showing that therapeutically relevant concentrations of many marketed AEDs, and of experimental compounds with antiepileptic properties, increase extracellular levels of dopamine and/or serotonin in brain areas involved in epileptogenesis. Preliminary results from our group also indicate that potent anticonvulsant effects can be achieved by increasing noradrenergic signaling in the hippocampus.

Genetic approaches have been increasingly coupled with physiological and pharmacological approaches in epilepsy research. Transgenic and knockout mice with modified monoaminergic signaling have provided new models for the study of the monoaminergic system in epilepsy. Currently, the role of at least D_1, D_2/D_3, $5HT_{1A}$, $5HT_{2C}$, $5HT_3$, 5HT7, α_1, α_2, and β subtypes of dopamine, serotonin and noradrenalin receptors in epileptogenesis and seizure propagation has been described using such models. However, discrepancies between the different experimental approaches, as well as differences in experimental results, hamper the interpretation of the relationship between neurochemical alterations and seizure activity – and this relationship merits further research. It appears that each modification of the factors that characterize excitation and inhibition is model-, time-, area-, and pathway-dependent. Some modifications appear as proepileptic within one brain region, experimental model or neuronal pathway, while others will appear as antiepileptic. Due to the large number of factors that can be modified, the resulting behavior of the neuronal network is difficult to predict.

Methods

Upon its introduction in the early 1970s, the microdialysis technique rapidly became a widely accepted method in

the neurosciences. This technique has made the extracellular brain compartment experimentally approachable without causing major physiological and anatomical disturbances in neuronal tissue.

Microdialysis probes can collect endogenous neurotransmitter substances, released from nerve endings in the vicinity of the probe, as well as drugs that reach the brain following systemic administration (**Figure 1**). This process takes place at the distal end of the microdialysis probe where the physiological perfusion fluid equilibrates with the surrounding extracellular tissue fluid via passive diffusion down a logarithmic concentration gradient. As the direction of the concentration gradient is governed by the difference in concentration through the membrane, microdialysis can also be used to administer exogenous substances (local delivery), while endogenous compounds are concurrently recovered. These features make the in vivo intracerebral microdialysis technique an elegant tool to unravel unidentified mechanisms of action and identify 'the mechanisms that matter' of marketed AEDs. Since the extracellular space is the biophase for drugs acting on cell-surface-bound drug targets, the microdialysis technique enables the investigator to continuously monitor the effect of AED administration – e.g., on extracellular endogenous components such as neurotransmitters, neuropeptides, etc. Insertion of a microdialysis probe into the seizure focus allows the investigator to characterize the neurobiological and metabolic changes that accompany seizures. The dialysate is obtained before, during and after drug treatment, so that each animal serves as its own control. Brain accessibility and blood-brain barrier passage of systemically applied drugs can be studied, giving crucial information concerning target site drug pharmacokinetics. Simultaneously their pharmacodynamic effects can be evaluated.

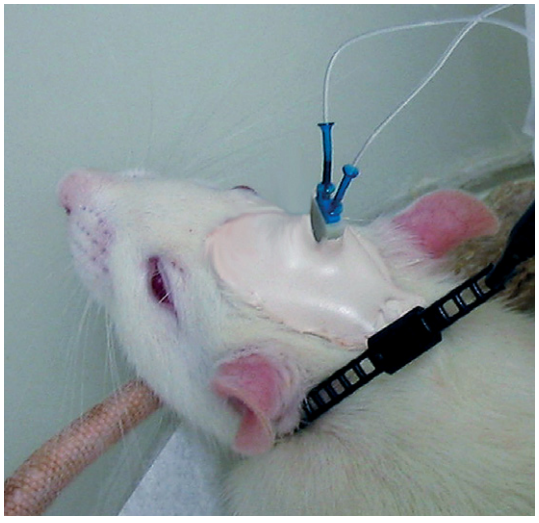


Figure 1 Freely moving rat with stereotactically implanted microdialysis probe in the dorsal hippocampus.

The term 'pharmacokinetics' (PK) refers to the time-concentration relationship of a drug in the body, and encompasses absorption, distribution, metabolism and excretion processes. Pharmacodynamics (PD) describes the mechanisms by which drugs exert their biochemical and physiological effect in the living organism. Although the magnitude of the pharmacological effect is related to the concentration at the site of action, in the past PK and PD were often studied and used separately for optimizing dosing recommendations. However, the usefulness of these disciplines is limited if viewed in isolation. PK–PD modeling links the concentration-time profile to the intensity of a pharmacological response, usually expressed as a biomarker or surrogate end point. It therefore allows better description of the complete time course of the desired and/or undesired effects in response to drug therapy. It enables the elucidation of causative relationships between drug exposure and response, and the characterization of the sequence of events leading to the observed drug effect. Mechanistic and empirical PK–PD models are available. These models can be descriptive and/or predictive of the time course of PD effect. Predictive models, when properly developed, may be used to guide or adjust doses and regimens, add evidence to the certainty of the decision to allow market access, and provide a better understanding of outcomes from clinical benefit studies. Population PK–PD modeling is primarily based on the nonlinear mixed-effects regression models, and allows the characterization of dose-concentration-effect relationships in populations rather than individuals, thereby providing investigators with the opportunity to identify and account for sources of inter-individual PK and/or PD variability.

Recent Results

New Pharmacological Targets and Approaches

The growing understanding of the pathophysiology of epilepsy, including the breakthroughs in genetics that have defined the fundamental defects of many epilepsy syndromes, and the structural and functional characterization of the molecular targets, provide new opportunities for rational AED development. Potential antiepileptic targets include voltage-gated ion channel subunits (α2-δ Ca^{2+} channel subunits, A- or M-type voltage-gated K^+ channels, α Na^+ channel subunits, hyperpolarization-activated cyclic nucleotide-gated cation channel subunits), SV2A synaptic vesicle protein, connexins, plasma membrane and vesicular glutamate and GABA transporters, and glutamate and GABA receptors. We screened different subtype-selective ionotropic and metabotropic glutamate receptor ligands in animal epilepsy models, and monitored the concomitant neurochemical changes in extracellular hippocampal

glutamate and GABA levels as biomarkers for increased network activity. The subtype-selective GLU_{K5} kainate receptor and metobotropic mGlu1a and mGlu5 antagonists were shown to completely suppress complex partial seizures, while mGlu2/3 agonists only offered partial anticonvulsant effects.

Neuropeptides are appealing targets for the study of basic mechanisms of seizure initiation and arrest, and for the development of novel approaches for epilepsy treatment. They are modulators of the traditional neurotransmitters, contained in large dense-core vesicles within neurons and in brain areas involved in epileptogenesis. Since neuropeptides are preferentially released in response to bursts or high frequency firing neuronal firing, neuropeptide systems seem extremely relevant targets for epilepsy research. Seizure activity causes important region-specific temporal variations in expression of virtually every neuropeptide (and related receptors), as seen dramatically in the hippocampal formation. In a recent pharmacological study, we reported the anticonvulsant properties of angiotensin IV and somatostatin-14. The effects of both angiotensin and somatostatin were mediated via somatostatin receptor-2 activation. In accordance with our earlier data showing the importance of the monoaminergic system in seizure suppression, hippocampal monoaminergic transmission was demonstrated to be an important constituent of the anticonvulsant mechanism of action of both peptides. A substantial amount of evidence has implicated neuropeptide Y in the control of seizure activity. Its anticonvulsant activity is generally accepted to arise from a negative modulatory effect on glutamatergic synaptic transmission via presynaptic Y_2 receptor-mediated inhibition of voltage dependent Ca^{2+} currents. In a recent study we demonstrated that neuropeptide Y-mediated increases in hippocampal dopamine is a complementary anticonvulsant mechanism of action. The hippocampal dopaminergic effect was shown to be mediated via sigma 1-receptors activation. During the past decade, many other neuropeptides, such as galanin and dynorphin, have emerged as powerful endogenous anticonvulsant peptides and/or neurotropic factors that modulate seizure-associated neurodegenerative and neuronal plastic changes. Due to degradation by peptidase-catalysed breakdown and sparse blood-brain barrier permeability, peptides are generally poor candidates for future therapeutic agents. However, non-peptidergic ligands for peptide receptors may offer new opportunities as potential antiepileptic treatment strategies.

One of the mechanisms that has emerged as a credible potential factor in refractory epilepsy is impairment of drug penetration into the brain by efflux transporters. It has been hypothesized that over-expression of efflux transporters at the blood-brain barrier prevents AEDs from reaching sufficiently high brain concentrations, despite adequate plasma levels. Certain efflux transporters have been found to be transiently upregulated (over-expressed) following experimentally-induced seizures. It is of major clinical interest to evaluate whether AEDs are substrates for these transporters. If that is the case, over-expression of multidrug transporters in epileptogenic brain tissue is likely to result in reduced drug penetration into brain parenchyma and could critically contribute to pharmacoresistance in epilepsy. We evaluated the impact of p-glycoprotein and multidrug resistance-associated protein blockade on the brain distribution of oxcarbazepine and its active metabolite. Efflux transporter impairment resulted into increased extracellular brain levels of both compounds, as measured by intracerebral microdialysis. Also, the pharmacodynamic effects on hippocampal monoamine levels were potentiated, resulting in increased anticonvulsant activity. Over the last years, most of the marketed antiepileptic compounds were proven to be efflux transporter substrates (with exception of levetiracetam). Thus, co-administration of AEDs and efflux transporter blockers may be a new potential treatment strategy.

Alteration of Drug Efficacy in Epileptic Animals

Two hypotheses have been put forward to explain the phenomenon of pharmacoresistance. The first states that the drug does not reach the target in sufficient quantity, as has been ascribed above. The second states that drug targets in the brain have been altered in a way that they do not respond to the administered drug. Indeed, ample evidence can be found in the literature for changes in ion channel properties, as well as in GABA, glutamate and other receptors. If the target has disappeared altogether or has changed so drastically that it does not respond to the drug under investigation, it is obvious why the drug does not exert an antiepileptic effect. However, it should be noted that in every type of epilepsy, whether responsive to the current armentarium of AEDs or not, there may be differences – compared to the non-epileptic brain – in properties of receptors, ion channels, and other targets. These differences may appear small, and only moderately affect interaction of the drug with its target. However, their net effect on brain excitability may nevertheless be significant.

Unfortunately, most of our AED drug candidates are screened primarily in models of acute seizures induced in essentially non-epileptic brain, and the results from these tests may be misleading when extrapolated to the types of epilepsy for which the drugs were designed. Pharmacological investigations should preferably be carried out in animal models that properly reflect the type of epilepsy of interest. In fact, the same principles apply when mechanisms of drug action are investigated in in vitro preparations such as hippocampal slices. When selecting an animal model for pharmacological studies, or perhaps

even developing a new model, the relevance of the model for the type of epilepsy of interest is receiving increasing attention. Several interesting models now exist. The most widely used model is induction of status epilepticus by chemical or electrical means in rodents, which develops in a matter of weeks into spontaneous epilepsy. The associated neurochemical changes and synaptic reorganization in the animals closely mimic those seen in mesial temporal lobe epilepsy in humans. But there are other important model systems. Genetic models (e.g., GAERS and Wag/Rij rats) display spontaneous seizure resembling absence epilepsy and exhibit a similar pharmacology. Hyperthermia induced in immature animals can elicit complex febrile seizures. And rat pups exposed in utero to methylazoxymethanol acetate (MAM) develop dysplasia-associated epilepsies. Validation of a model is an important issue. Therefore, the selected animal model should display a profile of responsiveness to AEDs similar to the human type of epilepsy under study.

The ultimate proof of efficacy of a drug is its capacity to suppress spontaneous seizures. However, reliable determination of a change in seizure frequency can pose a major problem. For example, it is known that during status epilepticus, the anticonvulsant effects of diazepam and phenobarbital rapidly decrease. Whether this process continues in the latent period following status epilepticus is an important question, but measuring changes in frequency is not an option because, by definition, seizures do not occur in this period. To allow assessment of progressive decreases in anticonvulsant effect of a drug during the latent period, one needs to elicit seizures artificially or use a quantitative method that reflects the mechanism underlying the anticonvulsant action (e.g., enhancement of GABAergic inhibition).

An attractive method for determination of seizure threshold is to stimulate the brain with ramp-shaped electrical pulse trains. Stimulation up to the threshold determination lasts only 10–15 s and the procedure can be repeated at intervals as short as 5 min. Therefore, this method allows determination of the time course of drug action. An alternative is the use of a biomarker that either reflects the interaction of the drug with the target or the seizure susceptibility of the brain. The intrinsic efficacy and potency of benzodiazepines to enhance $GABA_A$-mediated inhibition, for example, is reflected in the increase in the β-frequency band in the EEG. This effect provides an extremely convenient measure of pharmacological effect, because the EEG can be recorded continuously, without the need to elicit seizures. Using this method, it was possible to demonstrate that midazolam almost completely looses its efficacy during the latent period following status epilepticus. Other useful biomarkers include endogenous molecules or metabolites that can be measured in the brain by microdialysis, MRI, or another appropriate method. It is important that, when using this type of biomarker, its relation to the anticonvulsant effect is thoroughly validated.

Characterization of Concentration-Effect Relationships of AEDs: Pharmacokinetic and Pharmacodynamic Modeling

It seems trivial to state that the efficacy of a drug does not only depend on the interaction with its target but also on the PK of the drug. Nevertheless, it is surprising how little attention is paid in the literature to this important aspect of drug efficacy. Searching for synergistic drug combinations revealed that more than half of the studies ignored brain concentrations altogether. Absorption from the gut, metabolism in the liver or elsewhere, renal excretion, distribution to various tissues, passage over the blood-brain barrier (and vice versa) determine how much or how little of the AED will reach its target in the brain. PK can be affected by interaction with other drugs, chronic medication, or disease progression. Induction of liver enzymes and upregulation of efflux transporters at the blood-brain barrier are obvious examples of mechanisms that contribute to a drug's PK. Thus, it is in general insufficient to determine the time of peak drug effect in a control animal and assume that this value is also valid under pathological conditions.

Because of these obvious difficulties, an integrated study of the relationship between the PK and PD (PK–PD) of a drug can be a powerful tool in characterizing drug effects. First of all, PK–PD modeling provides quantitative information about the range of concentrations over which a drug is effective. More interestingly, PK–PD modeling can be used to compare efficacy in different models of epilepsy, to establish the influence of age and gender, to study the influence of disease progression or the efficacy of drug combinations, to confirm or reject whether effects observed in vitro indeed contribute to the in vivo effect, and so on. Advanced PK–PD-modeling is a discipline in itself, but it begins with well-controlled dosing, and measuring effects and concentrations in plasma simultaneously. If possible, target tissue concentrations should be measured as well.

The concentration-effect relations of drugs acting on $GABA_A$-receptor-mediated inhibition have been studied in this way, using the increase in β-activity as the pharmacodynamic endpoint. By giving a bolus dose sufficient to saturate all receptors, and measuring EEG during the elimination of the drug, the whole range of concentration and effect can be covered in a single experiment. For this purpose, the rapid elimination of drug in rodents is a considerable advantage. Using this approach, we found that the concentration-effect relation of benzodiazepines and tiagabine could be described by a sigmoidal E_{max} model, and that their in vivo potency (EC_{50}) and efficacy

(E_{max}) correlated well with the in vitro affinity and efficacy. In kindled animals and in rats with spontaneous absences, midazolam exhibited the same potency but the efficacy was 25–30% lower than in control animals. In rats subjected to status epilepticus by injection of kainic acid, the decrease in efficacy was more dramatic, progressing from 50% at day 4 after status to 75% at day 14. In contrast, tiagabine was equally effective in control animals, kindled animals (perhaps even more effective), and in kainic acid-treated rats. Alphaxalone, a neurosteroid also acting on $GABA_A$-receptors, even exhibited a slightly increased efficacy in kainic acid-treated rats. This result suggests that epilepsy (and/or its development) selectively modifies specific $GABA_A$-receptors by altering subunit composition and thereby the effect of different drugs. This idea is consistent with numerous histochemical and electrophysiological studies.

Another application of PK–PD modeling is to use parameters derived from the modeling in order to monitor disease progression. For example, using PET, we have been able to determine specific binding (B_{max}) and affinity (K_D) for flumazenil (**Figure 2**). As PET is a non-invasive technique, it offers the opportunity to monitor the time course of changes in receptor density and affinity in relation to epilepsy development, anti-epileptogenic treatment, etc. Also, it should be noted that effective concentrations in animals and man are often remarkably similar, despite huge differences in required dosages. In other fields, PK–PD modeling has been used successfully to predict efficacy in humans from pre-clinical data, and such approaches should also be used for epilepsy research. For inspiration the reader should consult the literature on PK–PD modeling.

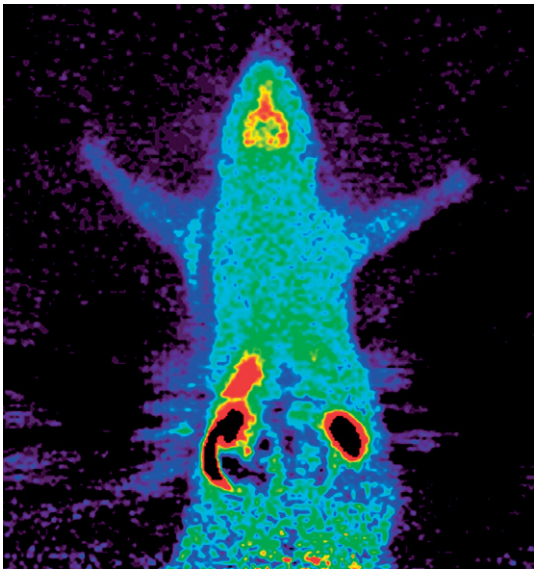


Figure 2 Positron Emission Tomography in a rat. Uptake of a tracer amount of ^{11}C-flumazenil in the brain can be easily seen and reflects mainly binding to the $GABA_A$ receptor. Combined with plasma concentration data, PET data have been used to model plasma and brain pharmacokinetics of flumazenil and to determine K_D and B_{max}.

Synergistic and Antagonistic Interactions of AED Combinations

From a therapeutic point of view, selective compounds might be interesting because of their potentially limited side-effect profile. However, it has been suggested that the failure of several rational therapeutic strategies, aimed towards more selective drugs, is related to the fact that absolute selectivity for one target may not be desirable for a multifactorial disease such as epilepsy. Most clinically efficacious AEDs act by a multitude of mechanisms, so that a rational combination of mechanisms in a single drug – e.g., generated by combinatory chemistry – may be a more successful strategy for creating novel broadly-acting AEDs than the development of highly selective compounds.

Nevertheless, highly selective compounds remain important tools for investigation. To design single drugs combining multiple mechanisms, one should ideally know which mechanisms to combine. On theoretical grounds, it can be expected that combining compounds acting at different targets provide the highest chance of creating an effective interaction, where the benefit would reflect a synergism for the anticonvulsant effect or antagonism for adverse effects. For this purpose, examining the combined effect of highly selective compounds would be ideal, because it will allow identification of the proper targets.

Characterization of the efficacy of combinations of two drugs, let alone three or more, is an experimental challenge, and interpretation of the data is fraught with pitfalls. Combinations may be synergistic only over a limited concentration range, or even more complex, be synergistic over one range and antagonistic over another. Consequently, complete characterization of the interaction requires investigation of the entire concentration range over which the drugs are active. This task may become prohibitive when only one effect point can be measured in an individual animal. Furthermore, pharmacokinetic interactions, choice of the animal model, and method of analysis can influence the outcome of the study. However, if it is possible to determine multiple effect points and concentration data in individual subjects, complete characterization becomes feasible. Furthermore, sophisticated study designs, developments in methodological tools such as surface response analysis (which allows examination of interactions over a range of concentrations), and population PK–PD analysis (which takes into account, and actually takes advantage of, various sources of variability) have come to the investigators' aid.

Given the fact that many patients clearly benefit from combination therapy, it is likely that favorable

interactions exist. Nevertheless, sound clinical evidence to support this view is still lacking. In experimental studies, the number of combinations that can be made is unlimited, and many claims for synergistic anticonvulsants can be found in the literature. In about half of the experiments, the PK are not considered and it is therefore impossible to conclude whether pharmacodynamic interactions occurred in these experiments. Considering only the more properly designed studies, it is not yet entirely clear which properties in an anticonvulsant drug favor synergism. Nevertheless, some trends have become visible. The majority of studies, in which AMPA antagonists (and also NMDA antagonists) formed part of the combination, reported synergistic effects. As the AMPA and NMDA antagonists were combined with agents either acting on multiple targets or blocking Na^+-channels, there is still no definitive proof that postsynaptic glutamate receptors favor synergism, but these receptors are certainly good candidates. Similar results were obtained for compounds acting on presynaptic glutamate receptors, but only a few studies have been carried out with these compounds.

There is accumulating evidence that synergism can occur in a restricted range of concentrations. This understanding was demonstrated quite early for a number of registered AED combinations. Increasingly, brain concentrations are also taken into account in such combinatorial analysis. In a number of cases where favorable interactions were observed, the synergism had to be attributed to an increase in brain concentration, rather than a pharmacodynamic interaction. Such results indicates that brain PK (regional distribution, metabolism, blood-brain barrier transport) may have a decisive influence. Combinations may not only suppress seizures in an all-or-none manner. In a study with tiagabine and lamotrigine, we demonstrated by quantitative behavioral analysis that over a limited concentration range, certain seizure components were selectively suppressed. This result illustrates that the nature of seizures, and thereby their severity, can be modified by drug combinations. Finally, optimization of rational drug synergism may be more effective in controlling seizures than either drug alone. It is encouraging that increasing attention is paid to potential adverse effects when using drug combinations. This concern is important, because combinations will be successfully introduced into clinical practice only when synergistic anticonvulsant effects are not spoiled by increased adverse effects.

Conclusion – Directions for the Future

Despite the substantial progress in understanding the pathophysiology of the epilepsies, many patients remain refractory to currently available pharmacological treatments. More effective, innovative therapeutic agents are therefore urgently needed. In the future, screening in animal models will probably remain the engine that drives AEDs discovery. Inclusion of truly epilepsy models, which closely mimic the human disease state in a battery of tests for early stage characterization, is necessary for the further unraveling of the processes involved in epileptogenesis and the identification of new potential pharmacological targets. Additionally, a complete characterization of the primary and secondary mechanisms of action of existing AEDs, and the recognition of the antiepileptic 'mechanisms that matter' may offer new opportunities for rational (combinatory) drug design and optimization of rational drug synergism. The development and validation of biomarkers, which can be used to monitor the natural course of epileptogenesis as well as anticonvulsant activity of drugs, and advances in pharmacokinetic-pharmacodynamic modeling, are indispensable for optimizing the analysis of the in vivo efficacy of new pharmacological interventions and treatment strategies.

See also: **Antiepileptic Drugs**: Antiepileptic Drug Mechanisms; Pathways of Drug Diffusion into the Epileptic Brain; Preclinical Drug Development in Epilepsy; **Models**: Seizure Models in Acute and Organotypic Slices; **Pharmacoresistance**: Animal Models of Drug-Resistant Epilepsy; Multidrug Resistance.

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Preclinical Drug Development in Epilepsy

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Introduction

Despite the introduction of 11 new antiepileptic agents over the past 20 years, and unprecedented advances in our knowledge of how those drugs work at the cellular level, at least 30% of epilepsy patients continue to experience seizures on otherwise optimal therapy. Even amongst those patients who achieve seizure control, a significant proportion can expect dose-related adverse effects to impact upon their quality of life. For the most part, modern antiepileptic agents are associated with fewer and less severe side effects; they have simpler pharmacokinetics and fewer drug interactions; but they have largely failed to improve the outcome, at least in terms of efficacy. As a result, still more new antiepileptic drugs are required.

Background – Drug Discovery

In order to understand why recent drug development in epilepsy has been relatively unsuccessful, it is important to review the strategies employed in each case. Starting with vigabatrin, which was first licensed in the UK in 1989, and ending with rufinamide, the most recent addition to the antiepileptic armamentarium, three principal approaches are evident: target-orientated design, structural modification, and random screening.

Amongst modern antiepileptic agents, vigabatrin and tiagabine are the sole products of target-orientated design, fulfilling their intended purpose of enhancing γ-aminobutyric acid (GABA) mediated neurotransmission by selective inhibition of GABA metabolism via an action on the enzyme GABA-transaminase, and of GABA re-uptake via an action on the GAT-1 transporter, respectively. Other new drugs are products of a structural modification strategy which is based on chemical adaptation of both exogenous and endogenous neuroactive compounds. For example, gabapentin and pregabalin are analogs of the inhibitory neurotransmitter GABA, and oxcarbazepine is a carbamazepine derivative, designed to bypass the epoxide metabolite believed to be responsible for many of the adverse effects of the parent compound. Finally, there are several new antiepileptic agents (such as topiramate, levetiracetam, zonisamide, and rufinamide) that have arisen from random screening of libraries of molecules. This category also includes lamotrigine, which might more accurately be defined as the product of a candidate screening program, albeit based on the incorrect assumption that antifolate compounds might prove useful in the treatment of epilepsy.

Target-orientated design is likely to yield a single mechanism drug with high specificity which can be tailored to defects in the epileptic brain (where known), but may be inadequate for a heterogeneous disorder such as epilepsy. To date, efforts at target-orientated drug design for epilepsy have foundered, most likely because targets were selected on the basis of a hypothesis rather than on direct evidence. Structural modification relies on the established activity of the parent molecule and provides an opportunity to improve on specific aspects of efficacy and/or tolerability. It could be argued that this approach is unlikely to change the spectrum of activity, unlikely to benefit those who are unresponsive to the medications upon which any novel compound is based, and employed predominantly to limit perceived commercial risk. Random screening has been the most successful drug development strategy in recent years, identifying several agents

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with a broad spectrum of clinical activity. However, this approach is laborious and expensive. As far as screening is concerned, mechanism of action is largely irrelevant (and may remain undiscovered), with success dependent on the models employed in the screening protocol, and with a significant potential for useful compounds to be discarded prematurely.

Methods

Models

Irrespective of the method employed for compound discovery, preclinical evaluation of efficacy and toxicity typically follows a standardized, if not specifically regulated, path. The efficacy and neurotoxicity of new antiepileptic agents are initially established in a series of experimental models. The most commonly employed models for evaluation of efficacy include the maximal electroshock (MES) and subcutaneous pentylenetetrazol (sc PTZ) tests, and the kindled rat. More recently, the 6-Hz psychomotor seizure test has gained favor as a high throughput model which can identify those compounds that are inactive in both of the two primary screening modalities (MES and sc PTZ).

Maximal electroshock

In the MES test, animals receive a short, supramaximal electrical stimulus sufficient to induce tonic extensor seizures of the hind limbs. Electrical stimuli are applied through either ear-clip or corneal electrodes and are delivered by a constant current apparatus. Typical stimulation parameters are 0.2 s of rectangular positive (or square-wave) pulses at 60 Hz, with a pulse width of 0.4 ms. A current of 50 (mice) or 150 mA (rats) is generally sufficient to induce tonic extension of the hind limbs in the majority of naive animals, although some rodent strain differences are observed. Tonic hind limb extension is an 'all or none' phenomenon against which the effectiveness of test compounds can be evaluated by measuring the percentage of protected animals in any given treatment group.

Pentylenetetrazol

PTZ is a chemical convulsant agent which produces seizures following systemic administration and is used extensively for the investigation of the anticonvulsant effects of novel drugs. The sc PTZ test involves the subcutaneous administration of PTZ at a dose (CD_{97}) which is known to elicit convulsions in 97% of naive animals. The CD_{97} varies according to species and strain, and is routinely determined prior to any new series of drug studies. The most common CD_{97} in mice is 85 mg kg^{-1}. PTZ-induced seizures are characterized by an initial 'absence-like' immobility, followed by brief myoclonic jerks, sustained myoclonus, and finally generalized clonic seizures with a loss of the righting reflex. Use of higher doses can result in tonic extension of the hind limbs. Evaluation of anticonvulsant efficacy is most commonly performed by comparing the time to the first generalized seizure with loss of the righting reflex in drug- and vehicle-treated animals.

Kindled rat

Kindling is a phenomenon by which daily electrical stimulation of a specific brain region (usually amygdala or hippocampus), with a fixed and initially subconvulsive current, results in progressive sensitization and precipitation of epileptic seizures. The procedure involves stereotaxic, surgical implantation of a unilateral, bipolar stimulating and recording electrode, followed by daily stimulation for upwards of 15 days. The behavioral response to kindling evolves through five stages (stages I–V): stages I and II are characterized by immobility and twitching of the vibrissae, and by facial clonus and head nodding; stage III is characterized by unilateral forelimb clonus; and stages IV and V are associated with rearing and bilateral forelimb clonus, and with generalized clonic seizures and a loss of the righting reflex, respectively. Once animals reach stage V (after approximately 15–21 stimulations) they can be considered fully kindled, and their increased sensitivity to stimulation prevails for several months. Antiepileptic efficacy can be assessed against various components (threshold, amplitude, duration) of the electrographic response to kindling stimuli (otherwise known as the after-discharge), or more commonly by measuring protection against fully kindled, stage V seizures. Antiepileptogenic activity, or the ability to retard or prevent the development of seizures, can also be investigated by drug treatment in the kindling acquisition phase and comparison of the time to nth stage V seizure.

6-Hz psychomotor seizures

The 6-Hz psychomotor seizure model is a variation on the MES test. It employs a more modest current (32 mA) applied, via corneal electrodes, at lower frequency (6 Hz) over a longer period (3 s). The 6-Hz test generates a much less severe response than the traditional MES, with only brief clonic seizures followed by stereotyped behaviors (jaw-chomping, whisker-twitching) against which the efficacy of candidate compounds can be assessed. As discussed earlier, this model has recently been incorporated into screening protocols in order to identify compounds (such as levetiracetam) with antiepileptic potential but which are inactive in the conventional MES and sc PTZ tests.

Other efficacy models

Additional experimental models may be employed to examine the spectrum of preclinical efficacy and thereby

facilitate comparison with established antiepileptic drugs. These models may include (but are not necessarily restricted to): the audiogenic-seizure sensitive mouse (DBA/2 or Frings), which experience generalized clonic seizures on exposure to loud bell-ringing; the bicuculline and picrotoxin tests, both of which are chemical convulsant agents administered subcutaneously to elicit generalized convulsions in a similar manner to the sc PTZ test but with different mechanisms of epileptogenic action; and the intravenous pentylenetetrazol (iv PTZ) threshold test, which can be employed to investigate the ability of candidate compounds to elevate (or lower) the seizure threshold as achieved by a slow, intravenous infusion of PTZ, with the time to first appearance of seizure manifestations recorded.

Neurotoxicity models

Neurotoxicity is most often determined by the rotarod test, which involves placing experimental animals (usually mice) on a 2.5-cm diameter rod, rotating continuously at 6 rpm. Control animals are able to maintain their equilibrium on the rotating rod for prolonged periods of time, whereas those treated with neurotoxic doses of candidate compounds are more likely to fall. Neurological impairment is generally indicated by loss of equilibrium (falling) in three consecutive tests performed over a 1-min period. Additional investigations of drug-related neurotoxicity include the horizontal screen test (where animals are placed on an inverted horizontal wire mesh), the positional sense test (where one hind-limb is deliberately lowered over the edge of the table), the gait and stance test (where animals are observed for overt signs of ataxia, abnormal gait, stupor, somnolence, etc.), and the muscle tone test (in which experienced investigators can identify a lack of skeletal muscle tone during simple handling).

Formulations

Solubility of test compounds is an issue in preclinical studies and can influence the dose range, route of administration, and choice of vehicle. Antiepileptic drugs are necessarily lipophilic, in order that they cross the blood-brain barrier; however, this characteristic can limit their solubility in aqueous solutions. For informal studies, individual investigators may choose to dissolve candidate compounds in either distilled water or 0.9% saline or in organic solvents such as polyethylene glycol, dimethyl sulfoxide, or Tween 80. Ethanol should be avoided as it possesses anticonvulsant properties in its own right. For more formal investigations, such as those described under the Antiepileptic Drug Development (ADD) program (below), candidate compounds are routinely dissolved or suspended in a standardized vehicle, such as 0.5% methylcellulose solution, to ensure reproducibility and comparability of findings. Test compounds are most often administered either orally or intraperitoneally in a volume adjusted according to both body weight and species (e.g., mice, 0.01 ml per gram body weight; rats, 0.04 ml per gram body weight). Chemoconvulsant agents (such as PTZ or bicuculline) and test compounds should be administered by different routes to avoid unforeseen interactions at the level of absorption.

Analyses

Data derived from preclinical screening are generally expressed in a series of values, such as the median effective dose (ED_{50}; the dose at which 50% of animals are protected from seizures), the median toxic dose (TD_{50}; the dose at which 50% of animals exhibit neurotoxicity), the time of peak effect (time since drug administration at which maximal efficacy or neurotoxicity is observed), and the protective index (PI; ratio of median toxic dose to median effective dose). All of these measures allow comparison with previously reported data. PIs give an indication of the relative separation between effective doses and those at which side effects are likely to be observed. They may be useful in predicting whether a novel compound is likely to possess clinical utility; for example, a PI of around 1.0 is often thought to be predictive of little or no efficacy at tolerable doses. It is important to note, however, that PIs can be manipulated to show novel drugs in a preferential light by selecting the efficacy model in which the candidate compound is most potent and the neurotoxicity model in which it is least potent.

The ADD Program

In response to changes in legislation governing the licensing of new medications, and the resulting hiatus in antiepileptic drug development in the 1960s and early 1970s, the ADD Program was instituted by the United States National Institute of Neurological Disorders and Stroke in 1974. Since then, the ADD program has screened more than 30 000 compounds submitted by academic and pharmaceutical company chemists from across the globe. It is currently based at the University of Utah, under the direction of Professor H. Steve White.

The ADD program currently comprises three principal evaluation stages – termed: identification, quantification and differentiation – and so-called 'further studies,' investigating proconvulsant potential, and mechanisms of action (**Table 1**). Initial identification of anticonvulsant efficacy is undertaken in the MES and sc PTZ models, performed at 30 min after intraperitoneal (mice) or oral (rats) administration of 30–300 mg kg^{-1} doses of the candidate compound. Those agents which fail to demonstrate any efficacy under these conditions are further evaluated in the 6-Hz psychomotor test, 30 min after a single intraperitoneal dose of 100 mg kg^{-1} in mice. Compounds which fail this initial identification stage are generally discarded.

Table 1 Overview of testing procedures in the Antiepileptic Drug Development program

1. Identification
   a. Mouse (i.p.)
      Dose range: 30, 100, and 300 mg kg^{-1}
      Tests: MES, sc PTZ, rotarod
      Time of tests: 30 min and 4 h
   b. Rat (p.o.)
      Dose: 30 or 50 mg kg^{-1}
      Tests: MES (30 mg kg^{-1}) or sc PTZ (50 mg kg^{-1}) plus minimal neurotoxicity
      Time of tests: 15 min, 30 min, 1 h, 2 h, and 4 h
   c. Mouse (i.p.) – only performed for compounds inactive in 'a' and 'b' above
      Dose: 100 mg kg^{-1}
      Test: 6 Hz
      Time of test: 15 min, 30 min, 1 h, 2 h, and 4 h
2. Quantification
   a. Mouse (i.p.)
      Determination of TPE: MES, sc PTZ, 6 Hz, rotarod
      Determination of ED_{50}: MES, sc PTZ, 6 Hz
      Determination of TD_{50}: rotarod
   b. Mouse (p.o.)
      Determination of TPE: MES, sc PTZ, rotarod
      Determination of ED_{50}: MES, sc PTZ
      Determination of TD_{50}: rotarod
   c. Rat (p.o.)
      Determination of TPE: MES, sc PTZ, minimal neurotoxicity
      Determination of ED_{50}: MES, sc PTZ
      Determination of TD_{50}: minimal neurotoxicity
3. Differentiation
   a. Mouse (i.p.)
      Determination of ED_{50} in sc bicuculline model
      Determination of ED_{50} in sc picrotoxin model
      Determination of ED_{50} in Frings audiogenic seizure-sensitive mouse
   b. Rat (i.p.)
      Determination of ED_{50} against stage V seizures in amygdala kindled rat
      Determination of ED_{50} against spike-wave discharges in γ-hydroxybutyrate model of absence
4. Further studies
   a. *Proconvulsant potential*
      Mouse (i.p.)
      Dose: Single dose of investigational agent at TPE and ED_{50}
      Test: Determination of seizure threshold following timed i.v. infusion of PTZ
   b. *Mechanism of action*
      (i) Patch-clamp electrophysiology studies (voltage-gated sodium and calcium channels and ligand-gated ion channels; NMDA, AMPA/kainate, GABA)
      (ii) Electrophysiological recording from synaptically coupled neuronal pairs

6 Hz, 6-Hz psychomotor seizure test; AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid; ED_{50}, median effective dose; GABA, γ-aminobutyric acid; i.p., intraperitoneal; i.v., intravenous; MES, maximal electroshock; NMDA, *N*-methyl-D-aspartate; p.o., oral; PTZ, pentylenetetrazol; s.c./sc, subcutaneous; TD_{50}, median toxic dose; TPE, time of peak effect.
Adapted from White HS, Woodhead JH, Wilcox KS, *et al*. (2002) Discovery and preclinical development of antiepileptic drugs. In: Levy RH, Mattson RH, Meldrum BS, and Perucca E (eds.) *Antiepileptic Drugs*, 5th edn., pp. 36–48. Philadelphia: Lippincott, Williams & Wilkins.

Those drugs which demonstrate efficacy in one or more of the initial experimental screens then undergo more detailed quantification of their efficacy and neurotoxicity. First, time of peak effect is established in both mice (intraperitoneal and oral administration) and rats (oral administration only), with a single dose of candidate drug evaluated in multiple models (MES, sc PTZ, 6 Hz, rotarod) at a variety of time points. Thereafter, ED_{50} in MES, sc PTZ and 6-Hz tests, and TD_{50} in the rotarod test, are determined at time of peak effect, again using two species (mice and rats) and two administration routes (intraperitoneal and oral). Finally, compounds are submitted for differentiation studies where their efficacy (ED_{50}) in a host of experimental models (such as the kindled rat, bicuculline and picrotoxin tests, audiogenic-seizure sensitive mouse, and γ-hydroxybutyrate-induced spike-wave seizures) is determined at the time of peak effect following intraperitoneal administration.

Novel drugs may undergo additional studies of their proconvulsant potential using the iv PTZ threshold test. The likelihood of their being associated with pharmacological tolerance (pharmacokinetic or pharmacodynamic) is also investigated using a repeated administration protocol. Mechanisms of action are explored using patch-clamp analysis focused on multiple voltage- and ligand-gated ion channels, such as sodium channels, calcium channels, GABA receptors, and glutamate receptors – which represent the primary pharmacological targets for existing antiepileptic agents. Paired-pulse recording in monosynaptically connected neurones, to investigate in vitro effects on membrane potential, action potential generation, and neurotransmitter release, can also be performed.

This extensive characterization provides information not just about whether the submitted compound is active or inactive, but also about the extent and range of its efficacy and neurotoxicity in comparison with other antiepileptic drugs, an indication of its PI, preliminary evidence regarding its mechanism of action, basic pharmacokinetic knowledge, and an indication of possible doses for use in initial human studies. The ADD program, as described earlier, has been instrumental in the development of all eleven modern antiepileptic drugs.

Additional Investigations

In addition to assessment of efficacy and neurotoxicity in animal models, all new candidate compounds for epilepsy are required by regulatory authorities to undergo detailed toxicological investigation (**Table 2**). This procedure generally involves acute tests using single drug doses, and two series of prolonged tests employing daily oral administration of three different drug doses to two different species (usually rats and dogs) for a minimum period of 14 days (short-term toxicology) and between 6 and

24 months (long-term toxicology). Routine toxicology investigations attempt to uncover biochemical and morphological abnormalities associated with prolonged drug exposure; additional, so-called 'special tests' specifically focus on behavioral, teratogenic and reproductive effects, and the potential for carcinogenicity. Animals in long-term studies undergo regular blood analyses, electrocardiograms, neurological and ophthalmological assessment, and a full postmortem at study termination. Teratogenicity is evaluated in detail, by physical and behavioral examination of the offspring of chronic drug-exposed females – from delivery, through weaning, growth, pubescence, and sexual maturity. Mutagenicity, as an indicator of potential carcinogenicity, is evaluated in vitro using the Ames test, in which mutagens can induce a reverse mutation in a specific strain of Salmonella bacterium, allowing it to grow on histidine-free medium.

Predicting Clinical Activity from Preclinical Models

Attempts have been made to use efficacy in preclinical models as a predictor for the potential spectrum of clinical activity, with the correlation based largely on pharmacological sensitivity to established antiepileptic drugs (Table 3). The MES test, responsive to carbamazepine and phenytoin, is reported to indicate activity against generalized tonic–clonic seizures. In contrast, the sc PTZ model is believed to reflect efficacy against generalized absence and/or myoclonic seizures and is principally sensitive to ethosuximide, sodium valproate and benzodiazepines. Finally, the kindled rat, proposed to predict utility in partial epilepsies, identifies a wide range of established antiepileptic agents (including carbamazepine, phenytoin, sodium valproate, barbiturates and benzodiazepines) with efficacy against various electrographic and behavioral components of the kindling response, including protection against fully kindled, stage V seizures.

Experience with modern antiepileptic drugs has, however, called this simplistic extrapolation of preclinical seizure protection into question. Several newer compounds, including topiramate, levetiracetam, and zonisamide, possess a broad spectrum of clinical activity, including at least anecdotal efficacy in absence epilepsies – and yet all are inactive in the sc PTZ test. In contrast, tiagabine has potent anticonvulsant effects in the sc PTZ model, but can make clinical absence seizures worse. Thus, drugs that are active in multiple preclinical models do not necessarily have clinical efficacy against multiple seizure types or in multiple epilepsy syndromes. This experience most likely reflects the fact that the majority of preclinical models currently used for drug development purposes are models of a single seizure – and as such, do not adequately mirror the chronic, recurrent and unpredictable nature of the clinical disorder.

Table 2 Routine toxicological assessment during drug development

1. Acute treatment
   a. Median lethal dose (LD_{50} or equivalent) in rodents
   b. Pyramiding single dose studies in dogs
   c. Local effects on rabbit skin (only for agents to be used topically)
2. Repeated treatment
   a. Subchronic (up to 3 months) using rats and dogs and three dose levels
   b. Chronic (6 months to 2 years) using rats and dogs and two or three dose levels
3. Special investigations
   a. Effects on fertility and reproduction
   b. Teratogenicity
   c. Carcinogenicity and mutagenicity
   d. Behavioral effects
   e. Interactions with other chemical agents

Adapted from Plaa GL and Willmore LJ (1995). Toxicology. In: Levy RH, Mattson RH, and Meldrum BS (eds.) *Antiepileptic Drugs*, 4th edn., pp. 51–60. New York: Raven Press.

Future Studies

Current screening procedures in the ADD program (and elsewhere) may not be adequate to identify all potentially useful antiepileptic drugs. For example, levetiracetam was originally discarded by the ADD program due to its lack of efficacy in both MES and sc PTZ models; its

Table 3 Correlation between the efficacy of established antiepileptic drugs in selected preclinical models and their clinical spectrum of activity

	Clinical seizure type		
	Generalized tonic–clonic seizures	*Generalized absence/myoclonic seizures*	*Partial seizures*
Maximal electroshock test	PB, PHT, CBZ, VPA	–	–
Subcutaneous pentylenetetrazol test	–	ESM, VPA, BZDs	–
Kindled rat (stage V seizures)	–	–	PB, PHT, CBZ, VPA, BZDs

BZDs, benzodiazepines; CBZ, carbamazepine; ESM, ethosuximide; PB, phenobarbital; PHT, phenytoin; VPA, sodium valproate.

effectiveness was only revealed after subsequent screening in the kindled rat and audiogenic-seizure sensitive mouse. This failure prompted the subsequent incorporation of the 6-Hz psychomotor test into the ADD screening protocol, for those compounds which are unsuccessful in the initial identification phase. Whether other such compounds have slipped through the net, or are likely to do so in the future, is unclear. It is almost impossible, however, given the costs and high throughput nature of screening, to guard against all such eventualities.

The relatively modest impact of modern antiepileptic agents, at least in terms of efficacy, has lead to the suggestion that the clinical utility of novel compounds is unlikely to improve, unless the models used for their identification and characterization are updated. This is the so-called 'old models identify old drugs' argument, which questions the almost exclusive continued use of experimental seizure models such as the MES test, first developed in the 1930s and employed in the identification of phenytoin, in modern screening protocols. From a pharmacological perspective, using a limited number of traditional models as the primary screen for drug development may indeed favor certain mechanisms of action at the expense of novelty. Current evidence, however, does not support this assertion. Prior to the development of modern antiepileptic agents, there were no compounds with selective effects on high-voltage activated calcium channels (i.e., gabapentin) or on GABA transporters (i.e., tiagabine). These agents are examples of structurally novel drugs, with unique and selective pharmacological effects, that are active in at least one traditional seizure model. Thus, old models can identify new drugs; whether those drugs have better efficacy than their more established counterparts is another issue.

Drug resistance is one of the most significant problems in the clinical management of epilepsy. At least 30% of patients fail to respond to treatment with currently available medications. New drugs are required to meet this therapeutic deficit, but whether they are likely to be identified by traditional screening protocols is debatable. It may be necessary to introduce a second tier of screening, using specific models of drug resistant epilepsy, to distinguish generic anticonvulsants from those with potential efficacy in refractory epilepsies. Several such models have been described in recent years. The 6-Hz psychomotor seizure model has, on occasion, been promoted as a model of therapy-resistant epilepsy, on the basis that it is relatively insensitive to sodium channel blockers (such as phenytoin and lamotrigine), but more responsive to compounds with multiple mechanisms of action (such as sodium valproate and levetiracetam). Other models of drug resistant epilepsy, such as the phenytoin-resistant kindled rat, are more technically demanding and less suitable for high throughput screening. This model is an extension of the traditional kindling model, with animals selected and bred on the basis of response to phenytoin against fully kindled stage V seizures. If phenytoin nonresponders are employed in subsequent drug studies, they are invariably less sensitive to treatment with the majority of established and modern antiepileptic drugs than their responsive counterparts. Whether such models can be accommodated in screening protocols, and perhaps more importantly, whether they genuinely represent human refractory epilepsy, remains to be determined.

Another target for drug development in the epilepsy field, albeit somewhat further removed from contemporary practices, is epileptogenesis or the process of acquiring epilepsy following some unspecified neuronal insult. The search for such a drug is, to some extent, the holy grail of epilepsy therapy – focusing on the notion that 'at risk' individuals might be treated in a prophylactic manner to prevent the development of chronic seizures. Several models of epileptogenesis already exist, despite the fact that the clinical problem is yet to be delineated. The ability of a drug to prevent or retard the development of stage V seizures in the kindled rat model has been suggested to demonstrate antiepileptogenic potential. More clinically representative models are those in which recurrent, unprovoked seizures develop after a short period of acutely provoked status epilepticus, induced by either chemical (e.g., pilocarpine) or electrical stimulation. Understanding the processes ongoing in the 'latent period' between initial insult and seizure onset, which in these models may be weeks or months and which in humans may be twenty years and more, has the potential to identify novel drug targets. Several current antiepileptic agents can retard kindling acquisition, and some have been proposed to possess disease-modifying effects in post-status epilepticus models. As yet, however, there is no drug which can effectively prevent the development of chronic seizures in animal models.

Irrespective of the direction of drug development in the epilepsy field in the coming years, it is imperative that screening protocols remain robust and reliably predictive of clinical activity in general. They must also be sufficiently sensitive to identify compounds with novel mechanisms of action and sufficiently flexible to accommodate technically demanding models of drug resistant epilepsy and epileptogenesis. The recent and ongoing re-evaluation of drug development strategies in epilepsy should help to ensure that future treatments are targeted at the areas of greatest need.

Summary

Despite the introduction of eleven new antiepileptic drugs in the past 20 years, drug development in the field of epilepsy continues apace. All new compounds undergo rigorous examination in a series of experimental models prior to first human use. Preclinical studies are designed

to identify anticonvulsant activity, and thereafter to quantify the experimental efficacy and neurotoxicity of individual agents. Knowledge of potency and range of efficacy can facilitate comparisons with established drugs, and additional investigations may yield information about pharmacology, pharmacokinetics, and possible clinical activity. Preclinical toxicology is a statutory requirement of drug development and provides important information about long-term safety. The drug development process in epilepsy must constantly evolve to ensure that future generations of antiepileptic drugs are superior to those currently available, and to prioritize those areas of clinical epilepsy and those patients in greatest need.

See also: **Antiepileptic Drugs**: Antiepileptic Drug Mechanisms; Clinical Drug Development in Epilepsy; Pharmacological Approaches for the Assessment of Antiepileptic Drug Efficacy in Experimental Animal Models; **Models**: Model Characterization in Relationship to Human Disorders; Seizure Models in Acute and Organotypic Slices.

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C

CELL DAMAGE/EXCITOTOXICITY

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Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus

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Introduction

Epilepsy is one of the most common neurological conditions, occurring in up to 1% of the adult and 2% of the pediatric populations. In addition, ~40% of patients with epilepsy have seizures that are refractory to treatment. This large population of patients is having recurrent seizures on a regular basis, despite the best of neurological care. It is essential to understand the potential risks to the brain of episodic seizures and to determine whether repeated seizures in this population of patients can lead to neuronal damage and brain injury. This article evaluates the evidence supporting the question of whether clinical and in vitro recurrent seizures or epileptiform events can cause neuronal damage. The results of this article indicate that recurrent seizures do not cause neuronal damage.

Status epilepticus (SE) is a prolonged seizure condition representing a major medical and neurological emergency, and is associated with a significant morbidity and mortality despite aggressive treatment protocols. In vivo studies are complicated by compensatory environmental changes in the brain that are characteristic of living systems. Changes in glucose utilization, hormonal production, extracellular fluid composition, and the involvement of other physiological processes, for example, may vary under different conditions that induce SE. These changes alter the environment of neurons under study, complicating the mechanistic assessment in vivo of whether continuous seizure discharges alone – or other related physiological effects – cause neuronal damage and death. Evaluating the effects of epileptiform activity per se on electrophysiological and metabolic functions is very difficult at the single neuron level in vivo, since it is not possible to carefully control the neuronal environment during seizure activity in the whole animal. Studies from our laboratory have investigated the effects of electrographic epileptiform discharges on neuronal viability in an in vitro model, in order to elucidate the mechanisms of seizure-induced cell death. Although the neuronal cultures do not have anatomical connections as seen in the intact brain, and do not display clinical seizures, epilepsy and SE in hippocampal neuronal cultures have been routinely used to characterize biochemical, electrophysiological, and molecular mechanisms underlying these conditions.

Background

It has been well established that SE is associated with neuronal death. This neuronal damage is dependent upon multiple variables, including the location of seizure

foci, seizure duration, age, and genetic susceptibility; there are, in addition, many exacerbating factors, such as the underlying etiology of the seizures – e.g., hypoxia and ischemia. Pioneering studies by Meldrum and coworkers, in ventilated baboons, provided the first evidence that prolonged electrographic seizures (SE) alone, in the absence of underlying systemic physiological complications, could cause neuronal death. There is now strong evidence that SE can cause significant neuronal damage, especially in the limbic system, in both animal models and human patients. Evidence is now accumulating especially from in vivo models of epilepsy, suggesting that neuronal death is associated with an initial epileptogenic insult and not with later spontaneous seizures that do not develop into SE. Understanding the effects of epileptiform discharges on neuronal viability and pharmacology of neuronal death is important in developing therapeutic strategies for treating SE and epilepsy.

The results presented in this paper provide direct evidence that self-limited seizure activity does not injure neurons, but that SE can cause neuronal injury and death if the electrographic epileptiform activity continues for a significant duration. These studies confirm the findings and implications of human and animal studies, and provide the first direct documentation in an in vitro model that the electrographic epileptiform activity associated with periodically recurring seizures does not cause neuronal injury or death.

Methods

Hippocampal Neuronal Cultures

We conducted studies on primary mixed hippocampal neuronal cultures prepared as described previously (see Further Reading), with slight modifications. In brief, hippocampal cells were obtained from 2-day postnatal Sprague-Dawley rats (Harlan, Frederick, MD) and plated at a density of 2×10^5 cells cm^{-2} onto a confluent glial bed on 35-mm grid cell culture dishes (Nunc, Naperville, IL) previously coated with poly-L-lysine ($0.05\,mg\,ml^{-1}$). Cultures were maintained at 37 °C in a 5% CO_2/95% air atmosphere and fed twice weekly with neuronal feed. These mixed cultures were used for experiments from 13 days in vitro following neuronal plating.

Hippocampal Neuronal Culture Model of SE

Continuous epileptiform activity, identical to electrographic discharges recorded from neurons from whole animal models of SE, were induced in the neuronal cultures by exposing the cultures to a solution containing no added $MgCl_2$ (low Mg^{2+}). Culture maintenance medium was replaced with physiological recording solution with or without $MgCl_2$. In low Mg^{2+} condition, all neurons manifest continuous high voltage epileptiform discharges at a frequency of 8–12 Hz; this activity meets the criteria for electrographic SE. Using this model of SE, we directly compared cultures that manifested continuous epileptiform discharges with cultures maintained under control conditions. SE was observed in the experimental cultures until restoration of physiological recording solution (containing 1 mmol $MgCl_2$), giving the investigators precise control of the duration of SE. Cultures were studied during constant perfusion of fresh low Mg^{2+} or control solutions over the culture plates, using a gravity feed perfusion system (Warner Instrument Corp., Hamden, CT) at the rate of $2\,ml\,min^{-1}$. Continuous perfusion did not disrupt the neurons and provided a complete exchange of the experimental media every 1 min.

Hippocampal Neuronal Culture Model of Epilepsy (SREDs)

The hippocampal neuronal culture model of spontaneous recurrent seizures (SREDs) manifests spontaneous recurrent synchronized epileptiform activity that is essentially identical to that seen in intracellular recordings from neurons in a seizure focus of an intact animal. In this in vitro model of epilepsy, SREDs were induced in the neuronal cultures by exposing them for 3 h to a solution containing no added $MgCl_2$ (low Mg^{2+}). At the end of this 3-h period, low Mg^{2+} treatment was terminated by gently washing the cultures with 3×1.5 ml of minimum essential medium, and the cultures were returned to the maintenance medium (incubated at 37 °C under 5% CO_2/95% atmosphere). SREDs developed in these neuronal cultures after ~12–24 h and lasted for the lifetime of the culture.

Whole-Cell Current-Clamp Recordings

Whole-cell current-clamp recordings were obtained from individual neurons in culture. Briefly, cell culture dishes were mounted on the stage of an inverted microscope (Nikon Diaphot, Tokyo, Japan). Patch electrodes with a resistance of 2–4 mΩ were pulled on a Brown-Flaming P-80C electrode puller (Sutter Instruments, Novato, CA), fire-polished, and filled with a solution containing (in millimoles): 140 K^+ gluconate, 1.1 EGTA, 1 $MgCl_2$, and 10 Na-HEPES; pH 7.2; osmolarity adjusted to 290 ± 10 mOsm with sucrose (pBRS). Whole-cell recordings were carried out using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) in current-clamp mode. Data were digitized via Digidata 1322A (Axon Instruments, Foster City, CA) and transferred to VHS tape, using a PCM device (Neurocorder, New York, NY), and then played back on a DC-500 Hz chart recorder (Astro-Med Dash II, Warwick, RI).

Neuronal Death Assay

Neuronal death at various time points after low-Mg^{2+} or control media exposures was assessed morphologically as well as determined using fluorescein diacetate (FDA)–propidium iodide (PI) microfluorometry. Neurons labeled with FDA or PI were quantified using Ultraview (Perkin Elmer Life Sciences), and percent neuronal death was calculated as the number of neurons labeled by PI divided by the sum of the number of neurons labeled by PI and FDA.

Data Analyses

One-way analysis of variance (ANOVA), followed by post hoc Tukey test or Kruskal–Wallis variations of ANOVA, were performed where appropriate to evaluate neuronal cell death. Data are reported as mean ± SEM. Paired *t*-tests were utilized to compare differences in neuronal death data between the fluorescent techniques and phase contrast microscopy. Each neuronal culture plate was treated as $n = 1$; a minimum of three determinations were obtained on each plate, and a mean cell death was obtained. Net neuronal death was calculated by comparing neuronal death in the experimental conditions with that observed in sham control groups in order to adjust for cell death because of handling artifacts. Data were analyzed using SigmaStat 2.0 and plotted using SigmaPlot 8.02 (SPSS Inc., Chicago, IL).

Results

The SE and SRED models have allowed us to carefully control the external environment of the neuronal networks and thus rigorously determine if SE or SREDs alone can induce neuronal death. This experimental approach provides a careful evaluation of the effects of different patterns of electrographic activity on the viability of a population of neurons, compared with neurons under identical conditions without seizure activity.

SE in Hippocampal Neuronal Cultures

Exposing hippocampal neurons in culture to low Mg^{2+} caused the induction of large, long-duration synaptic potentials with multiple action potentials, evolving into high-frequency burst discharges. Figure 1(b) illustrates a 30 min recording from a neuron after 3 h of exposure to low Mg^{2+}. The resultant spike discharges were sustained for the entire low Mg^{2+} exposure. Expansion of the time scale indicated that spike frequency occurred at 8–15 Hz. This high-frequency spiking continued throughout the 9-h period of low Mg^{2+} treatment. Following retoration of the normal Mg^{2+} level to the media, these spontaneous discharges immediately stopped. Recording from a control neuron showed occasional spontaneous action potentials (Figure 1(a)).

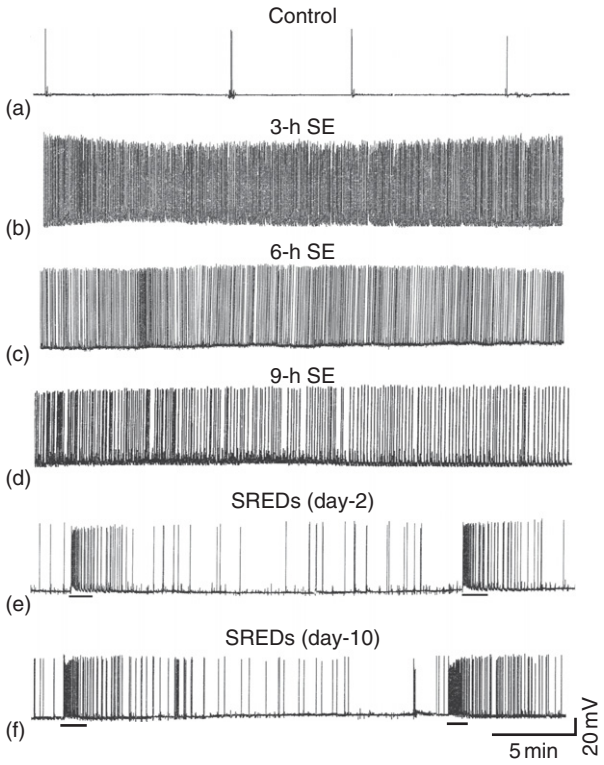


Figure 1 Continuous high-frequency epileptiform discharges and SREDs in cultured hippocampal neurons. (a) Representative current-clamp recording from a control neuron showing occasional spontaneous action potentials. (b–d) Representative current-clamp recordings during low Mg^{2+} treatment showing induction of tonic high-frequency epileptiform bursts (abbreviated SE in the figure) from neurons subjected to 3, 6, and 9 h of low Mg^{2+} exposure respectively. Tonic high-frequency spiking was observed at all the timepoints. Although a little slowing of spike activity is sometimes observed at later time points, the spike frequency was still >3 Hz and meets the criteria of SE. (e) A continuous 30-min recording from a representative neuron 2 days following an initial 3-h exposure to low Mg^{2+} solution. Two SREDs or spontaneous seizure episodes lasting ~60–80 s can be seen in this time frame (denoted by black bars beneath each episode in the trace). (f) A continuous 30-min recording from a representative neuron 10 days following an initial 3-h exposure to low Mg^{2+} solution. These SREDs at 10 days after initial injury with low Mg^{2+} are identical to the SREDs seen 2 days after low Mg^{2+}-induced high-frequency spiking (e) and demonstrate that the SREDs phenotype lasts for the life of the neurons in culture in this model. Reproduced from Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S and DeLorenzo RJ (2007) In vitro status elipticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Research* 75: 171–179, with permission from Elsevier.

SREDs in Hippocampal Neuronal Cultures

SREDs or seizure events were observed, using whole-cell current-clamp recording techniques, in neurons in cultures 2 days after a 3-h, low-Mg^{2+} treatment. Two SREDs lasting between 1 and 1.5 min are shown in Figure 1(e) and were observed in a continuous 30-min recording. These SREDs are characteristic of the epileptiform phenotype and were observed for the life of the neurons in

culture after the initial insult with low Mg^{2+}. Thus, SREDs were also observed even at 10 days after initial SE insult (Figure 1(f)). SREDs started and stopped spontaneously had durations between 1 and 3 min, and occurred at frequencies of up to 2–6 episodes every hour. We have shown previously that SREDs produced in cultured neurons manifest electrographic properties, neuronal population synchronicity, and anticonvulsant sensitivity analogous to comparable features observed in vivo. Age-matched control neurons revealed 'normal' baseline activity consisting of intermittent action potentials. SREDs were never observed in control neurons, and there were no significant differences in membrane potential and input resistance between control and epileptic neurons.

Increasing Durations of SE and It's Effect on Neuronal Cell Death

Experiments were initiated to investigate the effects of increasing durations of SE on hippocampal neuronal cell death (Figure 1). In order to standardize the method for cell death assessment, in the first set of experiments low Mg^{2+} exposure was continued for 0 or 12 h, at the end of which neuronal cultures were washed with solution containing normal Mg^{2+} concentrations (2 mmol) and cell death was measured using FDA–PI staining. Figure 2(a) shows representative fluorescent images from neurons before (0 h) and after (12 h) exposure to low Mg^{2+}-induced SE. Intravital staining with FDA–PI in cultures that

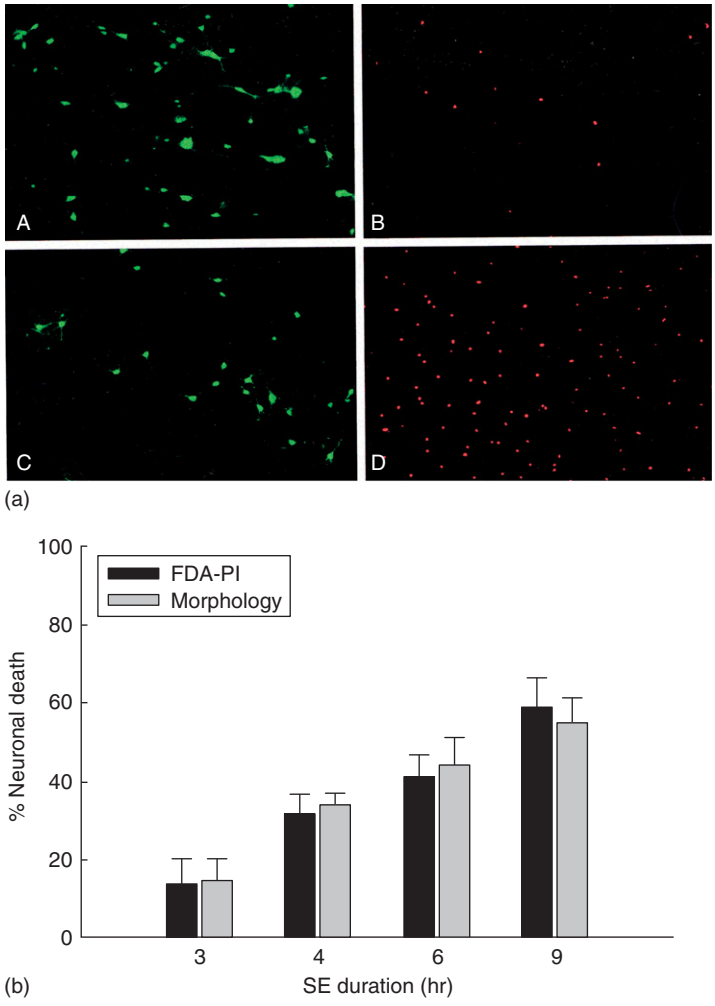


Figure 2 Neuronal cell death as a function of seizure duration. (a) Representative images of neurons at 0 and 12 h of low Mg^{2+}-induced high-frequency spiking. Frame A: FDA staining of viable neurons at 0 h ($n = 49$). Frame B: PI staining of nuclei of nonviable neurons in the same field as A at 0 h. Frame C: FDA staining of viable neurons at 12 h of low Mg^{2+} exposure ($n = 32$). Frame D: PI staining of nonviable neurons in the same field as C at 12 h of low Mg^{2+} exposure. (b) Comparison of neuronal cell death after 3, 4, 6, and 9 h exposure to low Mg^{2+} solution (abbreviated SE in the figure). Percentage neuronal cell death is dependent on the duration of exposure to low Mg^{2+} solution in culture. Gray bars represent cell death assessed by visual examination of neuronal morphology. Black bars indicate cell death examined using FDA–PI intravital staining as described in Materials and Methods. Differences between the two techniques were not statistically significant (paired *t*-test). FDA–PI staining was performed 24 h after exposure to low Mg^{2+} solution for these studies. Reproduced from Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S and DeLorenzo RJ (2007) In vitro status elipticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Research* 75: 171–179, with permission from Elsevier.

were not exposed to low Mg^{2+} conditions exhibited a preponderance of cells fluorescing green and few fluorescing red (Figure 2(a), panels A and B). Cultures that were exposed to low Mg^{2+} conditions displayed a decrease in FDA (green) staining brightness and corresponding increase in PI (red) fluorescence (Figure 2(a), panels C and D).

Neuronal cultures were then subjected to varying durations of exposure to low Mg^{2+} (3, 4, 6, and 9 h), after which time cell death was quantified using the FDA–PI staining. To rigorously control for the validity of the FDA–PI staining method, we also quantified neuronal viability by morphological assessment in the same fields of neurons. Paired *t*-tests were used to compare differences in neuronal death data between the fluorescent techniques and phase contrast microscopy. As shown in Figure 2(b), the results from both the FDA–PI fluorescent dye staining and the neuronal morphology techniques were consistent (not statistically divergent). Since the FDA–PI technique was accurate and required less time to perform, it was used for the subsequent neuronal viability measurements.

Increasing durations of exposure to low Mg^{2+} caused the percentage of neuronal death to increase correspondingly (Figure 3(a)). Cell death increased from ~10–15% after 3 h of SE to 55–60% after 9 h of exposure. After 12 h of SE, ~80% of the neurons appeared to have died. Control culture neuronal death was determined to be ~18%. This amount of cell death represented neuronal injury because of handling effects of the cultures; the control cultures were subjected to the same handling procedures (including removal of media, washing with pBRS, and maintenance in pBRS) and for the same durations that were used for the low Mg^{2+} time course cultures. The cell death values reported in Figure 3(a) were adjusted for control cell death in order to reflect net cell death occurring from low Mg^{2+} treatment alone, in the absence of these handling artifacts. Differences in neuronal death between control and low Mg^{2+} conditions were not significant at 0, 1, and 2 h time points, but were significant for low Mg^{2+} durations of 3–12 h ($p < 0.001$, Figure 3(a)). In order to quantify the correlation of cell death and duration of SE, a linear regression analysis was performed. As shown in Figure 3(b), the percent neuronal cell death correlated significantly with the duration of exposure to low Mg^{2+} ($r^2 = 0.972$, $p < 0.001$). These results demonstrate a significant relationship between duration of SE and the degree of neuronal death.

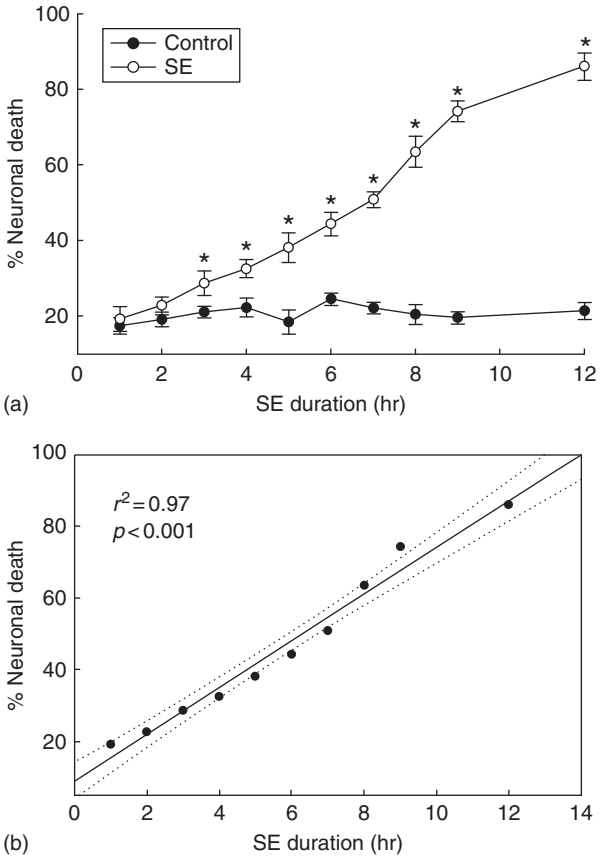


Figure 3 Correlation between duration of low Mg^{2+}-induced high-frequency spiking and neuronal cell death. (a) There was a time-dependent increase in neuronal cell death in cultures exposed to increasing durations of low Mg^{2+}-induced high-frequency epileptiform discharges (abbreviated SE in the figure) ranging from 0 to 12 h (n = 6–9 at each time point tested). Black circles (●) indicate sham controls. Open circles (○) represent percent cell death from low Mg^{2+}-exposed cultures. Statistically significant differences were observed for all the time points starting at 3 h following exposure to low Mg^{2+} solution (*$p < 0.001$, one-way ANOVA). (b) Linear regression analysis of neuronal cell death and duration of exposure to low Mg^{2+} solution gave an r^2 value of 0.97 ($p < 0.001$). Dotted lines represent 95% confidence interval limits. Reproduced from Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S and DeLorenzo RJ (2007) In vitro status elipticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Research* 75: 171–179, with permission from Elsevier.

Accumulation of Glutamate or Other Metabolites into the Media do not Cause Neuronal Cell Death

It is important to rule out the possible neurotoxic effects of the accumulation of glutamate or other metabolites in the media on the time-dependent low Mg^{2+}-induced neuronal cell death. Therefore, experiments were performed using a perfusion system to continuously perfuse fresh low Mg^{2+} medium over the culture plate. Glutamate was measured before and during low Mg^{2+}-induced SE. The glutamate concentration in the media alone was not detectable, and when media was replaced with pBRS, the glutamate concentration in neuronal culture was estimated to be $<1\ \mu mol\ l^{-1}$ (also essentially not detectable). The glutamate concentration was then measured at various time points in the presence of the low Mg^{2+} solution. The concentration of glutamate steadily rose to a maximal level of 5–6 $\mu mol\ l^{-1}$ at the longest durations of SE. Upon continuous perfusion with a low Mg^{2+} solution, the glutamate concentration throughout the duration of

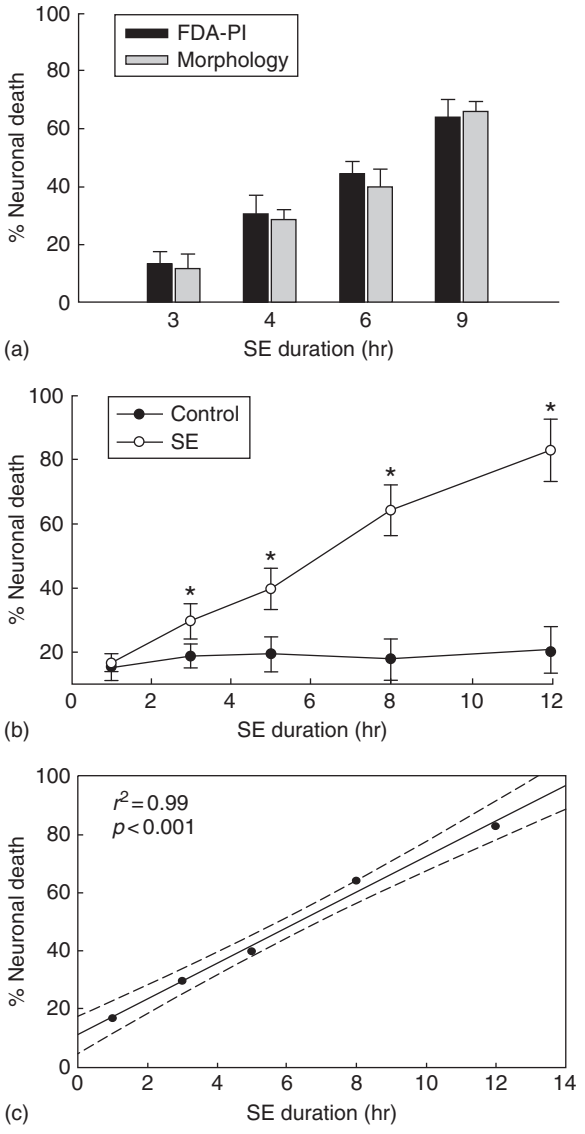


Figure 4 Accumulation of glutamate or other metabolites into the media do not cause cell death. Low Mg^{2+}-induced possible accumulation of glutamate or other metabolite in the culture media was prevented by continuously perfusing culture plate with fresh low Mg^{2+} solution throughout the duration of experiment using gravity feed perfusion system. (a) Both FDA–PI (black bars) and morphological assessment (gray bars) procedures still revealed a time-dependent increase in neuronal cell death as the result of continuous high-frequency epileptiform discharges (abbreviated SE in the figure), despite continuous perfusion of low Mg^{2+} perfusion media. (b) Time-dependent increase in neuronal cell death with increasing durations of exposure to low Mg^{2+} solution despite continuous perfusion of fresh low Mg^{2+} perfusion media. Low Mg^{2+} solution was perfused throughout the duration of experiment ranging from 0 to 12 h (n = 6–10). Black circles (•) indicate sham controls. Open circles (○) represent percent cell death from low Mg^{2+}-exposed cultures (abbreviated SE in the figure). Both control and low Mg^{2+}-exposed neurons were subjected to similar perfusion protocol. Significant differences were observed for all the time points starting at 3 h following exposure to low Mg^{2+} solution (*$p < 0.001$, one-way ANOVA). (c) Linear regression analysis of neuronal cell death and duration of high-frequency epileptiform discharges under constant low Mg^{2+} perfusion conditions (abbreviated SE in the figure) gave an r^2 value of 0.99 ($p < 0.001$). Dotted lines represent 95% confidence interval limits. Reproduced from Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S and DeLorenzo RJ (2007) In vitro status elipticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Research* 75: 171–179, with permission from Elsevier.

experiment was maintained at not detectable levels (i.e., <1 μmol l^{-1}). Although our perfusion system cannot effectively remove synaptic glutamate, it prevented the build up of glutamate (or other materials) in the neuronal culture media and thus maintained a constant environment for the neurons during SE.

Figure 4 demonstrates that despite the continuous perfusion of fresh low Mg^{2+} solution, neuronal death increased with increasing durations of SE (in comparison with control neurons). Similar to the nonperfusion experiments (Figure 3), both FDA–PI staining and morphological methods demonstrated an increase in neuronal death as a function of time (Figure 4(a) and 4(b)). We did observe a small decrease in cell death with increasing durations of SE when fresh low Mg^{2+} solution was continuously perfused throughout the duration of the experiment (compared with the nonperfusion conditions). This decrease in cell death was highest at the longer time points; however, this reduction in cell death (<3–5%) did not reach statistical significance. As previously shown, there was a significant correlation between percent neuronal death and duration of SE in the continuous perfusion experiments ($r^2 = 0.99$, $p < 0.001$, Figure 4(c)). These experiments indicate that SE alone, and not the build up of glutamate or other seizure-released metabolites, was responsible for neuronal death during low Mg^{2+}-induced SE.

SREDs do not Cause Cell Death

These studies were conducted to evaluate the effects of SREDs that occur several times per hour, for the life of the neurons in culture, on neuronal survival. Neuronal cultures were first shown to generate SREDs (using patch clamp electrophysiology); SREDs were observed in the cultures at all the time points tested (Figure 1(e) and 1(f)). To allow the cells to recover from the initial 3-h low Mg^{2+} exposure, we conducted experiments starting 2 days after the initial insult. Figure 5 presents the effects of SREDs on neuronal death. Using both the FDA–PI staining and morphological assessment methods, we observed that SREDs did not cause a significant increase in cell death in comparison with age-matched control cultures (Figure 5(b)). Moreover, there was no significant correlation between percent neuronal death and time of exposure to SREDs ($r^2 = 0.26$, $p = 0.3$, Figure 5(c)). Cell death in age-matched control cultures was 4–6%; this percent cell death was lower than that observed for the

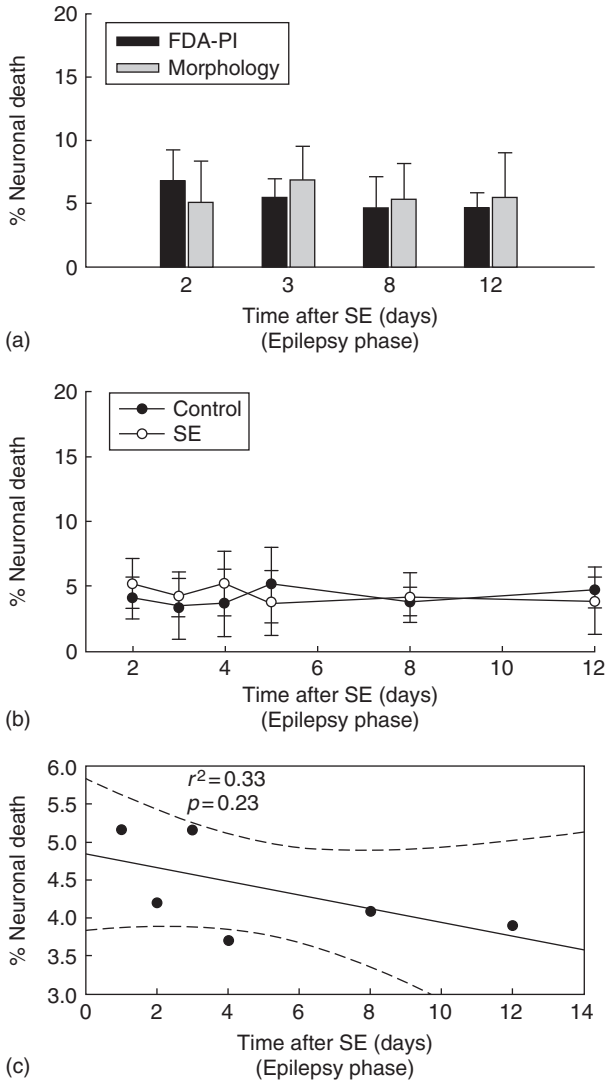


Figure 5 Spontaneous recurrent seizures do not cause neuronal cell death. (a) FDA–PI staining (black bars) and morphological assessment (gray bars) procedures revealed no increase in percent cell death with longer exposure to SREDs. (b) The amount of cell death in SREDs cultures was similar to age-matched control cultures and did not increase with time. Thus there was no increase in neuronal cell death in SREDs phase with time. The cell death in control cultures was around 4–6% and was lower than the control conditions for the pBRS-washed cultures due to the lack of washing and handling of the neurons and the direct assessment of the cell death in culture maintained in media without manipulation. (c) Linear regression analysis revealed no significant correlation ($r^2 = 0.26$, $p = 0.3$) between neuronal cell death and time of exposure to SREDs in cultures. Reproduced from Deshpande LS, Lou JK, Mian A, Blair RE, Sombati S and DeLorenzo RJ (2007) In vitro status elipticus but not spontaneous recurrent seizures cause cell death in cultured hippocampal neurons. *Epilepsy Research* 75: 171–179, with permission from Elsevier.

control conditions in the pBRS-washed cultures, presumably due to the lack of washing and handling of the neurons (i.e., we measured cell death in the control culture that were maintained without manipulation).

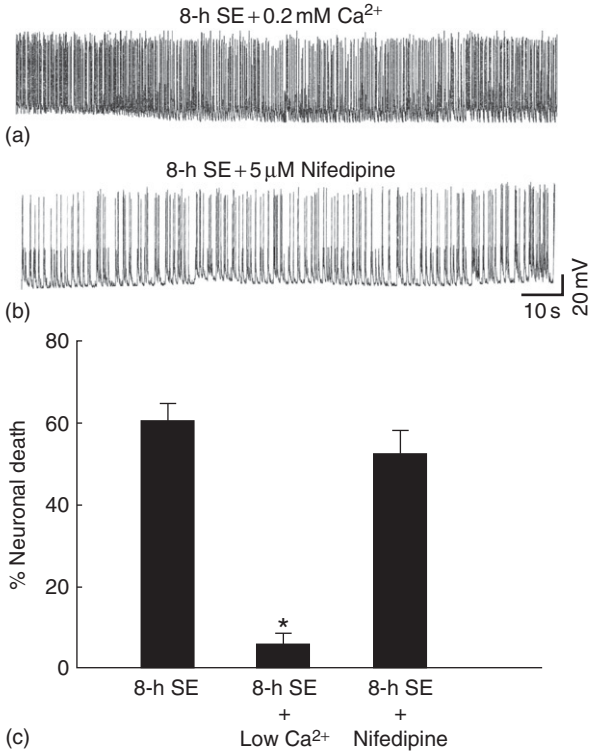


Figure 6 In vitro SE-induced neuronal death is dependent upon extracellular calcium. (a) Representative current-clamp recording from a neuron subjected to low Mg^{2+}-induced SE in the presence of low Ca^{2+} (0.2 mmol). Lowering extracellular Ca^{2+} had no effect on low Mg^{2+} induced high-frequency spiking. (b) Representative recording from a neuron treated with nifedipine (10 μmol) during low Mg^{2+}-induced SE. Similar to low Ca^{2+} condition, nifedipine treatment did not affect low Mg^{2+}-induced high-frequency spiking. (c) Neuronal death assessment revealed that lowering extracellular Ca^{2+} significantly attenuated net neuronal cell death compared to 8-h SE alone group (*$p < 0.001$, one-way ANOVA). In contrast, percent neuronal death was not significantly different between 8-h SE and nifedipine-treated cultures. Modified from Deshpande LS, Lou JK, Mian A *et al*. (2008) Time course and mechanism of hippocampal neuronal death in an in vitro model of status elipticus: Role of NMDA receptor activation and NMDA dependent calcium entry. *European Journal of Pharmacology* 583: 73–83.

Role of Ca^{2+} in SE-Induced Neuronal Death

Reduced extracellular Ca^{2+} (0.2 mmol) during low Mg^{2+} treatment significantly attenuated net neuronal death when compared with 8-h SE controls (**Figure 6(c)**). Lowering extracellular Ca^{2+} had no effects on the expression of low Mg^{2+}-induced SE (**Figure 6(a)**). SE durations of 8 h with 2 mmol Ca^{2+} ($n = 9$) resulted in 60.2% ± 5.03% neuronal death; in contrast, in cultures treated with low Ca^{2+} and low Mg^{2+} solution for 8 h ($n = 6$), net neuronal death was determined to be 4.05% ± 3.03% (when controlled for sham treatment). These experiments indicate that SE-induced neuronal death is dependent upon influx of extracellular Ca^{2+}.

To investigate the route of this Ca^{2+} entry, we used concentrations of well-characterized voltage gated Ca^{2+} channels inhibitors shown to be effective under our conditions. Treatment with nifedipine (5 μmol, $n = 6$) appeared to slow down low Mg^{2+}-induced high-frequency spiking (**Figure 6(b)**), but it did not significantly reduce neuronal death compared with untreated 8-h SE cultures (**Figure 6(c)**). Similarly, the T-type voltage-gated Ca^{2+} channel blocker ethosuximide (1 mmol) did not significantly prevent cell death due to SE in this model (data not shown). It was not possible to evaluate the effects of some of the other voltage-gated Ca^{2+} channel inhibitors, such as ω-conotoxin, because electrophysiological studies showed that these agents effectively blocked synaptic transmission and also prevented low Mg^{2+}-induced SE (data not shown). However, the lack of effects of nifedipine and ethosuximide on reducing cell death from SE supports the interpretation that Ca^{2+} entry through voltage-gated Ca^{2+} channels is not playing a significant role in causing cell death.

Role of Glutamate Receptor Subtypes in SE-Induced Neuronal Death

Treatment with the AMPA/kainate glutamate receptor antagonist CNQX (10 μmol, $n = 6$) had no significant effects on percentage of neuronal death compared with 8-h SE cultures (**Figure 7(d)**). Exposure to the metabotropic glutamate receptor antagonist mCPG (250 μmol, $n = 6$) did not significantly reduce neuronal death after 8-h of SE (**Figure 7(d)**). Treatment with the AMPA/kainate glutamate receptor antagonist NBQX (10 μmol, $n = 6$) did result in a small decrease in neuronal death from of 8-h SE ($p < 0.05$). In the current-clamp studies, we found that NBQX considerably slow down low Mg^{2+}-induced high-frequency epileptiform spiking (**Figure 7(a)–7(c)**). Thus, its reduction of the severity of SE may have contributed to its minor, but significant effect in reducing neuronal death.

Treatment with the NMDA receptor antagonist MK-801 (10 μmol, $n = 6$) produced a marked reduction in net neuronal death to 9.5% ± 3.36%, a figure that represented approximately an 80% decrease in neuronal death compared with 8-h SE cultures not treated with MK-801 (**Figure 8(c)**). Treatment with D-APV (25 μmol, $n = 6$) also reduced net neuronal death, to 13.2% ± 2.02%. Treatments with APV or MK-801 had no effects on the expression of low Mg^{2+}-induced SE (**Figure 8(a) and 8(b)**). These results demonstrate that during in vitro SE, extracellular Ca^{2+} that enters neurons through the NMDA receptor pathway is coupled to the molecular cascades that initiate neuronal death.

Discussion and Future Directions

This study provides a direct demonstration, in a controlled environment, that continuous electrographic epileptiform activity, but not spontaneous recurrent epileptiform discharges, caused cell death in hippocampal neurons in culture. We observed a strong correlation between duration of SE and subsequent neuronal death. However, there was no significant correlation between the time of exposure to SREDs and neuronal death. In fact, aside from the cell death associated with the initial exposure to low Mg^{2+}, there was no increase in neuronal death in cultures manifesting SREDs compared to age-matched control cultures for the life of the neurons in culture. SE-induced neuronal death was also found to be dependent upon extracellular Ca^{2+}, since reducing the driving force for Ca^{2+} entry, by lowering extracellular Ca^{2+}, significantly attenuated neuronal death. SE-mediated neuronal death was reduced by NMDA receptor channel blockade, while

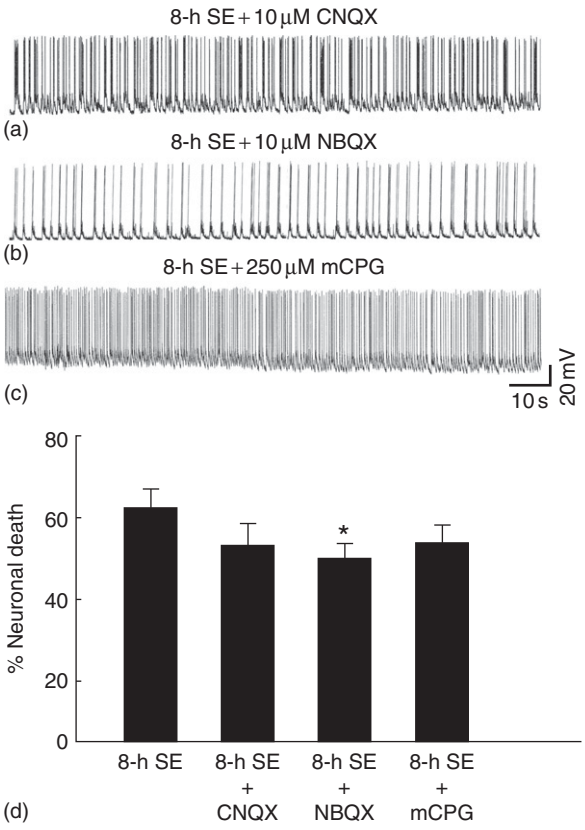


Figure 7 Role of glutamate receptor subtypes in SE-induced neuronal death. (a–c) Representative current-clamp recordings from neurons subjected to treatment with various glutamate receptor antagonists during low Mg^{2+}-induced SE-like activity. Treatment with AMPA/KA receptor antagonist CNQX (10 μmol) or NBQX (10 μmol) did slow down low Mg^{2+}-induced high-frequency spiking. But the spike frequency was still >3 Hz that meets the criteria of SE. Similarly, the metabotropic glutamate receptor antagonist mCPG (250 μmol) also had no effects on SE-like activity. (d) Neuronal death assessment revealed that there were no statistically significant differences between CNQX and mCPG-treated cultures and 8-h SE alone cultures. However, a small but significant reduction in neuronal death was observed upon NBQX treatment (*$p < 0.01$). Modified from Deshpande LS, Lou JK, Mian A *et al*. (2008) Time course and mechanism of hippocampal neuronal death in an in vitro model of status elipticus: Role of NMDA receptor activation and NMDA dependent calcium entry. *European Journal of Pharmacology* 583: 73–83.

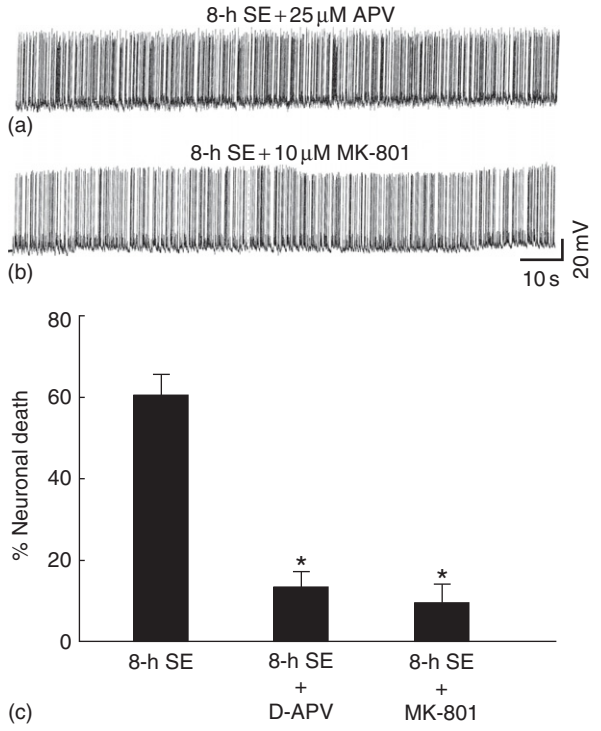


Figure 8 SE-induced net neuronal death is NMDA receptor dependent. (a, b) Representative current-clamp recordings from neurons subjected to treatment with NMDA receptor antagonists during low Mg^{2+}-induced SE. Treatment with APV (25 μmol) or MK-801 (10 μmol) had no effects on low Mg^{2+}-induced high-frequency spiking. (c) In contrast, there was a significant reduction in net neuronal death when these NMDA receptor antagonists were present throughout the duration of SE as compared with 8-h SE controls (*$p < 0.001$). Modified from Deshpande LS, Lou JK, Mian A *et al*. (2008) Time course and mechanism of hippocampal neuronal death in an in vitro model of status elipticus: Role of NMDA receptor activation and NMDA dependent calcium entry. *European Journal of Pharmacology* 583: 73–83.

neuroprotection afforded by blockade of non-NMDA glutamate receptor subtypes or voltage-gated Ca^{2+} channels appeared to be minor.

The results are generally consistent with animal and human data that demonstrate that neuronal cell death is associated primarily with SE and not with recurrent seizures. For example, in vivo studies using rat models of SE-induced temporal lobe epilepsy suggest that cell death is only associated with the initial injury from SE, but not with the subsequent spontaneous recurrent seizures. However, there are data showing that even a few evoked seizures in kindled rats produce TUNEL-positive responses in hilar neurons, and that hippocampal neuronal loss increases with increasing numbers of kindled seizures. Clinical studies have demonstrated conflicting results, with reports of increased serum and CSF levels of neuron-specific enolase, a marker of neuronal injury, after spontaneous seizures. However, a recent population-based, longitudinal quantitative MRI study of 179 patients with epilepsy showed that progressive regional or global cerebral damage was not a consequence of epileptic seizures. Furthermore, a separate quantitative postmortem study of the human hippocampus (from patients with chronic epilepsy) showed that seizures do not inevitably cause neuronal loss. Our studies support these findings and indicate that epileptic seizures do not cause neuronal damage by themselves.

Neuronal injury secondary to SE is the consequence of excessive neuronal excitability and synaptic activity, with high-frequency epileptiform discharges resulting in depolarizing shifts of membrane potential, free-radical production, neuronal metabolic overload, and elevations in extracellular glutamate. Although several microdialysis studies in humans and animals have shown significant elevations in extracellular glutamate during SE, others studies have failed to show such increases in extracellular glutamate during SE. It is well established that prolonged exposure to glutamate can kill neurons, and it has been suggested that increased glutamate in the extracellular space may account for neuronal death associated with SE. Although our experiments with continuous perfusion throughout the duration of high-frequency spiking cannot lower the synaptically trapped glutamate, our measurements do show that this perfusion manipulation can lower and prevent buildup of glutamate in the extracellular media. Under these conditions, the possible neurotoxic effects of extracellular accumulation of glutamate (and other substances) are eliminated. The cell death that occurs under these perfusion conditions is, therefore, a result of excessive synaptic activity. These findings are consistent with the observation that prolonged SE caused by the electrical stimulation of the perforant pathway (presumably involving excessive synaptic activity) can damage postsynaptic neurons. In addition, it has also been reported that increasing durations of kainic acid-induced SE – to the point of neuronal injury – cause cell death in the absence of a measurable increase in extracellular glutamate concentration. Taken together, these results further stress the importance of limiting seizure duration during SE to prevent brain injury.

Our data support the previous research that showed neuronal death after SE to be excitotoxic in nature. Thus, glutamate-mediated activation of NMDA receptors results in excessive influx of Ca^{2+}, which triggers various NMDA receptor-coupled neurotoxic signaling cascades, some of which (e.g., the activation of proteases, neuronal nitric oxide synthase, ceramides and poly(ADP-ribose) polymerase-1) cause cell death. The induction of these pathways may contribute to a positive feed-forward cycle that results in increased levels of intracellular Ca^{2+}, with subsequent energy failure and neuronal death. The neuroprotection we observed upon NMDA receptor antagonism or lowering extracellular Ca^{2+} could possibly result from inhibition of these neurotoxic cascades. These results are consistent with earlier in vivo studies from our laboratory, and those reported by others, that while NMDA receptor inhibition does not stop on-going SE

activity, the blockade of NMDA receptor signaling pathways is sufficient to attenuate SE-induced neuronal death. Interestingly, prolonged activation of the NMDA receptor transduction pathways during SE is also thought to underlie the permanent plasticity changes in neurons responsible for the expression of epilepsy.

Determining the effects of recurrent seizures in humans or animals is complicated by several factors, such as age, type of convulsant stimuli or seizure type, treatment with antiepileptic drugs, and numerous other clinical variables. The results from this study demonstrate that the hippocampal neuronal culture system provides a powerful in vitro model that allows for careful evaluation of the effects of seizures on neuronal viability – in the absence of various confounding factors. Understanding the molecular mechanisms mediating neuronal death due to continuous epileptiform may provide new insights into the development of novel therapeutic agents to prevent neuronal injury during SE. Future studies should be directed at not only controlling seizure activity, but in preventing the damaging manifestations of prolonged electrographic seizure activity. The results of our study provide a pharmacological framework within which to develop novel therapeutic interventions to prevent neuronal injury during seizure activity. Development of agents that can protect the brain during prolonged seizure activity offer new hope for the prevention of brain injury from SE.

See also: **Cell Damage/Excitotoxicity**: Excitotoxicity and Neurodegenerative Disease; Molecular Mechanisms of Cell Death After Seizures; Neuronal Injury from Seizures: Influence of Models, Development and Genetics; The Ontogeny of Seizure-Related Programmed Cell Death in the Developing Brain; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Plasticity**: The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis.

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Excitotoxicity and Neurodegenerative Disease

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Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS). Almost all excitatory neurons in the CNS are glutamatergic, and it is estimated that over 70% of all cortical nerve-endings release this excitatory amino acid (EAA). Glutamate is one of the most abundant chemicals in the mature human brain at an average intracellular concentration of 11 mM. Excessive levels of glutamate can be toxic to neurons,

activity, the blockade of NMDA receptor signaling pathways is sufficient to attenuate SE-induced neuronal death. Interestingly, prolonged activation of the NMDA receptor transduction pathways during SE is also thought to underlie the permanent plasticity changes in neurons responsible for the expression of epilepsy.

Determining the effects of recurrent seizures in humans or animals is complicated by several factors, such as age, type of convulsant stimuli or seizure type, treatment with antiepileptic drugs, and numerous other clinical variables. The results from this study demonstrate that the hippocampal neuronal culture system provides a powerful in vitro model that allows for careful evaluation of the effects of seizures on neuronal viability – in the absence of various confounding factors. Understanding the molecular mechanisms mediating neuronal death due to continuous epileptiform may provide new insights into the development of novel therapeutic agents to prevent neuronal injury during SE. Future studies should be directed at not only controlling seizure activity, but in preventing the damaging manifestations of prolonged electrographic seizure activity. The results of our study provide a pharmacological framework within which to develop novel therapeutic interventions to prevent neuronal injury during seizure activity. Development of agents that can protect the brain during prolonged seizure activity offer new hope for the prevention of brain injury from SE.

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Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS). Almost all excitatory neurons in the CNS are glutamatergic, and it is estimated that over 70% of all cortical nerve-endings release this excitatory amino acid (EAA). Glutamate is one of the most abundant chemicals in the mature human brain at an average intracellular concentration of 11 mM. Excessive levels of glutamate can be toxic to neurons,

which in culture can be killed by bathing in as little as 10–40 μM of glutamate. Excitotoxicity is the prolonged or overstimulation of glutamate receptors (GluRs), subsequently allowing the excessive influx of calcium (Ca^{2+}). Cytoplasmic Ca^{2+} is then responsible for the activation of a myriad of Ca^{2+}-dependent enzymes – including protein kinase C, phospholipase A2 and C, Ca^{2+}/calmodulin-dependent protein kinase II, nitric oxide synthase, and other proteases and endonucleases – all of which have the potential to destroy neurons. During excitatory synaptic transmission, the levels of glutamate can rise 1000 fold in the synaptic cleft. Glutamate is cleared from the synapse mainly by astrocytic EAA transporters (EAATs). The EAATs prevent excessive amounts of glutamate remaining in the synapse to overstimulate GluRs. A balance of excitatory and inhibitory neurotransmission is also necessary to protect neurons from potentially excitotoxic insults. Inhibitory neurotransmitter receptors mainly act to hyperpolarize the membrane, preventing propagation of the excitatory postsynaptic current. γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter of mammalian CNS.

Glutamatergic neurotransmission is complex and multifaceted, and perturbations in any aspect of glutamate or GABA release, signaling, clearance, or cycling may lead to excitotoxic neurodegeneration. Both the GABAergic and glutamatergic neurotransmitter systems are implicated in epileptogenesis, as well as other neurodegenerative diseases where excitotoxicity is purported to play a role in the pathogenesis of neuronal death. Examples of such diseases are Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), Parkinson's disease (PD), stroke, and hypoxia/ischemia.

AD is the commonest neurodegenerative disease. AD patients have an increased risk of epilepsy, particularly in younger onset cases. It is estimated that 10–22% of AD patients suffer from seizures. Although the pathogenesis of the two diseases may be different, the pattern of disease pathology of AD and temporal lobe epilepsy (TLE) affects similar regions of the brain, early stages manifesting in the hippocampus. The pattern of neuronal damage is also very similar, affecting pyramidal neurons and causing axon-sparing lesions and dendritic pruning. The ingestion of domoic acid from blue mussels can induce seizures and produces region-specific neuronal damage similar to AD and epilepsy. Domoic acid is able to act as a neurotoxin probably because of its structural similarity with glutamate. The common features of AD and TLE pathologies with those produced by domoic acid could indicate a link in the pathogenesis of two separate neurodegenerative diseases through excitotoxicity. It has also been postulated that the link between the two diseases (i.e., the high frequency of seizures among AD patients) may arise because of the neuron loss in AD. Loss of pyramidal neurons in the hippocampus and neocortex may lead to the development of a seizure focus. The loss of normal balance between excitation and inhibition plays a role in the pathogenesis of both diseases. Published work reporting evidence of this imbalance, and future experimental strategies to delineate a synaptic role, will be discussed.

Background

The assessment of glutamate in autopsy tissue is problematic as it is both a neurotransmitter and a metabolic intermediate. Glutamate is a major product of the Krebs cycle in brain, and immunohistochemistry (IHC) does not distinguish transmitter and metabolic glutamate. Delineation of glutamatergic neurons by IHC can only be performed on perfusion-fixed tissue, and so a biochemical correlate of pyramidal neuron dysfunction in postmortem brain is difficult to establish. Reports of reduced levels of glutamate and aspartate in AD cerebral cortex and hippocampus are confounded by the rapid rise in glutamate levels that occurs after death. The ability to distinguish one pool of glutamate from the other in experimental systems currently remains elusive. For this reason, studies have mainly focused on molecules directly affected by glutamate, such as receptors and transporters. Excitotoxicity can occur through excessive glutamate release, implicating a synaptic dysfunction as is postulated to occur in epilepsy, through inefficient glutamate clearance as is evident from ALS, or through changes in GluR composition allowing hyperexcitation of neurons leading ultimately to excitotoxic cell death. In some neurodegenerative disorders with a more complex etiology, such as AD, it is possible that the pathogenesis involves a combination, if not all, aspects of excitotoxic glutamate build-up. Common to all neurodegenerative diseases, where excitotoxicity is the putative cause of neuronal death, is regional-specific neuronal damage as part of the pathology. It is possible that the intrinsic regional balance between excitatory and inhibitory systems may be the determinant.

Transporters

There are five major types of glutamate transporter, or EAAT. Transporters localized to astrocytes include EAAT1 and EAAT2. The latter is responsible for the majority of glutamate uptake in the CNS. EAAT3 is localized to somata and dendrites of neurons but in extrasynaptic sites. EAAT4 is found in cerebellar Purkinje cells and EAAT5 is specific to retina.

The EAATs are responsible for the fast efficient clearance of glutamate from the synaptic cleft, where glutamate is converted to glutamine via glutamine synthetase. Glutamine is then transported back into neurons, where it is reconverted into glutamate and packaged into synaptic

vesicles by the vesicular glutamate transporters (VGLUTs), of which three have been isolated and characterized.

Glutamate transporters and glutamine synthetase have been implicated in the pathogenesis of epilepsy and other neurodegenerative diseases, particularly AD and ALS.

Glutamate uptake in AD-affected cortex is reduced than in controls. However, attempts to correlate this reduction in transport efficacy with distribution and abundance of the different EAATs by IHC or immunoblotting techniques has led to conflicting reports. EAATs have been more directly implicated in other neurodegenerative diseases, particularly epilepsy and ALS. Mice lacking EAAT2 displayed spontaneous epileptic seizures, and other mice treated with antisense probes to EAAT3 or EAAT1 showed increased epileptic activity. Reduced glutamate transport and EAAT2 expression has been observed in ALS motor cortex. Some studies have found higher levels of EAAT2 splice variants in ALS that may contribute to the reduction in EAAT2 expression. Overrepresentation of EAAT2 splice variants present in hippocampus and temporal cortex has also been found in TLE patients. Aberrant EAAT1 expression has been found to occur in neurons that form neurofibrillary tangles as evidenced by tau expression in AD brain. These studies implicate abnormal glutamate transporter expression or function in the pathogenesis of neurodegeneration.

Receptors

GluRs play a more direct role in excitotoxic neurodegeneration typical of epilepsy and other neurodegenerative disorders. Many of the current pharmaceuticals available to treat epilepsy act on either the glutamate or GABA receptors. GABA is the major inhibitory neurotransmitter in the mammalian CNS. There are two major classes of GABA receptors. $GABA_A$ receptors are ligand-gated ion channels made up of many subunits that are responsible for rapid inhibitory effects by allowing the influx of Cl^-. $GABA_B$ receptors are G protein-coupled receptors that hyperpolarize the neuron by increasing K^+ conductance. $GABA_B$ receptors are also able to decrease Ca^{2+} entry and have a slow inhibitory effect. Both types of GABA receptor have the ability to counterbalance the effects of GluR activation. Disruptions to GABAergic transmission favoring excitation may lead to seizures and excitotoxic neuronal death.

There are two major classes of GluRs: metabotropic and ionotropic. Metabotropic GluRs (mGluRs) are G protein-coupled receptors divided into three groups – group I, II, and III. Activation of mGluRs raises intracellular Ca^{2+} stores from the endoplasmic reticulum. mGluRs can contribute to excitotoxicity by raising Ca^{2+} concentration or by prolonging depolarization of the neuron, thereby activating *N*-methyl-D-aspartate (NMDA) receptors.

Ionotropic glutamate receptors (iGluRs) are the largest group, and are generally named after their specific ligands: kainate, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA), and NMDA. Of these receptors, the NMDA receptor plays the most direct role in excitotoxicity, due to the receptor's Ca^{2+} channel properties. Overactivation of any of these receptors leads to epileptic seizures, and also has the ability to kill neurons.

GluRs have been the most popular target for pharmaceutical intervention in neurodegenerative disorders. GluR agonists show proconvulsant properties, whereas antagonists have been found to have anticonvulsant properties. Similarly, with the glutamate transporters, attempts to correlate the distribution and abundance of GluRs with disease states have been met with mixed results. Anticonvulsant drugs that have been successful in treating and attenuating seizure activity commonly ameliorate aspects of hyperexcitability. GluR antagonists are important in this category, but agonists of GABA receptors also have anticonvulsant properties. Conversely, GABA receptor antagonists induce seizure activity. Severe alcohol withdrawal may produce seizures, supposedly due to its action on the $GABA_A$ receptor.

Previous studies measuring the levels of GluRs in human autopsy brain have shown varying results: some report an increase, some a decrease, and others no change. Splice variants have also been described for the NR1 subunit of the NMDA receptor. NR1 subunit mRNA transcript variants containing an N-terminal cassette were found to be more abundant in affected regions of the AD brain than areas of the brain relatively unaffected by AD (and controls). This result was supported by immunoblotting results detecting the NR1 N-terminal cassette variant protein. Areas susceptible to AD damage also showed lower levels of NR2A and NR2B subunits compared with the homotropic area in controls. The mRNA transcripts of the NR2A and NR2B subunits were concordantly lower in affected areas of the AD brain than in controls. This finding would seem to suggest that the composition of the receptor, not its abundance, is important for susceptibility to excitotoxic insults.

Synaptic Dysfunction

There is evidence of synaptic dysfunction in epilepsy and AD. Riluzole, a drug used to treat ALS, has been suggested as a candidate antiepileptic drug. One of the pharmacological effects of riluzole is the inhibition of G protein-dependent synaptic glutamate release. In AD, synaptic loss is the best correlate of cognitive decline that characterizes the clinical symptoms of the disease. Neurodegenerative disorders where excitotoxicity may underlie the neuronal loss all share synaptic pathology. A study to delineate and characterize the regionally specific pathology of these disorders by categorizing the aberrant function of excitatory or inhibitory synapses, or whether these changes are specific to pre- or postsynaptic

terminals, could focus efforts of pharmaceutical intervention of glutamate-mediated neuronal death.

Methods

In our laboratory, we aim to study the synaptic pathology of excitotoxicity in neurodegenerative diseases in human autopsy brain tissue. Frozen brain tissue obtained at autopsy can be stored and assayed for a variety of markers, together with IHC from formalin-fixed tissue. The extent of nerve-terminal loss may be assessed in situ by measuring markers specific to the synapse. We have initiated a study that uses sensitive and specific assays for pre- and postsynaptic markers for (a) nerve-terminals in general and (b) excitatory terminals specifically. If the study can be extended to selective markers of GABA terminals, a 'map' of excitatory and inhibitory terminals could be constructed. Most studies on synaptic proteins in neurodegenerative diseases have focused on presynaptic markers for densities of terminals in the brain areas studied. Since neurons may receive inputs from different brain areas, quantitating presynaptic markers could be misconstrued because the afferents may differ in their origins. Rarely have studies measured the abundance of postsynaptic markers in neurodegenerative diseases, which would provide a better measure of local neuronal loss. If both presynaptic and postsynaptic markers are assessed, the numbers of synaptic contacts might be quantitated, at least in principle. Also by categorizing markers specific to excitatory or inhibitory synapses, and identifying these markers with their subsynaptic localization, a complete picture of excitotoxic synaptic dysfunction in neurodegenerative disease could be obtained.

Recent Results

General Presynaptic Markers

Synaptophysin is an integral membrane protein localized to synaptic vesicles. It is thought to comprise part of the pore complex, which forms when the vesicle fuses with the presynaptic membrane. It is a specific and sensitive marker for synaptic terminals. Synaptophysin concentrations are reportedly reduced in certain brain areas of AD. However, our IHC and ELISA data suggest that the reduction is not great, certainly not to an extent consonant with local neuronal loss. This emphasizes the extrinsic origin of many terminals.

Dynamin I is enriched in the presynaptic terminal and accounts for over 0.4% of total brain protein. Ca^{2+} influx inactivates dynamin I during neurotransmitter release until the vesicles are recycled. We investigated the levels of dynamin I in AD brain concurrently with synaptophysin and found no difference compared with controls.

Glutamatergic Presynaptic Markers

A key to excitotoxicity will be the ability to delineate excitatory terminals and to quantitate them relative to the total. Three distinct isoforms of the VGLUT (VGLUT1–3) have been cloned, characterized, and had specific antibodies raised against them. VGLUT1 and 2 divide distinct subpopulations of excitatory synapses. The third isoform, VGLUT3, overlaps in distribution with the other two isoforms in some brain areas. While not as abundant as VGLUT1 and 2, VGLUT3 colocalizes with markers of cholinergic and serotonergic synapses in certain areas. VGLUT1 and 2 are diminished in AD frontal cortex, which correlates with cognitive decline. Animal models of epilepsy have shown an increase in VGLUT1 in the hippocampus. In a study of hypoxic ischemia in rats, VGLUT1 immunoreactivity was significantly elevated in both hippocampi following insult; the levels of VGLUT2 were unaltered. It is reported that 14% of patients with hypoxic brain injury develop epilepsy. The elevation in VGLUT1 could signify the onset of increased transynaptic glutamate release. This difference between the two diseases shows a difference in pathogenesis. The increase in VGLUT in epilepsy could signify an overload of glutamate or an increase in glutamatergic terminals. The opposite effect in AD, albeit in a different area, could signify a loss of glutamatergic terminals, implying a different mechanism for excitotoxicity.

Two protein isoforms distinguish inhibitory presynaptic terminals from excitatory presynaptic terminals. Complexin I regulates inhibitory neurotransmitter release and complexin II regulates excitatory neurotransmitter release. They are cytosolic, acidic membrane proteins associated with synaptic vesicle docking to the presynaptic membrane that decreases neurotransmitter release. Complexin I and complexin II have 84% amino acid sequence identity in mammalian brain tissue. Complexin I mRNA is preferentially expressed in axosomatic synapses, which are mainly inhibitory, while complexin II mRNA is mainly expressed in axodendritic and axospinal synapses, which are typically excitatory. We found that both complexins were significantly decreased in hippocampus, inferior temporal cortex, and occipital cortex in AD cases compared with those in controls. The loss of complexins in AD was in contrast to the unchanged levels of dynamin I and synaptophysin, markers of presynaptic terminals. The ratio of complexin II to complexin I was unchanged between AD cases and controls. This result indicates a loss of regulation of neurotransmitter release in AD in preserved presynaptic terminals.

Loss of regulation in vesicular glutamate release might also increase synaptic glutamate concentrations. The ratio of complexin II to complexin I in hippocampus favored complexin II highly; inferior temporal cortex also favored compexin II (but to a lesser extent than

hippocampus), but occipital cortex favored complexin I over complexin II. This pattern may signify a regional susceptibility to excitotoxic insults in neurodegenerative diseases, such as AD, which rarely affects occipital cortex, which is commonly one of the most spared brain regions. Similarly, hippocampus is the earliest and one of the most affected regions in AD. Inferior temporal cortex is also commonly affected in AD. Both these regions showed significantly lower levels of complexin I and II compared with the homotropic area in controls. This result shows the potential role of vesicular glutamate exocytosis regulation in excitotoxicity and neurodegenerative disease and is worth investigation in epilepsy.

N-cadherin belongs to a large family of cell adhesion proteins. It is implicated in neurite outgrowth, synaptic junctional complex formation, and maintenance of the synapse. It is located at the synapse both in the presynaptic terminal and on the postsynaptic membrane, where it colocalizes with synaptophysin, synapsin I, PSD95, and GluR1. N-cadherin interacts with the cytoskeleton via β-catenin. Immunological studies have shown the staining of N-('neural') and E-('epithelial') cadherin to be mutually exclusive; some synapses are negative for both. Hippocampal cell culture studies have shown the segregation of N-cadherin from general synapses to excitatory synapses during maturation of the cell, suggesting that N-cadherin is specific for excitatory synapses. In AD tissue (as compared with that of controls), we found higher levels of N-cadherin in hippocampus, inferior temporal, and occipital cortex, albeit not significantly overall. We then investigated its binding partner β-catenin. The levels of β-catenin were significantly more abundant in AD cases, especially in hippocampus. β-catenin is also able to translocate to the nucleus, where it activates transcription factors. The concordant increase in N-cadherin and its associate β-catenin in AD could signify dysfunction of excitatory synapses, or an increase in the density of excitatory synapses in AD. Such dysfunction would require confirmation by IHC. Similar results have been obtained investigating the levels of N-cadherin in animal models of epilepsy. An increase in both N-cadherin protein and mRNA were observed in the hippocampus of rodents after induced seizures. Interestingly, an increase in β-catenin was observed in the hippocampus of chemically-induced seizures in rodents.

The Postsynaptic Region

An electron-dense thickening at the synaptic junction is termed the postsynaptic density (PSD). Most postsynaptic proteins isolated from the PSD are associated with neurotransmitter receptors. Proteins enriched in the PSD include fodrin, tubulin, actin, Ca^{2+}/calmodulin-dependent kinase II (CaMKII), and the neuroligins 1–3. Neuroligins are brain-specific membrane proteins with biochemical and structural features indicative of heterotypic cell adhesion. They associate with various splice variants of β-neurexins. Drebrin is enriched at the postsynaptic terminal where it binds actin. Excitatory synaptic terminals in adult mammalian CNS are held in place by an asymmetric junctional complex, termed type I PSDs. The protein PSD95 (SAP90) binds to the NR1 subunit of the glutamate-NMDA receptor. It has been implicated in the assembly of some of the components of the PSD and in receptor targeting and localization.

A major component of the PSD is the brain-specific α subunit of CaMKII, which, with the β-subunit, has been estimated to comprise 2% of total protein in rodent hippocampus and 1% of total protein in forebrain. CaMKII interacts with the NR2B subunit of the NMDA receptor as well as with PSD-95. Other substrates of the enzyme include GluR1 of AMPA receptors, SynGAP, F-actin, and densin-180. These associations have implicated CaMKII in long-term potentiation (LTP), neuronal plasticity, and memory formation. The levels of αCaMKII were unaltered in AD hippocampus compared with those in controls. One study had reported an increased level of αCaMKII in epileptic dentate gyrus, but another study had suggested a decrease of αCaMKII in epileptogenic brain tissue in another form of epilepsy. Activation of receptors and enzymes by CaMKII is implicated in epileptic neuronal damage as this kinase is activated by Ca^{2+}. Increases of intracellular Ca^{2+} are a by-product of GluR activation.

Hippocampal drebrin levels were shown to be lower in AD cases than in controls. The concentration of synaptophysin in that study did not differ, in agreement with our own studies. This result is unexpected if it is supposed that neuronal loss should lead to retrograde degeneration of axons. One explanation for this finding is that an initial axonal degeneration might be followed by a florid regrowth, as a compensatory process in the diseased tissue. The failure of these regenerate terminals to re-form appropriate connections may be a substrate of the clinical dementia and language malfunctions that are characteristic of AD. As a marker of dendritic spines, investigation of drebrin in epilepsy and other neurodegenerative disorders affecting dendrites could provide valuable insight into the disease pathogenesis.

A tubulin binding protein, gephyrin, is associated with glycine and GABA receptors and anchors them in clusters at inhibitory postsynaptic membranes. Inhibitory postsynaptic terminals are structurally different from excitatory PSDs and are named type II PSDs. Investigation of gephyrin as a marker of inhibitory postsynaptic terminals would be a great asset to AD and epilepsy research, and is worthy of future investigation. Fewer gephyrin-labeled puncta have been reported in a rodent model of epilepsy, indicating a decreased inhibitory content in the disease.

Future Challenges

To date, these changes in synaptic proteins indicate a significant role for glutamatergic synapses in the pathology of neurodegenerative disorders. The markedly lower abundance of the complexins in AD, and the area-specific variation in their excitatory to inhibitory ratio distribution, demonstrate a potential predictor of area-specific damage in AD. Excitatory synaptic junction protein N-cadherin and its binding partner β-catenin are elevated in AD and epilepsy. This result could indicate a novel pathway of excitatory synaptic dysfunction in neurodegenerative diseases. Further investigation by IHC is required to determine the specific subcellular localization of the increase.

The approach outlined could also be applied to disease progression. Charting the disease severity or progression that occurs in many neurodegenerative diseases, with the distribution and abundance of specific synaptic proteins, could aid in the determination of the specific elements that correlate with disease symptoms. This strategy could isolate the initial stages of the pathogenesis, and direct focused pharmaceutical intervention.

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; Molecular Mechanisms of Cell Death After Seizures; Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model.

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Molecular Mechanisms of Cell Death After Seizures

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Introduction

Seizures, particularly when prolonged (status epilepticus) or repeated (pharmacoresistant epilepsy), can damage the brain. Injury is found acutely in humans after status epilepticus, and neuroimaging and neuropathology suggest hippocampal changes may progress over time in some temporal lobe epilepsy (TLE) patients. Experimental modeling shows cognitive deficits brought about by repeated seizures relate to the extent of seizure-induced neuronal death. Neuronal death may also contribute to the pathogenesis of acquired epilepsies. There appears to be a critical threshold of neuronal loss within the hilar or CA3 subfields that when breached leads to permanently enhanced hippocampal excitability and manifestation of epileptic seizures. Understanding how to protect the brain against neuronal death after seizures is, therefore, a key area of basic epilepsy research, with significant therapeutic implications.

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Excitotoxicity and Seizure-Induced Neuronal Death

In the early 1970s, Meldrum and colleagues demonstrated that evoking seizures in primates replicated patterns of damage found in the hippocampus of TLE patients. Other work, particularly by Olney's group, provided mechanistic explanations. The prolonged or sustained activation of ionotropic glutamate receptors during seizures gates pathologic sodium and calcium entry. Neuronal death may result directly from osmotic stress and cell lysis, or follow secondary signaling cascades. The latter include calpain- and cathepsin-mediated proteolysis of structural proteins and essential enzymes, and DNA damage resulting from DNase II and neuronal nitric oxide synthase activity. Consistent with this hypothesis of glutamate-initiated cell death pathways, studies have shown that administration of glutamate receptor antagonists block or attenuate seizure-induced neuronal death and epileptogenesis.

Background

Seizures and Apoptosis Signaling Pathways

Despite this progress, disrupting glutamatergic signaling has major side effects in rodents and humans, including psychosis and neurotoxicity; further, glutamate antagonists have failed in clinical trials as neuroprotectants in other disorders. In addition, not all cell death after seizures appeared to fit the necrotic/excitotoxic hypothesis. Seizure-induced neuronal death could be reduced by inhibition of new protein synthesis. Moreover, seizure-damaged neurons were found to be positive for DNA fragmentation, and glutamate receptor antagonists failed to influence the numbers of such cells after brief seizures. Furthermore, morphological features of apoptosis as well as necrosis were present in seizure-damaged hippocampus. Questions therefore emerged about whether seizures activated apoptosis signaling pathways, whether such activation contributed to neuronal death (or epileptogenesis), and the relevance of these cell death pathways to human epilepsy. Over the past decade, experimental modeling and analysis of clinical material by a number of laboratories have furnished answers to some, but not all of these questions.

Apoptosis proceeds via one of two main pathways. The intrinsic (mitochondrial) pathway is triggered by DNA damage, endoplasmic reticulum stress, serum withdrawal, or perturbed calcium homeostasis. These changes are 'sensed' by Bcl-2 homology (BH) domain 3-only proteins which target and neutralize antiapoptotic members of the Bcl-2 family, facilitating multidomain Bax/Bak to undergo conformational change/activation. This pathway culminates in release of cytochrome *c* from mitochondria, which binds and activates the apoptotic protease activating factor 1 (Apaf-1). In turn, this complex recruits and activates caspase-9, a member of the cysteine aspartate-specific protease family. Caspase-9 cleaves and activates caspase-3, which then cleaves key substrates, triggering DNA fragmentation and cell demise. Caspase activity may be constrained by inhibitor of apoptosis proteins (IAPs), a molecular brake that is overcome by another mitochondria-originated protein, Smac/Diablo.

The extrinsic pathway is mediated by surface-expressed death receptors of the tumor necrosis factor (TNF) family (e.g., Fas and the p55 TNF receptor). Intracellular adaptor proteins containing death domains such as Fadd and Tradd associate with the receptors and form a molecular scaffold (death-inducing signaling complex) that enables recruitment of caspase-8. Activated caspase-8 can directly cleave caspase-3 or trigger the mitochondrial pathway by cleaving and activating the BH3-only protein Bid.

Methodological Approaches

Our laboratory uses in vivo and in vitro models of seizure-induced neuronal death, alongside analysis of patient material. Our in vivo model of status epilepticus is based around a focal elicitation approach that was originally developed by Ben Ari et al. Kainic acid (KA) is injected into the rat (or more recently, mouse) amygdala to generate seizures and a focal, ipsilateral lesion within hippocampal CA3/CA1. We perform quantitative EEG in our studies and find this of value for identifying electrographic correlates of damaging versus benign seizure activity, or associations with gene responses. We also use an in vitro seizure model, where hippocampal neurons are chronically cultured in a glutamatergic block; wash-out of the blocker triggers seizure-like activity. This model allows us to exploit some experimental techniques not possible in vivo. Tissue from these models is used to characterize spatiotemporal changes in apoptosis-associated gene expression after seizures, and to measure enzyme activity, protein–protein interactions, and subcellular localization of key players in the cell death cascade. Histological approaches include analysis of neuronal loss or DNA fragmentation – and assessment of intervention efficacy. Finally, clinical material from TLE patients enables us to validate experimental model findings and gain insight into translational relevance.

Results

Important Results to Date

Key discoveries

Our first contribution to the field came from two simultaneous discoveries in the laboratory at the University of Pittsburgh in the late 1990s. Having observed DNA

fragmentation in CA3 neurons after seizures, we investigated cytochrome *c* release in our model and detected rapid translocation into the cytoplasm within ~2 h. The seizure-damaged hippocampus also contained activated caspase-3, and we found that in vivo infusions of a putative caspase inhibitor protected against neuronal death in the hippocampus. At the same time, we were collaborating with Robert Clark and Neurological Surgery to look at apoptosis-associated genes in human neocortex removed during surgery for intractable seizures in patients with TLE. We found patient samples contained higher levels of procaspase-3 and cleaved caspase-3. The inflammatory caspase-1 was also cleaved in these samples, and Bcl-2 was over expressed. We interpreted these observations to mean that caspases and apoptosis-associated signaling molecules were altered in patients most at risk of progressive neuronal loss in hippocampus, and this interpretation was supported by findings in experimental models showing that prolonged seizures recapitulated some of these responses.

Intrinsic or extrinsic?

Studies moved next to the question of the route to caspase-3 activation. We expected the mitochondrial/caspase-9 pathway would be involved because elevated intracellular calcium, a consequence of seizure activity, had been shown to directly release cytochrome *c* from isolated brain mitochondria. We found that seizures induced formation of the so-called apoptosome complex, of cytochrome *c* with Apaf-1, and caspase-9 was cleaved and activated. However, so too was caspase-8. In fact, the early onset of the caspase-8 pathway and the protection afforded by its inhibition suggested an important role, and one we later found was conserved during seizure-induced neuronal death in vitro. The mechanism of caspase-8 activation after seizures remains puzzling, but probably is death receptor mediated because death-inducing signaling complexes form rapidly after experimental seizures and are even detectable in a TLE patient's hippocampus. Results from independent laboratories have since supported this unexpected mix of the intrinsic and extrinsic pathways in experimental models, and the robust protection afforded by several caspase inhibitors. Our studies of resected hippocampus from TLE patients have shown that both cleaved caspases 8 and 9 are present.

Other roles for caspases?

Not all laboratories have confirmed our findings. Fujikawa's group has shown DNA fragmentation can be weak and caspase processing absent in some models of generalized seizure-induced neuronal death. In addition, even where independent laboratories have confirmed seizures cause processing or activation of caspases, the temporal profile can vary. While our group detected responses within the first 4 h postseizure, other groups (e.g., Pitkanen's laboratory at the University of Kuopio) have shown later changes that extend into the period of epileptogenesis. These differences may be partly due to technical and model-specific factors. Whatever the basis for such differences, these experiments have identified nonlethal roles for caspases. Limited proteolysis or restriction of activated caspases to specific cell sites appears to enable sublethal activity, and this activity may underlie restructuring and the mechanism of ischemic tolerance. Such sublethal activity could explain the presence of cleaved caspases 2, 3, and 9 within neuronal processes in hippocampal sections from patients with TLE. Experimentally, caspases 2 and 6 are also overexpressed in regions of neuronal restructuring during epileptogenesis. Thus, roles for caspases extend beyond regulation of cell death into other areas of potential importance in epileptogenesis.

Bcl-2 proteins: Upstream determinants of seizure-induced cell death?

The significance of Bcl-2 family proteins for seizure-induced neuronal death (see **Figure 1**) was first indicated by the finding that neurons in *bax* knockout mice were resistant to glutamate and KA. However, convincing evidence that Bax is activated by seizures in vivo has not yet been presented, although we have detected mitochondrial clustering of Bax after seizures in vivo. Instead, BH3-only protein responses have been more completely explored. Seizures activate Bad, which dissociates from 14–3–3 and binds antiapoptotic Bcl-xl, an event thought to enable Bax activation. Interestingly, the neuroprotective effect of the ketogenic diet was recently linked to suppression of the Bad pathway. Seizures also induce Bim upregulation, a much more potent BH3-only protein, one that appears to target antiapoptotic Bcl-w in rat and mouse hippocampus. Bid is also an early responder after seizures in vivo, although calpain can also cleave Bid during excitotoxic insults. Reports from other laboratories support broad and early involvement of BH3-only proteins after seizures.

Transcriptional control?

Several transcription factors associated with apoptosis are activated by seizures. The most studied is p53, although the specific genes involved in effecting p53-induced neuronal death after seizures are still unknown. Mice deficient in p53 are protected in the short term against cell death after seizures, although when followed at later recovery points this protection may dissipate. Protection is also seen using pharmacologic approaches to target p53. Interestingly, levels of p53 are elevated in hippocampus from TLE patients.

We had rather discounted a role for transcription factors after studying the Bad-Bcl-xl pathway because all the components were constitutively expressed in hippocampus and activated without expressional changes. However,

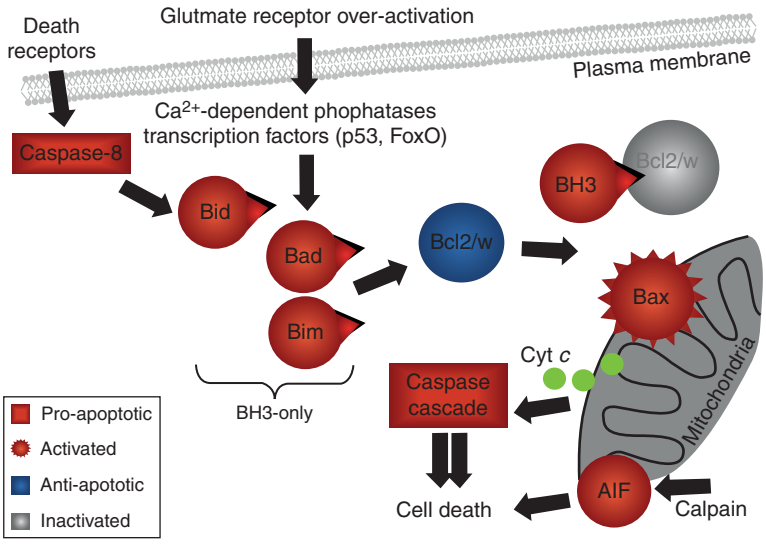


Figure 1 Cartoon depicting possible triggers and routes for apoptosis signaling after status epilepticus. Glutamate activates BH3-only proteins via calcium (Ca^{2+})-dependent phosphatases or transcription factors. Death receptors are also activated by damaging seizures, leading to caspase-8 and Bid cleavage. BH3-only proteins target and neutralize antiapoptotic Bcl-2 family proteins and enable Bax activation. Thereafter, apoptogenic substances including cytochrome *c* (cyt *c*) and AIF are released from mitochondria, activating caspase-dependent or independent cell death.

subsequent studies on Bim showed seizures rapidly activated FoxO transcription factors, and blocking this activation was found to be neuroprotective. Also interesting, protein kinase B/Akt protected cortical regions in this model by suppressing FoxOs such that when Akt was inhibited, cortical Bim expression increased and more cell death resulted. Significantly, Akt activity is elevated and FoxOs/Bim suppressed in patient hippocampus, suggesting potentially adaptive responses that could limit vulnerability to cell death. New data also suggest additional complexity in transcriptional control of Bim involving c-Jun *N*-terminal protein kinase.

AIF and caspase-independent pathways

An interesting development has emerged for calpains, traditionally linked to neuronal necrosis, as effectors of caspase-independent apoptosis. Chen's group at the University of Pittsburgh recently showed that activated calpain I can trigger apoptosis inducing factor (AIF) release from mitochondria and neuronal death, bypassing Bcl-2 proteins and caspases. This finding is relevant to seizure-induced injury, since mice overexpressing the calpain inhibitor calpastatin or deficient in AIF are partly protected against seizure damage in vivo. However, we found that the nuclear fraction from human TLE hippocampus contained significantly elevated levels of the caspase-activated DNase (CAD) compared with matched autopsy controls, while nuclear levels of AIF were not different between TLE patient samples and controls. This result suggests that caspase-dependent rather than caspase-independent apoptosis pathways are more important for ongoing pathogenic processes in chronic TLE.

Recent Results

Findings from Bcl-w knockouts

Since a pharmacological approach in the area of Bcl-2 family proteins was limiting functional studies, we developed relevant mouse models. We had found that intraamygdala KA caused seizure damage in mice that involves ipsilateral CA3 and CA1, strong DNA fragmentation, and some morphological features of programmed cell death. Early cytochrome *c* release and caspase processing were also conserved. We next carried out studies on antiapoptotic Bcl-w, since this gene had been identified as a target of Bim and we had other experience with Bcl-w. Seizures in normal mice caused bidirectional regulation of Bcl-w, depending on the severity of the seizure; Bcl-w increased after brief seizures that did not cause neuronal damage, but decreased/inactivated after damaging seizures. Levels of Bcl-w in hippocampus from our TLE patient cohort were also higher than autopsy controls, suggesting frequent seizures in humans also alter expression. Critically, mice deficient in *bcl-w* displayed significantly more hippocampal cell death after seizures than mice with normal *bcl-w*. Unexpectedly, we detected another difference between *bcl-w* knockout and wild-type mice: earlier polyspike seizure onset in *bcl-w* knockout mice. The difference was small, a few minutes, and not sufficient to explain the differences in histological injury. However, it implied that Bcl-w protein may have novel functions to explore. We next overexpressed Bcl-w in hippocampal neurons in vitro and performed patch clamp recordings which determined that Bcl-w overexpression significantly enhanced fast γ-amino-butyric acid (GABA) currents. These data suggest that Bcl-w can promote inhibitory neurotransmission.

This makes Bcl-w an attractive target in epilepsy, being both neuroprotective and potentially seizure-suppressive.

Other findings in experimental models

Recent work has focused on assessing the functional significance of BH3-only proteins on epileptogenesis. In *bim* deficient mice (from the Villunger laboratory), brain development appears normal (Figure 2a) and the mice undergo seizures after intra-amygdala KA as their wild-type (Figure 2a) littermates. Our early results suggest that loss of this gene influences seizure-induced neuronal death, with fewer DNA damage-positive cells in the vulnerable CA3 subfield compared with wild-type mice (Figure 2a). We have now used video and EEG telemetry to follow the emergence of epileptic seizures in wild-type mice in our model (Figure 2b). Our experiments reveal all mice

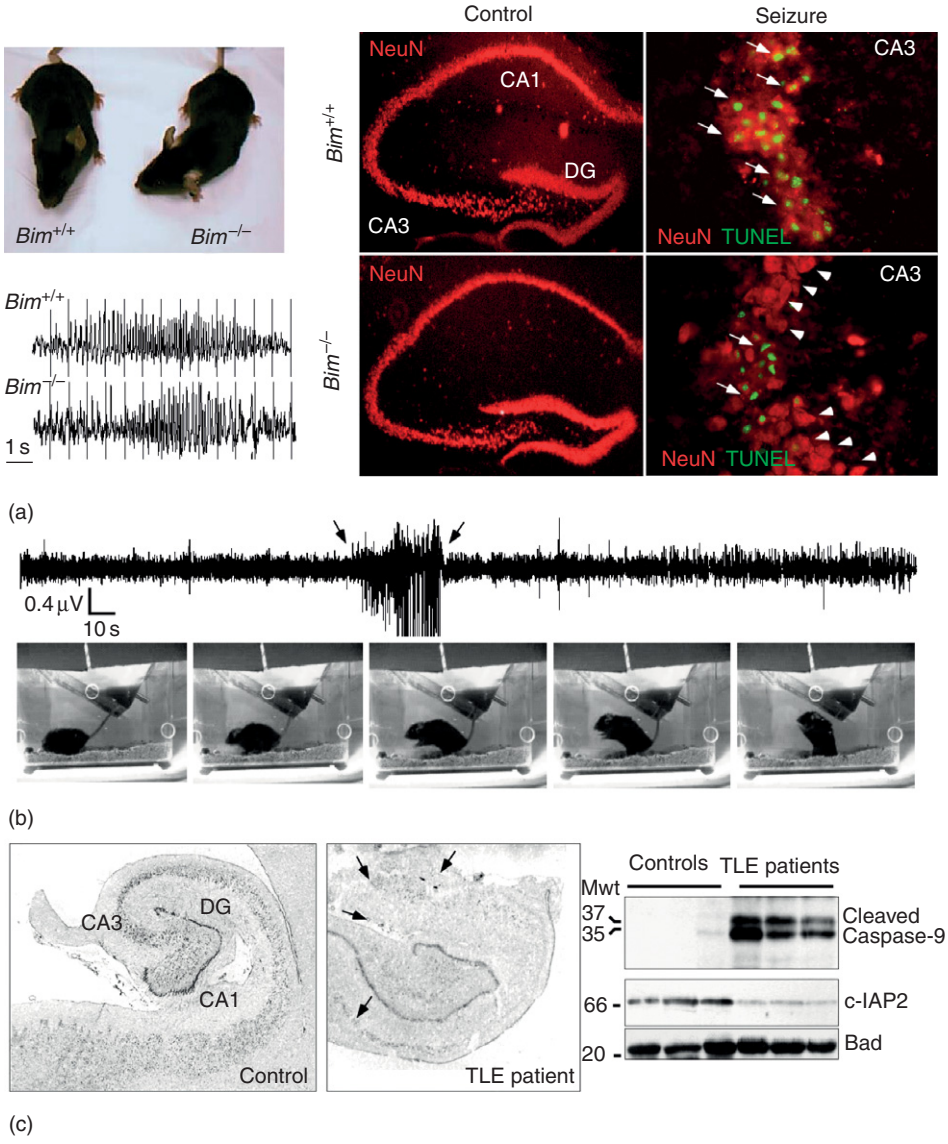


Figure 2 (a) Photographs of wild-type (*bim*$^{+/+}$) and *bim*-deficient (*bim*$^{-/-}$) mice and below, representative EEG traces of seizures following kainic acid (KA). Immunohistochemistry panel to the right shows hippocampi from (left panels) control and (right panels) seizure-damaged wild-type and *bim*-deficient mice. Wild-type mice display extensive TUNEL-positive neurons (arrows) in the CA3 subfield, while a proportion of neurons survive in *bim*-deficient mice (arrowheads). (b) An epileptic seizure captured using EEG-telemetry in a mouse that underwent prolonged seizures induced by KA. Panels below show sequential video stills depicting behavioral features. (c) Representative images of a nissl-stained hippocampus from an autopsy control (provided by the University of Maryland Brain & Tissue Bank) and a pharmacoresistant TLE patient. Note the evident neuronal loss (arrows) in the patient specimen. Western blot panel to the right shows expression of mitochondrial/intrinsic pathway proteins in the cytoplasmic fraction from three controls and age/gender-balanced TLE samples. Note presence of cleaved caspase-9 and lower antiapoptotic c-IAP2 levels. The Bad levels confirm loading. The authors gratefully acknowledge the unpublished data, courtesy of Hatazaki S, Mouri G, Murphy B, Paucard A, Schindler C, Thompson S, and Woods I.

injected with intra-amygdala KA developed epilepsy within 5 days. This resource will support screening mice deficient in apoptosis-associated genes for epileptic phenotype and it will be interesting to determine whether a different (perhaps milder) form of epilepsy develops in *bim* or other BH3-only deficient mice post *status epilepticus*.

Recent human material analysis

Some questions remain from our studies of TLE patient hippocampus. While we had detected some caspase-9 cleavage in the microsomal fraction of patient hippocampus, we were aware that this was not the compartment in which the protein was likely to be most enriched. Data in **Figure 2c** show representative autopsy control and TLE patient hippocampi and recent analysis of the cytoplasmic fraction. Western blots show there are significant levels of cleaved caspase-9 in TLE patient brain compared with autopsy controls (**Figure 2c**). Moreover, levels of c-IAP2, a potential negative regulator of caspase activity, are lower in the TLE samples than autopsy controls (**Figure 2c**). Levels of Bad in this fraction are similar between the TLE group and autopsy controls. These new findings extend the case that caspase activity may be present in hippocampus from refractory TLE samples.

Future Goals

Progress is still required in several areas. The cell type(s) in which apoptosis signaling is modulated in human TLE hippocampus is poorly characterized. Is pro- and antiapoptotic signaling occurring in the same or different cells, and are these mainly neurons? Beyond this, we must test whether blocking proapoptotic signaling has any protective effects against epilepsy development after *status epilepticus*. Using long-term EEG recordings we have found that mice neuroprotected by a brief (preconditioning) seizure given a day before *status epilepticus* developed five times fewer epileptic seizures than nonpreconditioned mice subject to *status epilepticus*. The degree of neuroprotection in BH3-only protein deficient mice after status epilepticus could be similar, so we would expect to observe antiepileptogenic effects. In summary, research on the molecular mechanisms of cell death after seizures has identified roles for apoptosis-associated signaling in experimental and, probably, human epilepsy. This has provided important insights into the pathophysiology of seizure damage and led us toward discovering potential routes to antiepileptogenesis.

Acknowledgements

The authors are grateful for funding support from the National Institutes of Health (RPS, DCH) and from Science Foundation Ireland, Health Research Board Ireland, and the Wellcome Trust (DCH).

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; Neuronal Injury from Seizures: Influence of Models, Development and Genetics; The Ontogeny of Seizure-Related Programmed Cell Death in the Developing Brain; **Epileptogenesis**: Specific Gene Expression Before and After Seizure in Epileptic EL Mice.

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Neuronal Injury and Progressive Cell Damage

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Introduction

Life and death are not dichotomies, but the extremes of a continuum. At the cellular level, a wide range of phenomena that are often forgotten and not investigated temporally interconnects these two poles. The complete picture of these phenomena requires analysis on a time scale different from that used to analyze the changes that take place after an insult is inflicted to injure tissue and cells. The biochemical, morphological, and functional consequences of an injury can be assessed quickly – at the millisecond level – as well as over the course of many years or decades. Cellular, molecular, and network changes in various neurons and glial cells have been proposed as critical factors for the development of epileptogenesis, and may also contribute to the progression of the seizure disorder (maintenance of recurrent spontaneous seizures).

Cause or Consequence Debate

The 'chicken or egg' debate on whether seizures are a cause or a consequence of brain damage is one of the oldest issues in epilepsy research. The association of epilepsy with brain damage and hippocampal sclerosis – a characteristic pattern of lesion which includes neuronal cell loss and gliosis in the hilus of the dentate gyrus and CA1 and CA3 regions – was first described by Bouchet e Cazauvieilh in 1825. On one hand, these neuropathological findings related to epilepsy have been extensively studied; on the other hand, the trigger of these neurobiological events, and whether/how these initial pathological findings are associated (or not) with the subsequent network plasticity changes, still remain unsolved. Just as epileptogenesis is perceived as a dynamic process, developing with variable latency after an identifiable insult (such as head trauma, status epilepticus (SE), brain tumor, infection, or anoxia), the processes of cell and tissue injury (and death) should also be viewed not as static but rather as dynamic processes.

Background

For a long time, assessment of the changes taking place after an epileptogenic or epileptic (ictal) events was mostly based on histology. Typically, after the death of a patient or upon the termination of a laboratory experiment, brain tissue would be processed with various histological techniques. Early work in this area usually relied on Nissl-stained sections of brain tissue, and provided cell counts of specific brain areas. The results from such analysis provide information about the number of remaining cells and thus, by comparison with cell counts from normal tissue, indicate the amount of cell loss. This approach, although able to establish whether or not there has been damage to the tissue, does not provide any information about when this damage occurred. In particular, in human epilepsy patients, this approach does not indicate whether tissue damage is cause or consequence of the epileptic process. For that insight, investigators have turned to experimental models of epilepsy, since animals can be examined histologically at various time points after injury. Furthermore, with the advent of the surgery as a clinical treatment for epilepsy, tissue from patients – at different ages, with different lengths of seizure history, with different seizure types, and seizure frequencies – became available. It was shown that hippocampal cell densities in mesial temporal lobe were not reduced in psychomotor epileptic patients with extrahippocampal foci consisting of tumoral tissue. Variables in seizure histories were not correlated with Ammon's horn cell densities, suggesting that sclerosis preceded the seizures, and that the seizures did virtually no significant further damage to hippocampus.

This new era of investigation has provided a wealth of information, but has generated only indirect evidence to shed light on the chicken or egg dilemma. It was only with the application of magnetic resonance imaging (MRI), to visualize the brains of living persons (and laboratory animals) over time, which finally gave rise to direct evidence on this issue. Particularly, with respect to experimental studies on small animal models, the limited spatial resolution of MRI has been problematic. The emerging clinical picture from imaging studies is that there is a small but progressive process of neural damage associated with length of seizure history and with seizure frequency. MRI data, while providing important insights, has also generated additional previously unimagined possibilities. For example, it was found that some close relatives of patients with familial mesial temporal lobe epilepsy show MRI changes, including atrophy of specific brain structures – and yet not have electroencephalographic abnormalities or seizures. The authors of that study concluded that evidence of hippocampal sclerosis is not necessarily related to seizure severity, and may occur in individuals who never had seizures. In addition,

these observations strongly indicated that hippocampal sclerosis in familial mesial temporal lobe epilepsy is not a consequence of recurrent seizures and is determined by a strong genetic predisposition. Given the heterogeneous nature of even a seemingly uniform condition such as mesial temporal lobe epilepsy, it is clear that the relationship between neuronal damage and seizures is likely to be determined by the specific nature of each distinct epileptic syndrome.

Similarly, initial biochemical analyses of epileptic tissue provided only static pictures of the ongoing changes. The possibility of addressing these changes in the same subject over time allows further advances in our understanding of the complex relations between epilepsy and neuronal damage. Neuron-specific enolase (NSE) is a marker of neuronal injury; elevated NSE correlates with neuronal death (as seen with histology) in animal models of SE. Studies using NSE support the idea that seizures (and not only overt SE) can cause neuronal injury.

As expressed in the opening of this article, not all cells will die immediately as a consequence of an epileptic seizure. Rescuing those cells that are killed at short latency after a seizure may be impossible. Conversely, it may be feasible to repair, or bring back to normal function, cells that have been injured but not lethally wounded. Indeed, while most studies have concentrated on the presence of cell loss, few are dedicated to the issue of cell injury. Evaluating what cells are injured by repeated seizure-related events – and particularly assessing what biochemical changes take place in those cells – might give rise to novel measures for halting the progressiveness of an epileptic condition. That is, it is as important to assess cell injury as to describe cell degeneration (death).

Methods

In our laboratory, we have taken advantage of a highly sensitive silver staining technique (Gallyas or argyrophil III) for the assessment of cell injury. This technique stains cells that have been injured in the preceding hours – only some of which will die in the near future. As expected, what cells will die and what cells will survive is a function of damage type and intensity. For example, not all rodent models of SE lead to degeneration of dentate granule cells. Indeed, silver staining technique stains dentate granule cells in rats subjected to pilocarpine-induced SE but not those subjected to kainic acid-induced SE.

Given the Golgi-like nature of most of the elements stained with Gallyas technique, it is often possible to describe the cell type that has been damaged (e.g., basket, pyramidal, mossy cell, fusiform cell). This technique, therefore, provides not only information about the number of damaged elements (neurons or glia) and their topographic distribution, but also on cell morphology. In addition, the level of contrast that is obtained allows us to detect even a single injured cell in a brain section. The fact that the technique does not rely on fluorescent dyes provides an additional advantage, as the marker is 'permanent.' Our work with this technique clearly demonstrates that it stains fibers and cell bodies of both glial and neuronal elements. A major concern is that this highly sensitive staining approach can easily provide false positive results if proper care is not taken with perfusion and with tissue processing.

Recent Results

We first compared the regional distribution of selectively vulnerable neurons, and the temporal evolution of neuronal injury, after pilocarpine- and kainate-induced SE. Pilocarpine SE leads to greater damage at shorter latencies than kainic acid. Few neurons were silver-stained two hours after kainate-induced SE, as compared to many silver-stained cells in most neocortical, hippocampal, amygdaloid, and hypothalamic structures in pilocarpine SE animals. For both models, most damage took place from 8 to 24 h after SE onset. Importantly, although some silver-stained cells do not eventually die as a consequence of SE, it is not clear that surviving cells would show full functional recovery. That is, after a brain injury some neurons would die acutely, whereas other neurons might die over a protracted period of time, during which they could contribute to seizure genesis. In fact, it was shown in pilocarpine-treated animals that surviving neurons in the dentate granule cell layer display substantial changes in their morphology; these changes vary over time, starting with an initial spine loss, followed by recovery and plastic changes in spine shape and density, and culminating in dendritic spine degeneration many months after SE induction. Similarly, we demonstrated distorted morphology of surviving interneurons in the hippocampus of animals subjected to pilocarpine-induced SE.

In slice studies, investigators have shown that orthodromic activation of CA1 neurons evoked only a single, stimulus-graded population spike in control slices, the same stimulus produced an all-or-none burst of population spikes in slices from epileptic animals (i.e., animals that underwent pilocarpine-induced SE). Assessment of the intrinsic firing patterns of CA1 pyramidal cells determined that in control slices, the vast majority (97%) were regular firing cells – in contrast to only 53% regular spiking cells in slices from epileptic animals. The remaining 47% of the pyramidal cells had become intrinsic bursters. The discharge of spontaneously active CA1 bursters anteceded the population discharge, whereas most other pyramidal cells began to fire conjointly with the first population spike in the field potential epileptiform burst. Thus, spontaneous bursters were suggested to be

the likely initiators of spontaneous population bursts in these slices. The authors concluded that the dramatic upregulation of intrinsic bursting in CA1 pyramidal cells, particularly the de novo appearance of Ca^{2+}-dependent bursting, could contribute to the epileptogenicity of the hippocampus in the pilocarpine model of epilepsy. Our data are consistent with this view, and further suggest that neurons that are silver-stained following SE might become those permanently altered bursters. By developing a means to pinpoint which cells will be altered by the lesional event, we hope to provide a way to more readily evaluate strategies to prevent these changes.

We then showed that even the much milder chronic spontaneous recurrent seizures that follow pilocarpine-induced SE are able to trigger neuronal injury. Animals were sacrificed from 1 to 6 h after either single or multiple seizures. Silver-impregnated dark neurons were never found in control animals or in epileptic animals that had no behavioral seizures in the 8 h prior to sacrifice. After spontaneous seizures, dark (injured) neurons were mostly interneurons and were present in hippocampus (CA1 stratum radiatum), amygdala, piriform cortex, and other limbic structures. Animals with multiple seizures had a higher number of dark cells than animals with single seizures. These results suggested that even single generalized spontaneous tonic–clonic seizures could induce long-lasting morphological changes, and are consistent with the idea that epilepsy is a progressive disorder where one seizure begets the next.

The smaller number of neurons stained after a single seizure led our group to question whether SE would trigger some sort of adaptative process, analogous to the so-called ischemic tolerance. Indeed, as hypothesized counts of silver-stained cells, using the Gallyas (argyrophil III) method, revealed a markedly lower neuronal injury in animals submitted to reinduction of SE as compared to rats exposed to a single episode of pilocarpine-induced SE. This effect could be explained by either (or both) of the following considerations: (1) The first SE would remove the vulnerable cells, leaving behind resistant cells not affected by the second SE; (2) The first SE would confer increased resistance to the remaining cells, analogous to the process of ischemic tolerance. Counting of Nissl-stained cells, to differentiate between these alternative mechanisms, indicated that different neuronal populations reacted differently to SE induction. For some brain areas, most if not all of the vulnerable cells were lost after an initial insult, leaving only relatively resistant cells and little room for further damage or cell loss. For some other brain areas, in contrast, our data supported the hypothesis that surviving cells might be modified by the initial insult, which conferred a sort of excitotoxic tolerance. As a consequence of both mechanisms, subsequent insults (regardless of their intensity) after an initial intense insult resulted in very little damage.

Evidence for resistance to damage conferred by previous epileptic activity has been shown in several experiments by different groups. However, in all of these studies, assessment of the effects of a second lesional event was always performed within a short time interval after the first insult. For example, preconditioning animals by intrahippocampal kainic acid administration produced significant neuroprotection against a second intrahippocampal kainic acid dose administered from 1 to 7 days later – but not 15 days after the first injection. In our study, however, we allowed a period of many months to pass between the initial major lesional event and the induction of a second SE. Under these conditions, the lack of an extensive lesion after the second SE was not due to a lower degree of the second insult compared to the first. Accordingly, our data could be seen as indicative of neuroprotection or excitotoxic-induced tolerance. The possibility that there was simply a lack of vulnerable cells to be damaged by the second SE was not supported by our counts of the number of silver-stained cells relative to the number of available neurons (Nissl-stained).

Thus, our data suggest that in some neuronal populations there is a tolerance to damage that is induced by pilocarpine SE and may extend for over 15 months after the initial insult. In addition, this 'tolerance' to further damage does not seem to increase or diminish as a consequence of repeated spontaneous seizures, irrespective of the frequency of these latter seizure events. The protection conferred by the initial SE event was able to partially withstand even a second SE bout 6 months later. Our findings may be relevant to the clinical studies that attempt to determine under which conditions neuronal damage is a cause or a consequence of seizures. Knowledge of the factors influencing such tolerance might be relevant to the development of neuroprotective strategies.

The lack of significant neuronal loss and neuronal damage by repeated seizures (when they occur) does not imply that those repeated seizures do not harm the patient. Indeed, not only might those repeated seizures have a tremendous impact on a person's ability to have a normal life, but also (more importantly within the context of this article) may negatively affect cognition. Even though repeated seizures do not necessarily lead to increasing overt neuronal injury, these events may alter synaptic plasticity and have consequences on other physiological functions (as extensively demonstrated in kindling studies).

Future Goals

The answer to the chicken or egg dilemma may vary depending on the epileptic syndrome or animal model considered. A major challenge ahead is to dissect the biochemical bases underlying these different responses to injury shown by different neuronal populations.

For example, how does this excitotoxic tolerance develop? In our previous experiments, we did not evaluate whether excitotoxic tolerance would develop after a less intense SE or even after just a single seizure. In the event that excitotoxic tolerance does develop after a single seizure, we must then evaluate how long the tolerance will last and to what extent different neuronal populations might be protected.

Another goal is to evaluate whether the modulation of neuronal injury would affect the epileptogenic process. To this end, as discussed earlier, there is evidence that multiple SE events, triggered in subsequent days and at a controlled level of intensity, result in very little damage. This model, however, has not yet been evaluated with the Gallyas silver staining technique for detecting injured neurons or with other means of assessing neuronal stress. The model of two controlled priming SE episodes, followed by one full-blown SE episode, is associated with no cell loss and yet with the emergence of epilepsy (e.g., spontaneous recurrent seizures). Is neuronal injury (and not only of cell death) also blocked in the repeated SE model? Presumably not, as the animals do become epileptic. What is the biochemical cascade associated with this injury process, and what is its temporal progression? Given that, despite the lack of neuronal loss, animals subjected to the titrated model of SE induction still develop spontaneous seizures, it is relevant to investigate neuronal injury in this model and to determine whether we are able to modulate the injury – and thus modulate the epileptogenic process.

A basic concept in epileptology is that upon the death of certain cell populations the remaining cells reorganize, and this reorganization may contribute to epileptogenesis and ictogenesis. Is the loss of a nearby neuron, or the loss of a component in a neuronal circuit, what triggers the changes in the surviving neurons? To what extent is it relevant that the surviving neurons are injured (but not killed) by the initial precipitating injury? We are currently exploring several pathways (e.g., NMDAR-induced cell swelling, the recruitment of Ca^{2+} stores, and their modulation by BDNF) associated with the changes in the biochemical machinery of the cells that result from excessive excitation. Therefore, as important as understanding what cells degenerate in epilepsy, it is critical to understand what cells are damaged but do survive and serve as the locus of epileptogenic plastic changes.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Imaging**: Imaging Characterization of Familial Temporal Lobe Epilepsies; **Models**: Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; **Plasticity**: Synaptic Reorganization in the Hippocampal Dentate Gyrus.

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Neuronal Injury from Seizures: Influence of Models, Development and Genetics

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Introduction and Background

The earliest observation linking seizures to brain injury is generally attributed to Bouchet and Cazauvielh who reported on hippocampal abnormalities in an autopsy series on patients who had epilepsy. Decades later, Sommer described a characteristic pattern of neuronal injury in the hippocampus of epileptic patients that has been called Ammon's horn sclerosis (AHS). These descriptions preceded a detailed knowledge of the ultrastructure of the brain and involved gross inspections of brain specimens. The epilepsy-related lesion suggested in these early reports – now also referred to as mesial temporal sclerosis (MTS) – was first described in detail by Margerison and Corsellis based on a study of 55 autopsy specimens of patients with known epilepsy confirmed by clinical electrophysiology data. It involved cell loss and gliosis in the Sommer sector (CA1), along with a major but variable part of the end folium (previously referred to as CA4, but the current preferred appellation is 'hilus') and the dentate gyrus. An illustration from their classic paper is reproduced as Figure 1, to show the typical lesion as well as to mark the historic description of seizure-related hippocampal pathology. Their study could not clearly resolve the controversy as to whether hippocampal sclerosis is the cause or the result of temporal lobe epilepsy. Detailed discussion of the evidence supporting either point of view is not within the scope of this article, but we refer the reader to a review by Sutula and Pitkänen (see Further Reading section), in which the authors review important evidence from animal and human studies. They suggest that regardless of a possible etiologic role of pre-existing structural abnormalities, progressive seizure-induced neuronal injury can occur and that it may be modified by genetic and environmental factors. Improvements in high resolution neuroimaging modalities such as high field magnetic resonance imaging provide us with an opportunity to study patients prospectively and undertake careful documentation of seizure density and duration for correlation with quantitative analysis of findings from imaging.

Meldrum and colleagues were the first to demonstrate that seizures themselves, in the absence of systemic factors, produce brain injury. In their classic study, baboons were intubated, ventilated, and paralyzed – and then subjected to prolonged seizures. The brain damage seen subsequent to these seizures could not be attributed to respiratory arrest, and thus was interpreted as a direct response to seizure activity. Subsequently Nevander and colleagues confirmed similar findings in a study involving well-oxygenated rats. These observations were explained by the emerging concept of excitotoxicity; that is sustained and repetitive depolarization of neurons led to a massive influx of calcium into the cell, triggering a cascade of events leading to cell death. Theses studies showed that even careful clinical management of systemic

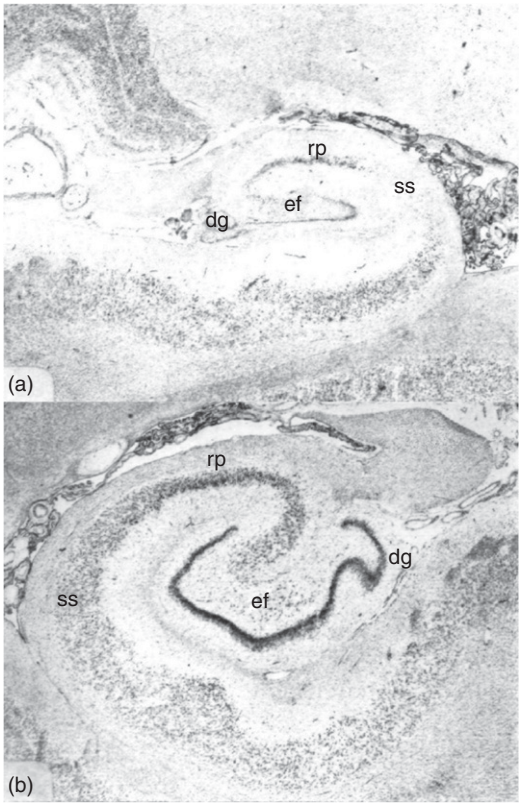


Figure 1 (a) A classical 'Ammon's horn sclerosis' showing the extensive loss of hippocampal nerve cells affecting particularly the Sommer sector, the end fohum, and the dentate gyms. This may be compared with: (b) the hippocampus from the opposite cerebral hemisphere of the same patient. There is a marked loss of nerve cells in the end folium but the other areas appear intact. ss, Sommer sector (HI field); rp, resistant part (H2 field); ef, end folium (H3, or H4 and 5 fields); dg, dentate gyms), (a, b) Cresyl violet ×10.

factors – for example, during status epilepticus (SE) – is insufficient to protect neurons. Aggressive management of on-going seizure activity, including meticulous detection of electrographic (but subclinical) seizures, is important for optimal outcome. In recent years, this concept of seizure management is also beginning to take hold with respect to seizures and SE in the neonatal settings.

While seizure-induced injury usually encompasses widespread regions of the brain, a great deal of research has focused on the hippocampus, no doubt in part triggered by the availability of surgically resected hippocampi from patients undergoing epilepsy surgery. In parallel, animal models were studied in an attempt to replicate the human lesion. There is considerable variability in the lesion among humans, but the diagnosis of hippocampal sclerosis is readily made – not only by the pathologist but also by the neuroradiologist – during the early evaluation of the epilepsy patient.

Methods

Experiments subjecting animals to SE produced by chemoconvulsants (like kainic acid or pilocarpine) can reliably produce hippocampal sclerosis in rodents. Kainic acid has been injected into the hippocampus (or amygdala) in some models, but systemic administration of these drugs is most common. A variation of the pilocarpine model involves pretreating animals with lithium chloride, which reduces the dose of pilocarpine needed to induce status epilepticus and to improve 'consistency' in results. Continuous electrical stimulation of the hippocampus has also been employed in different paradigms, including that involving perforant path stimulation to produce self-sustained status epilepticus; seizure activity is considered to be self-sustaining when seizures continue even after the provocative stimulus is withdrawn. Mazarati and colleagues showed that 30 min of electrical stimulation of the angular bundle resulted in seizures that continued for hours. This method has the advantage of not involving chemicals, that may be inherently neurotoxic, to produce experimental SE.

Neuronal injury has been demonstrated using a variety of histological techniques. Degenerating neurons can be identified by staining with hematoxylin and eosin or fluoro-jade blue. Tissue studies have also employed TUNEL staining and immunohistochemical studies for various caspase proteins to elucidate the mechanisms underlying neuronal injury

Investigators have shown that hippocampal sclerosis can also be demonstrated after repeated kindling stimulations, suggesting that even repeated brief seizures (rather than SE) can contribute to chronic loss of neurons and hippocampal sclerosis. These studies also highlighted the selective vulnerability of the hippocampal neuronal populations to seizure-induced injury. Neuronal loss was demonstrated in the CA1 and the hilus of the dentate gyrus after only three kindling-induced generalized tonic–clonic seizures, but there was no discernible cell loss in the somatosensory cortex even after 150 seizures.

These models have been studied extensively to demonstrate seizure-induced brain injury following SE or repeated seizure activity, as well as to evaluate subsequent molecular, cellular and network plasticity that may contribute to epileptogenesis. In addition, studies have been reported that attempt to evaluate the evolution of cognitive and behavioral comorbidities that follow this seizure insult, and if those are associated with seizure-related cell damage.

Recent Results

Seizure-Induced Neuronal Demise – Apoptosis or Necrosis?

A number of studies have attempted to define the seizure-related brain damage in terms of apoptosis or necrosis. Review of the literature suggests that this discussion is clouded by semantic inconsistencies. Original definitions of these distinct types of cell death were based on early morphologic changes accompanying tissue injury.

Many reports (see Henshall and Simon in Further Reading and in this volume) have described apoptotic cell death following both brief seizures as well as SE in rodent models. Most of these reports concluded that seizure-related cell death was apoptotic, based on the demonstration of a number of biochemical markers typically associated with apoptosis, including enhanced expression of either the message or protein product corresponding to such cell death-associated mediators as p53, Bax, activated form of various caspases, and the demonstration of oligonucleosomal DNA cleavage (as shown by DNA 'laddering'). The most common histological marker for apoptosis has been TUNEL staining, which reflects double-stranded DNA cleavage. Now we know, however, that TUNEL positivity is not specific to apoptosis.

The classical description of apoptosis as a distinct mode of cell death was based on morphological descriptions based on electron microscopy (EM). The EM findings for apoptosis are dramatically distinct from those for necrosis. As summarized by Fujikawa, the morphologic findings in most neurons after a bout of status epilepticus appear to correspond to necrosis, even though many biochemical markers reflecting the initiation of programmed cell death can be demonstrated. This form of cell death has therefore been referred to as 'programmed neuronal necrosis.' Interestingly, it does not appear to be unique to seizures as the provocative insult, since it has also been demonstrated in a hypoxic–ischemic model.

The molecular events pertaining to cell death following seizure(s) seem to be represented along a continuum, and depend upon the intensity/duration and/or frequency of seizures. Very brief seizures may induce a phenomenon that has been termed 'preconditioning' which affords relative neuroprotection from a subsequent event in the short term. With more prolonged or frequent seizures, cell death may ensue with prominent DNA laddering, suggestive of an 'apoptotic' mode of cell death. When the same provocation for producing seizures is titrated to yield a more rapid onset and continuous seizure activity, a necrotic appearing morphology and biochemical markers of death have been noted.

Some studies have noted that a given insult can produce an apoptotic morphology in the dentate granule cells but a necrotic morphology in the pyramidal cells of the Ammon's horn. This apparent paradox may be related to the fact that many of the affected dentate granule cells are newborn (i.e., there is ongoing neurogenesis in the dentate gyrus). It has been estimated that a large majority of the newly born cells undergo apoptotic death, and that this process is enhanced by seizure activity. Thus, at least in the dentate, apoptosis is correlated with the developmental immaturity of newborn cells, recapitulating the normal role of apoptosis during development.

Regardless of the morphology of seizure-induced death of neurons, the identification of a large number of molecular targets can contribute to the development of neuroprotective strategies. A number of targets involve the so-called 'intrinsic' pathways, set in motion by the dramatic seizure-related rise of intracellular calcium and the consequent disruption of intracellular organelle (mitochondria, endoplasmic reticulum) function. In addition, 'extrinsic' pathways – for example, involving the activation of surface receptors of the tumor necrosis factor (TNF) super-family can also play important roles. Ligands for the latter include TNF-α, Fas ligand, and the TNF receptor apoptosis-inducing ligand (TRAIL). There is a great diversity of potential targets for neuroprotective strategies, including the TNF receptors indicated earlier, the receptors for excitatory amino acids, the intrinsic pathway-associated organelles (e.g., mitochondria-related targets), the cellular elements involved in oxidative injury, etc. Indeed, this diversity suggests that effective neuroprotective strategies may have to address more than one specific target, and a 'neuroprotective cocktail' approach may emerge.

Seizure-Induced Neuronal Death in the Developing Brain – Influence of Models

Experiments involving kainic acid (KA)-induced status epilepticus in immature animals formed the basis for the initial impression that the developing (immature) brain does not undergo the classic seizure-induced injury to the dentate hilus and Ammon's horn that lead to hippocampal sclerosis. Investigators initially reported that rats younger than postnatal day 17 or 18 were relatively protected from seizure-induced injury (although injury from systemic KA-induced seizures was demonstrated in immature rabbits). However, it was noted that the administration of corticotropin-releasing hormone (CRH) to rat pups of postnatal age 10 (P10) resulted in severe seizures and extensive hippocampal injury. Such injury could not be reproduced in mature animals, even at much higher doses of CRH. Thus, the relative lack of vulnerability in immature rats appears to be both species- and model-specific.

Treatment of P10 rabbits with pilocarpine following pretreatment with lithium produced hippocampal injury similar to that produced by KA. Our laboratory undertook a systematic study of the lithium–pilocarpine model in rat pups of different ages. The pattern of injury varied with age, with CA1 most vulnerable in the P14 animals; injury to the hilus and CA3 became prominent by P21. Anatomic evidence of synaptic reorganization, as evidenced by mossy-fiber sprouting, was seen in animals several weeks after SE at P21 in this model, but not in those subjected to SE at P14. The vulnerability of CA1 in the very young animals (P14) in this model has assumed special significance, in the light of emerging human data confirming abnormal signal in the CA1 region of some children after prolonged febrile convulsions. With this model, we also observed neuronal injury outside the hippocampus – in the amygdala, thalamus, cortex, caudate, and other regions. Injury to the thalamus was clearly discernible even in the P14 pup, but involvement of the cortex and amygdala was minimal at this age and steadily increased with brain maturation.

Studies of electrically induced status epilepticus (stimulation of the perforant path) have also provided evidence for injury in immature animals, but have also confirmed that such injury in immature animals is substantially milder than that encountered in adult animals.

Inflammation could be an important modifier of seizure-induced neuronal injury in the developing brain. When P10 animals are subjected to experimental hypoxia, they develop seizures but do not display neuronal injury. However, the neurologic outcome of newborns with hypoxia–ischemia as measured by microcephaly, cerebral palsy, etc. was worse in the subgroup of infants that experienced electrographic seizures in a study that matched the groups for Apgar score, initial pH, and base deficit; these observations suggested that the presence of seizures added to the hypoxic–ischemic injury. Another study that investigated brain injury in hypoxic–ischemic newborns, using signal changes in magnetic resonance spectroscopy for *N*-acetylaspartate (NAA), a marker of neuronal viability, confirmed an association between neonatal seizures and brain injury. The apparent disconnect, between the lack of brain injury as seen in experimental hypoxia–ischemia with seizures and the clinical consequence of hypoxia–ischemia when accompanied by

seizures, may be explained by the role of inflammation that commonly accompanies hypoxia–ischemia and seizures in clinical situations.

Our laboratory subjected P7 and P14 rat pups to lithium–pilocarpine SE after exposure to the bacterial endotoxin, lipopolysaccharide (LPS), a common experimental provocateur of inflammation. The CA1 vulnerability we had demonstrated in this model in the P14 animals in our earlier study was robustly enhanced by pretreatment with LPS (Figure 2). However, the dentate granule cells, hilus, and neurons in sector CA3 were unaffected. Even the P7 pup, usually resistant to neuronal injury in this model, showed selective CA1 injury following the combined LPS pilocarpine treatment. This enhancement of neuronal injury in the CA1 occurred in LPS-treated animals undergoing SE, even though LPS treatment did not result in increased temperature or prolongation of SE. Animals treated with LPS alone, but not subjected to SE did not show any injury.

An important link in humans connecting inflammatory signaling, early febrile seizures, and hippocampal sclerosis (with temporal lobe epilepsy) is suspected to be a specific polymorphism involving the interleukin-1β–511T allele. Our data support important interaction between inflammation and seizure-induced neuronal injury in the developing brain.

Genetic Influences

Some understanding of the role played by the genetic background in determining the vulnerability of the brain to seizure-induced neuronal injury has emerged in recent years. Schauwecker and Steward showed that SE induced by KA produced excitotoxic cell death in the CA3 and CA1 sectors of the Ammon's horn in 129/SvEMS and FVB/N strains of mice, in the same pattern as seen in rats. However, C57BL/6 and BALB/c mice showed considerable resistance to seizure-induced neuronal injury (although they exhibited comparable seizures); they exhibited excitotoxic cell death only at very high doses of KA, and only in a very restricted brain region. The C57BL/6 and BALB/c inbred strains appear to possess gene(s) that confer(s) protection from KA-induced excitotoxicity. The results reported in some articles describing the contribution of specific genes to cellular vulnerability, based on gene disruption manipulations, may have been confounded by the back-crossing process involved in generating the null hybrids. Consequence of null mutations should best be studied in mice with a pure genetic background. This important discovery also raises the hope, however, that we may understand the role of human genes in determining susceptibility to seizure-induced neuronal injury. Findings by Schauwecker and colleagues

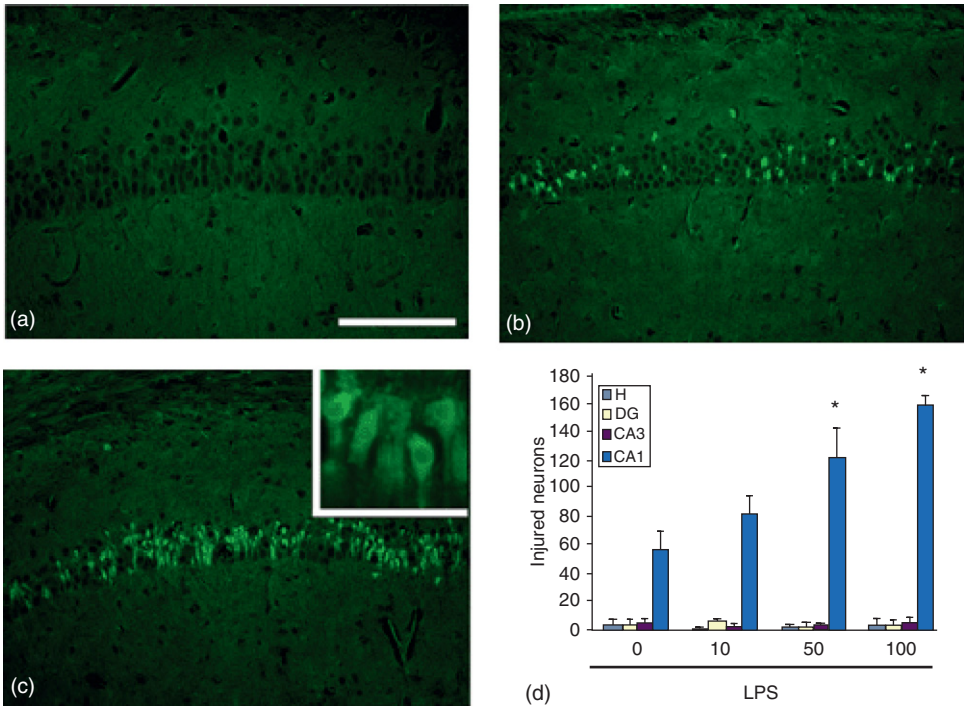


Figure 2 Lipopolysaccharide (LPS) plus pilocarpine (PC) induced hippocampal injury in 2-week-old rats. (a) LPS 100 μg kg^{-1} + vehicle lacks discernible CA1 neuronal injury; (b) vehicle + 60 mg kg^{-1} PC results in cell damage that becomes significantly more pronounced with (c) LPS 100 μg kg^{-1} + 60 mg kg^{-1} PC. High magnification inset in (c) demonstrates injured CA1 neurons with eosinophilic cytoplasm, irregularly shaped cell bodies and pyknotic nuclei. Scale bar is 200 μm for (a–c), 40 μm for inset. Graph (d) shows the mean hippocampal counts (± SEM) of damaged neurons with varying doses of LPS (0, 10, 50, and 100 μg kg^{-1}) + 60 mg kg^{-1} PC 24 h after SE. *$p < 0.05$ LPS0 vs. LPS50 or LPS100. H, hilus; DG, dentate granule cell layer.

provide a basis for reconciling at least some of the controversies in human data pertaining to the question of whether chronic epilepsy leads to ongoing neuronal injury.

Directions for Future Research

Knowledge gained from research on the mechanisms of seizure-induced neuronal injury is expected to influence the design of studies to investigate neuroprotection in the clinical setting. The diversity of targets, both extracellular (membrane receptors and ion-channels) and intracellular, suggests that multiple processes may have to be targeted simultaneously to achieve meaningful neuroprotection. Different clinical settings and different developmental stages may dictate distinct 'neuroprotective cocktails.' Laboratory studies should attempt to identify and include relevant variables that modify seizure-induced neuronal injury. Furthermore, increasing knowledge about the role of genetic background in conferring neuroprotection has the potential to be leveraged for the design of clinical interventions that could be based on the targeted activation of specific genes towards transcription and translation into neuroprotective factors.

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; Excitotoxicity and Neurodegenerative Disease; Molecular Mechanisms of Cell Death After Seizures; **Pediatric Epilepsy**: Developmental Aspects of Seizures; Epileptogenesis in Childhood.

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The Ontogeny of Seizure-Related Programmed Cell Death in the Developing Brain

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Introduction and General Overview

Due to controversies regarding the meaning of specific terms that are used to describe different forms of cell death, it is helpful to start by defining these terms.

Necrosis is a process involving cell death associated with the presence of specific cytoplasmic and inflammatory changes. These cytoplasmic changes consist of mitochondrial swelling, destruction of organelles, dilatation of endoplasmic reticulum (ER), and disruption of the plasma membrane leading to release of intracellular contents and inflammation. Nuclear chromatin is small, irregular, and dispersed within the intact nuclear membrane. Necrosis occurs as an independent process but may also occur in late stages of apoptosis, especially when energy reserves of the cell are depleted.

Apoptosis was originally described as a series of well defined and stereotyped morphologic changes in dying cells. It has features that are mainly specific to the nucleus. Typical features include cell shrinkage, phosphatidylserine exposure, formation of apoptotic bodies, and maintenance of organelle integrity with condensation and fragmentation of DNA. DNA fragmentation occurs in a two-step process. First, it is degraded into high molecular weight fragments, with chromatin condensation seen around the nuclear membrane. Second, DNA is degraded into smaller oligonucleosomal fragments (low molecular weight fragments). In its most classical form, apoptosis is seen when caspase 3 is activated. Detection of apoptosis is conventionally based on the use of two techniques, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) that detects double stranded DNA fragments, and the agarose gel electrophoresis that detects 180 bp DNA fragments in the pattern known as DNA 'laddering.' These techniques were thought at one time to be specific for apoptotic cell death detection. However, both TUNEL positivity and DNA laddering may be present in apoptotic and necrotic cells.

The classic apoptotic pathway (Type II apoptosis) of cell death involves cleavage and activation of caspases which lead to oligonucleosomal DNA fragmentation. Apoptosis inducing factor (AIF) mediates caspase-independent cell death with DNA fragmentation of the type that results in HMW fragments ~50 kb (Type I apoptosis) rather than LMW fragments 200–1 000 bp (Type II apoptosis). Unlike Type II, Type I apoptosis is not detected by TUNEL staining and on agarose gel electrophoresis DNA 'laddering' is absent.

'Apoptosis like programmed cell death (PCD)' is a form of PCD with chromatin condensation that is less complete than in apoptosis, and with appearance of phagocytosis-recognition molecules before lysis of the plasma membrane. Most published forms of caspase-independent apoptosis, as well as some of the caspase-dependent apoptosis – such as tumor necrosis factor (TNF)-induced death of MCF-7 cells – fall into this class (i.e., apoptosis-like PCD).

Programmed necrosis (or necrosis-like PCD) is a form of PCD that occurs in the absence of chromatin condensation. It is a passive process of cell death in which there is rapid neuronal death, necrotic morphology, and activation of terminal steps of PCD (e.g., caspase activation and/or double stranded DNA breaks). It usually involves caspase-independent signaling pathways. Seizure-induced neuronal death is morphologically necrotic, but also includes programmed processes such as DNA laddering. A subgroup of necrotic PCD models are often described as involving 'aborted' apoptosis; that is, a standard apoptosis program is initiated, then blocked at the level of caspase activation, and finally terminated by alternative caspase-independent routes.

Programmed necrosis-like death or necroptosis as defined by Degterev *et al.* (2005) is a caspase-independent cell death mechanism. It is initially stimulated by the activation of Fas/TNFR family of death domain receptors that can trigger either extrinsic pathway of apoptosis or necroptosis. But in necroptosis Fas/TNFR family can induce cell death even when caspases are inhibited. Cell death under these conditions lacks the typical features of apoptosis and looks more like necrosis.

Autophagy (Greek, to eat oneself) is an intracellular degradation of cytoplasmic constituents within lysosomes. A portion of cytoplasm, including organelles, is enclosed by a phagophore, an isolation membrane, to form an autophagosome. The outer membrane of the autophagosome fuses with the endosome first and then with the lysosome, and the internal material is degraded. Although autophagy is commonly induced by necroptosis, both processes are independently regulated and in general necroptosis is distinct from autophagy and independent of autophagy for cell death execution. Autophagy has also a role in cell survival pathway under some circumstances such as starvation and growth factor deprivation.

Programmed cell death is a term sometimes used interchangeably with apoptosis. However, programmed necrosis

and autophagy are also programmed events. Thus, PCD is divided into apoptosis (type I PCD), autophagy (type II PCD), and programmed necrosis (type III PCD).

Background

The commitment to cell death depends on the activation of many signaling pathways.

Caspase-Dependent Pathways

The 14 members of the caspase family have been identified to be closely involved in the apoptotic process of the cell. These caspases are categorized as 'initiator caspases' (caspases 1, 2, 4, 5, 8, 9, 10, and 14) and 'effector caspases' (caspases 3, 6, and 7) based on their specific role in the apoptotic pathway. The two best-documented caspase-dependent pathways include the intrinsic and extrinsic pathways.

Intrinsic Pathway

The intrinsic pathway is activated by cell stressors that disturb the intracellular organelle function, such as elevated intracellular Ca^{2+} levels, growth factor (GF) withdrawal, chemotherapeutic agents, irradiation, or reactive oxygen species. These stressors lead to the activation of the proapoptotic Bcl-2 family proteins (such as Bax) and then to the release of cytochrome-*c* from the mitochondrial intermembrane space into the cytosol. In the presence of dATP, cytosolic cytochrome-*c* binds to the apoptotic protease-activating factor-1 (Apaf-1) and recruits an initiator caspase – caspase 9 – to form the apoptosome complex. This complex gives rise to an activated caspase 9, which in turn activates caspase 3 that initiates the execution phase of the cell death pathway. Caspase 3 activates caspase-activated DNase (CAD), which cleaves the DNA into 180-bp fragments (DNA laddering). CAD was found to be activated 48 h after kainic acid (KA)-induced status epilepticus (SE) (for 2 h); however, DNA fragmentation was first observed 24 h after SE, suggesting that another DNase might be involved in the early DNA fragmentation. Interestingly, caspase 3 was found not to be activated in injured neurons after SE, suggesting that either another enzyme activated CAD or another DNase is involved in the DNA fragmentation. The release of cytochrome-*c* and the fate of the cell are determined by the balance between antiapoptotic proteins (Bcl-2, Bcl-x, and Bcl-w) and the proapoptotic proteins (Bid, Bad, Bim, Bax, and Bak) of the Bcl-2 family. After KA-induced SE, Bad is dissociated from the protein 14-3-3 and dimerizes with Bcl-x. Bax, which is normally bound to Bcl-x, is then displayed and translocates to the mitochondria to release cytochrome-*c*. KA-induced SE also upregulates the dephosphorylation (active form) of the transcription factors of the forkhead in rhabdomyosarcoma (FKHR) family, including FKHR and FKHR-like-1 (FKHRL-1), which are translocated to the nucleus to upregulate Bim expression. In turn, Bim dimerizes with Bcl-w, triggering the release and activation of Bax.

The ER is the second important site (mitochondria being the first) for the initiation of the intrinsic apoptotic pathway. The activation of inositol (1, 4, 5) tris phosphate/ryanodine receptor, or the blockade of the sarcoplasmic/endoplasmic Ca^{2+} ATPase (SERCA), initiates Ca^{2+} release that instigates Ca^{2+}-dependent protease activation and apoptosis. The ER is also known to be connected, both physically and biochemically, with the mitochondria, so that any ER stress can lead to mitochondrial Ca^{2+} accumulation and dysfunction.

In addition, other factors such as ceramide may be involved. Ceramide, which causes PCD when added to neuronal cultures in vitro and when stereotaxically injected in the hippocampus in vivo, has been shown to increase during SE (Table 1 and Figure 1). Data from our lab indicate that these increases are at least partially secondary to sphingomyelinase activation. Administration of C2-ceramide causes apoptotic cell death in cultured rat cortical neurons and in SH-SY5Y human neuroblastoma cells. This process is associated with upregulation of active

Table 1 Hippocampal TUNEL and Hoechst stains and ceramide levels in different models of SE

Model and age	*TUNEL positivity*	*Hoechst #33342 (DNA clumping)*	*Increased ceramide levels*
Adult KA	+	+	+
P35 KA	+	+	+
P7 KA	–	–	–
Adult KA + MK-801 (1 mg/kg)	–	N/A	–
Adult KA + MK-801 (5 mg/kg)	–	N/A	–
Adult LiPC	+	+	+
Adult LiPC + MK-801 (0.2 mg/kg)	–	–	–
P21 LiPC	+	+	+
P7 LiPC	+	+	+

Note that models in which PCD occurs (positive TUNEL fluorescence and Hoechst DNA clumping) are accompanied by an increase in ceramide levels.

TUNEL, Hoechst and Ceramide procedures were performed 30 h after onset of SE that lasted >3 h. '+' indicates that $p < 0.05$ compared to sham manipulated controls (original data were reported previously: Mikati *et al.* (2003); Daderian R, Rizk E, El Dada S, Zeinieh M, Kurdi R, Harb S, Rahmeh A, Usta J, Dbaibo G, El Sabban M, and Mikati MA (2005). NMDA receptor activation results in ceramide increases and in apoptosis in lithium pilocarpine SE. *Epilepsia* 46(Suppl 6): 207). N/A = not applicable.

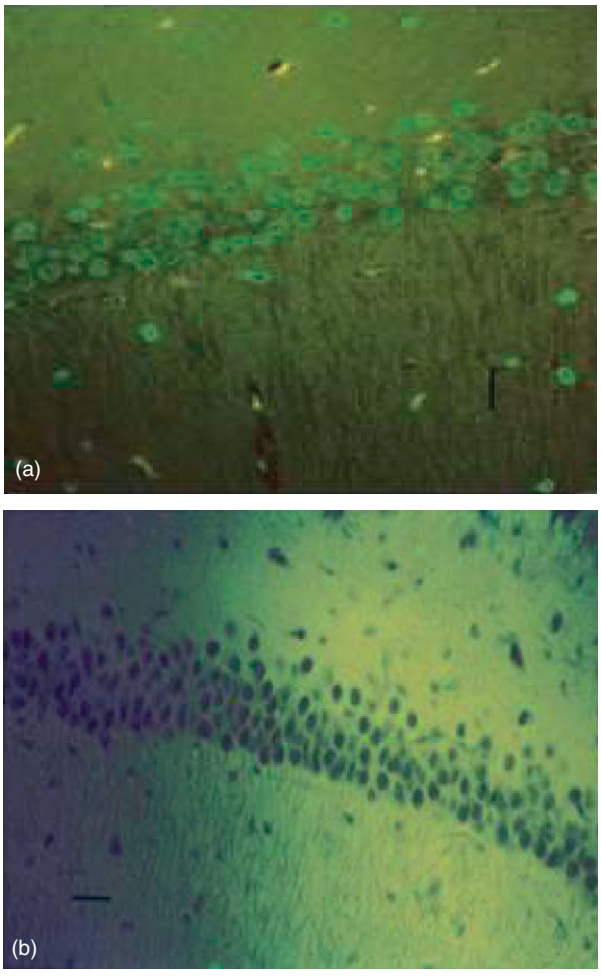


Figure 1 (a) TUNEL stain of the CA1 region of an adult rat injected with C2-ceramide (biologically active ceramide) as compared to (b) an adult rat injected with C2-dihydroceramide (biologically inactive ceramide). Scale bar, 20 μm. Note disruption of CA1 architecture and TUNEL positivity (yellow-light green fluorescence) induced by C2-ceramide (see also Mikati *et al*. 2003).

caspases 3 and 9, but without changes in active caspase 8 protein levels. The stereotaxic injection of the biologically active C2-ceramide results in increases in inositol triphosphate (IP3) levels in the hippocampus 2 h after injection (Figure 2). Ceramide has been shown to elevate IP3 levels in non-neural tissues. It has also been shown that IP3 increases in the hippocampus at 30 min, and peaks at 1 h following KA administration in mature animals. Type-III IP3 receptor activation contributes to PCD processes in T-lymphocytes and CHO cells by releasing intracellular Ca^{2+} stores from ER, and transmitting Ca^{2+} signals into mitochondria – thus activating the apoptotic mechanism. Thus, it is possible that ceramide exerts its effects through IP3 elevations. However, further studies are needed to prove or disprove this hypothesis, particularly since ceramide can have multiple other intracellular effects.

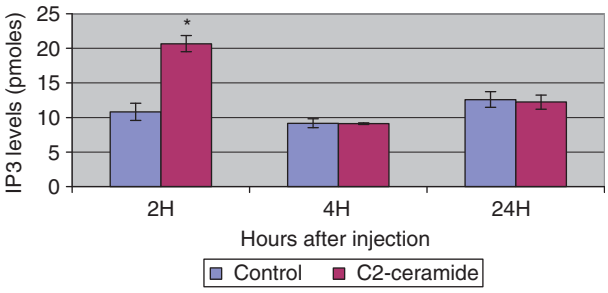


Figure 2 IP3 levels at 2, 4, and 24 h post vehicle and C2 ceramide injection. Data expressed as mean ± SE. $^*p < 0.05$ compared to control groups.

Extrinsic Pathway

The extrinsic pathway is initiated after the activation of 'cell death receptors', that is, after the binding of the ligands TNFα to TNFR1, FasL to Fas (CD95), and TNF receptor apoptosis inducing ligand (TRAIL) to TRAILR1 (DR4) and TRAILR2 (DR5). Once these receptors are activated, the cell death signal is propagated inside the cell by FADD (Fas associated protein with a death domain) recruitment in case of the Fas receptor, or TRADD (TNF-R1-associated death domain) recruitment in the case of the TNFR1. This recruitment then leads to activation of caspase 8, which will induce the apoptotic pathway either by cleaving and activating the effector caspases 3, 6, and 7 or by Bid. Cleaved or truncated Bid (tBid) dimerizes with Bax, resulting in the release of cytochrome-*c*, thus bridging the extrinsic and the intrinsic pathways.

Caspase-Independent Pathway

This cell death pathway, triggered by NMDA receptor activation, includes poly(ADP-ribose) polymerase-1 (PAPRP-1) activation, which produces AIF and endonuclease (Endo G) release and their translocation from the mitochondria to the nucleus, which in turn results in large-scale (50 kb) DNA cleavage and internucleosomal (180 bp) DNA fragmentation (DNA laddering), respectively. These changes are evident when KA is administered intra-hippocampally or systemically in mice. Also, calpain I, a cytoplasmic calcium-dependent cysteine protease, triggers Bid cleavage; tBid translocates to the mitochondria causing the mitochondrial release and translocation of AIF and EndoG to the nucleus.

Methods

Different techniques have been used in order to determine different types of cell death. The most common techniques used are terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL), ISEL, Hoechst 3342 staining, agarose gel electrophoresis

of DNA, and electron microscopy (EM). TUNEL is used to detect DNA fragmentation, but can only detect oligonucleosomal DNA breaks with 3′-OH ends (TUNEL and ISEL are similar in that they can only detect oligonucleosomal DNA breaks with 3′-OH/5′-P ends). TUNEL was initially thought to be specific for apoptosis; however, some studies have shown that TUNEL-positive cells had necrotic morphology – i.e., the case of cells dying through programmed necrosis. The Hoechst 33342 nuclear stain (DNA-sprcific dye bis-benzimide) allows the in situ detection of chromatin condensation, which constitutes one of the morphological features of apoptosis. Agarose gel electrophoresis of DNA is used to determine the type of DNA fragmentation: If oligonucleosomal bands separated by approximately 200 bp (also known as DNA 'laddering') is observed, one can conclude that the cell is dying either through apoptosis or programmed necrosis. If a DNA smear pattern is apparent, then necrosis is the mechanism by which cells are dying. EM gives a detailed characterization of the dying cell by analyzing the different compartments of the cell, particularly the nucleus and its components.

Recent Results – Ontogenic Differences in SE Induced Apoptosis/Necrosis

Several studies have shown that mature and immature rats are often affected differently by SE. Immature animals have often been shown to be resistant to the development of neuronal injury even after severe KA-induced SE. However, a number of recent studies have indicated that the immature brain does exhibit apoptosis and/or necrosis after undergoing different types of experimental SE. These results show that the relative resistance of the immature brain to SE-induced apoptosis/necrosis is model-dependent.

Pilocarpine and Lithium-Pilocarpine Models

In these models, the administration of convulsant doses of pilocarpine (with or without lithium) produces widespread apoptotic and necrotic neuronal injury in adult as well as in immature rats. Animals at different ages show region-specific differences in types of cell death. The immature brain appears to be more likely to manifest apoptotic cell death, whereas the mature brain is likely to manifest necrosis. The pattern, the localization, and the severity of neuronal injury in this model are age-dependent, as demonstrated in studies of 2-, 3-, and 4-week-old rat pups (and adult rats) 24 h after LiPC-induced SE. The damage seen in 2- and 3-week-old pups appeared to involve both necrosis (as evidenced by the eosinophilic cells) and apoptosis (as assessed by different techniques such as TUNEL staining, visualization of fragmented nuclei under fluorescence microscopy of ethidium bromide-stained sections, EM, and DNA electrophoresis). In the 2-week-old pups, necrotic cells were predominant in the CA1 subfield, whereas the apoptotic cells were predominant in the CA1 region, in subiculum and in thalamus. In the 3-week-old animals, necrotic cells were evident in the CA1 and CA3 hippocampal subfields, in the hilus, and in granule cells of the dentate gyrus; the apoptotic cells were seen only in the inner layer of the dentate granule cells and in a few thalamic neurons. In the 4-week-olds and in adult animals, only necrotic cells were apparent in the hilus, CA3, CA1 and granule cells. In adult rats at 24 and 72 h after 3 h of LiPC-induced SE, there was necrotic cell death in the hippocampus, amygdala, and piriform, entorhinal, and frontal cortices, despite the presence of DNA laddering in these regions 24 and 72 h after SE and the presence of positive TUNEL staining in some of the regions 72 h after SE. The results of these and other studies suggest that LiPC-SE produces neuronal injury with the morphological features of necrosis rather than apoptosis, and that internucleosomal DNA cleavage and other PCD phenomena can be activated by SE in neurons which become necrotic. Pups, 7–9 days old, subjected to three consecutive episodes of SE-induced by systemic pilocarpine injections showed apoptotic cell death, evaluated by TUNEL staining, in the hilus, granule cells, and thalamus 24 h after the last SE episode. In the LiPC model, the mechanism of SE-induced damage in the mediodorsal thalamic nucleus at P12 was shown to be necrotic rather than apoptotic. This conclusion was based on evidence using several different techniques, including EM, TUNEL staining (negative), caspase 3 and cytochrome-*c* immunocytochemistry (negative) in the thalamus, and OX-42 immunostaining (positive) of microglial cells in the central and lateral segments of medidorsal nucleus (supporting the idea of inflammation in the damaged area). After 2 h of seizures induced by LiPC in 2-week-old rat pups, semi-thin, toluidine blue-stained sections showed extensive neuronal injury in the CA1 region, and EM showed that most of these cells have a necrotic morphology. However, 24 h after 2 h of seizures, the majority of neurons in the CA1 had an apoptotic morphology. This result suggests that the same seizures kill different neurons in the same population of CA1 subfield at different rates and by different processes.

In another study, P19 and adult rats were treated with LiPC subcutaneously and intra-abdominally to evoke SE. The presence of the apoptotic neurons, using TUNEL staining, was detected in the CA3 of hippocampus, dentate gyrus, and cortex of the temporal lobe, starting 30 min (and lasting up to 8 h) after SE. Although the count of total dead neurons was higher in normal infant rats than in adult rats, the count of TUNEL-positive neurons in mature brain remarkably increased and exceeded that in immature brain at 30 min after hypoxia. There is also an increase in the expression of p53 in the

immature and adult rat following LiPC-induced SE. Induction of p53 occurred in the hippocampal subfields, piriform cortex, amygdala and thalamus at P15, P21 and in adult rats after LiPC-induced SE, but was maximal in P21 rats. Double-labeling experiments revealed that p53 was co-localized with NeuN and with TUNEL positivity. Our group demonstrated that LiPC-induced SE results in ceramide increases and in TUNEL positivity in the hippocampi of both adult and P21 rats (Table 1). LiPC-induced DNA fragmentation was maximal in the CA1 subfield in both age groups. These combined findings may be important because several studies have shown the relationship between ceramide and p53, both of which are involved in apoptotic signaling pathways. Even though this relationship has not yet been investigated in SE, ceramide has been shown to trigger p53-dependent apoptosis in fibrosarcoma tumor cells and in SKN-SH cells. In addition, in human glioma cells treated with etoposide (a DNA topoisomerase II inhibitor), p53 activation was shown to modulate ceramide generation by neutral sphingomyelinase activation.

Kainic Acid Model

The type of seizure in this model depends very much on the mode of administration of KA. Apoptosis commonly occurs in the immature brain after stereotaxic injection of KA into the brain, but this pattern is not the case after intraperitoneal injection (i.p.). The latter is probably a better model of SE, as the injury associated with systemic KA injection may be more closely related to SE rather than to the direct effects of KA itself. Whether KA is stereotaxically injected or given i.p., adult rats often show necrotic morphologic changes with DNA fragmentation probably representing programmed necrosis.

The types of neuronal loss after intracerebroventricular infusion of KA (10 or 50 nmol) to P7 rats have been investigated. Brain sections were examined at several neurodevelopmental time points (P8, P14, P25, P40, P60, and P75) to assess neuronal loss, and to search for evidence of necrosis and apoptosis. This analysis showed that KA administration caused both immediate and delayed hippocampal neuronal damage in the CA1 and CA3 subfields. The effect of KA was dose-dependent, and the delayed neuronal damage occurred through apoptosis. Another study investigated the effect of a single bilateral intracerebroventricular infusion of KA (50 nmol in 1.0 µl) on neurodegeneration in P7 pups. Progressive loss of neurons in the CA1 and CA3 subfields of the hippocampus, at P14, P25, P40, and P60, was detected. TUNEL staining showed apoptotic cells mainly in the CA3 subfield, and the TUNEL-positive cells decreased with age. Twenty-four and seventy-two hours after 3 h of KA-induced SE in adult rats produced necrotic cell death in the hippocampus, amygdala, and piriform, entorhinal, and frontal cortices, despite the presence of DNA laddering in these regions 24 and 72 h after SE and positive TUNEL staining in some of the regions 72 h after SE. The authors concluded that KA-SE induces neuronal injury with the morphological features of necrosis rather than apoptosis. Moreover, neuronal cell death with morphological features of necrosis was found at 12, 24, and 48 h after KA administration to adult rats, in the entorhinal and piriform cortices, basal amygdala, certain thalamic nuclei, and CA1 region of the hippocampus. In addition, a small number of apoptotic-like cells were observed in the same regions, using in situ labeling of nuclear DNA fragmentation. Typical necrotic cells were not stained with this method. A single intra-septal injection of kainate in adult rats induced apoptosis in the medial septum and amygdala, while the lateral septum exhibited a lower but significant incidence of apoptotic cells. TUNEL staining revealed a time-dependent sequential apoptotic cell death in hippocampal pyramidal cells. During the first two days postinjection, apoptosis in the hippocampus was only evident in the CA3 region. At 5 days postinjection, the apoptotic changes extended to the CA4 region, and at 10 days postinjection to the whole CA1 pyramidal cell region. Our group demonstrated that, in both adult and P35 rats, the administration of a convulsant dose of KA (15 mg/kg i.p) induces severe seizures and produces changes indicative of PCD (TUNEL positivity, DNA clumping in Hoechst 33342 staining) and ceramide increases in the hippocampus. The morphology of the injured cells was usually necrotic, and TUNEL positivity was present in only a few of the injured cells. These histological changes were the most severe in the CA3 and CA1 subfields. In immature P7 pups, the administration of a convulsant dose of KA (3 mg/kg) produced severe seizures, but did not induce increases in ceramide levels nor hippocampal PCD 30 h after KA injection, as assessed by TUNEL and Hoechst 33342 staining (Table 1). Of note is that young animals do not manifest the late stages of SE after KA (as seen in older animals) and require smaller doses to manifest seizures. These differences in the stages of SE may, thus, underlie the above reported differences in the presence or absence of injury and in the type of injury noted after KA-induced SE. Thus, the type of injury in the KA model depends on the mode of administration which may influence the severity of the seizures and mechanism of excitotoxicity.

Perforant Path Stimulation Model

In this model, the mature and the immature brains manifest similar types of cell death mostly consistent with programmed necrosis, although more apoptotic changes are probably seen in the immature brain. Perforant path stimulation, in P14-P16 rat pups, causes extensive hilar

cell and mild CA3 pyramidal cell necrosis evaluated at 2 and 24 h after stimulation. In situ end-labeling (ISEL) stain performed 2 h after the end of 16 h of stimulation reveals an intense band of positively labeled eosinophilic cells in the granule cells of the dentate gyrus, while control animals show no in situ end-labeling in that area. The positively labeled cells are not seen 24 h later, suggesting that they are rapidly scavenged apoptotic cells. In adult rat hippocampi, prolonged afferent perforant path stimulation produces neuronal death in all hippocampal subfields, particularly in the hilus. Degenerating hilar neurons and pyramidal cells exhibit the morphological features of necrosis, while degenerating granule cells exhibit all the morphological features of apoptosis after 24 h of perforant path stimulation.

Corticotropin Releasing Hormone Model

The morphologic alterations of neurons 16 h after SE induced in P10 and P11 rat pups by the neuropeptide, corticotrophin-releasing hormone were investigated. The morphological features of the degenerating neurons in the pyramidal cell layer of the CA3 region were suggestive of apoptosis, as assessed by EM and osmium tetroxide (showing dense staining of the degenerating neurons). In adult rats, CRH produces seizures, but does not cause any neuronal degeneration. In addition, the doses needed to induce seizures in adults are much higher than doses needed in infant rats.

Homocysteic Acid Model

The morphologic changes in the cerebral cortex and hippocampus of P12 pups, at 6 days after generalized clonic-tonic seizures induced by bilateral intracerebroventricular infusion of homocysteic acid (HCA) (600 nmol/side), were examined. In the granule cell layer of the dentate gyrus, neurons with fragmented nuclei in various stages of degeneration were detected (using Hoechst staining), and displayed the features of apoptotic death. This hypothesis was fully supported by results of the EM analysis.

Flurothyl Inhalation Model

In this model, the immature brain was shown to manifest both types of cell death – apoptosis and necrosis – whereas the mature brain manifested only necrotic cell death. When P20 rats were subjected to seizures by daily flurothyl inhalation for 6 days, edema, degeneration, and necrosis of nerve cells in cerebral cortex were observed, accompanied by inflammatory cell infiltration and apoptosis. Flurothyl-induced SE in adult rats leads to necrosis of the substansia nigra pars reticulate (SNPR), globus pallidus (GP), neocortex, thalamus, hippocampus, and amygdala. Histopathological damage was assessed by celestine blue and acid fuchsin stains.

Hyperthermia Model

In this model, in which, febrile seizures are induced by a hyperthermal bath with 40–45°C water, the immature brain was shown to manifest both types of cell death (apoptosis and necrosis) whereas the mature brain manifested only apoptotic cell death. Febrile seizure (FS) induced by hyperthermia results in necrosis in the hippocampal CA1 region in P21 rats, as assessed by EM. In contrast, 10 recurrent febrile seizures (induced once every 2 days) in P21 rats induced an increase in apoptotic cells in the hippocampal CA1 neurons as detected by TUNEL. P10 rats subjected to hyperthermia (up to 41–42 °C) by using a regulated stream of moderately heated air to induce seizures, show an abundance of argyrophilic neurons in the central nucleus of the amygdala and in the CA1 and CA3 pyramidal cell layers 24 h later. Neuronal injury in this model is not associated with significant DNA fragmentation (as detected by ISEL). Neurons in the hippocampal CA1 region and dentate gyrus of adult rats subjected to FS exhibited increased mitochondrial volumes with vacuoles, condensed matrix, and apoptotic bodies.

Future Directions

From all of the above data, we can conclude that both apoptosis and necrosis can occur in the developing brain following SE. In some models, including LiPC and intracerebroventricular KA, CRH and Homocysteic acid models, the developing brain is more likely to manifest apoptosis whereas the mature brain manifests necrosis. In these models the predominance of apoptosis in the developing brain may be related to its reliance on glycolysis (with a resultant resilience against energy failure), and to the constitutive and higher expression of many of the apoptotic factors (e.g., cyclin-dependent kinases, caspases, and immediate early genes) which reduce energy requirements for the apoptotic process and consequently favors apoptosis over necrosis. In other models, such as perforant path stimulation, flurothyl inhalation, and hyperthermia, necrosis – with or without apoptosis – has been observed in the immature brain. This variability suggests specific vulnerabilities of the developing brain to different types of SE.

Although significantly elucidated in the adult brain, the mechanisms of SE-induced apoptosis and necrosis are still not completely understood and remain to be investigated in the developing brain. Specifically, the Bcl-2 family proteins, cytochrome-*c*, caspases 3, 6, 7, 8, and 9, AIF, Fas receptor, and others that have been shown to be important in PCD in the adult brain, have not

been investigated in the developing brain. There is some evidence, as reviewed above, that PCD in the developing brain in the LiPC model, as in the adult brain, may involve activation of p53- and ceramide-related pathways. The mechanisms leading to the vulnerability of specific cell types, at specific ages, in specific SE models leading to apoptosis, are still also largely unknown and await further investigation. Additionally, one of the questions in need of further investigation in immature animals is whether – and more importantly, how – apoptosis and necrosis related mechanisms can coexist in the same degenerating cell in the brain? How does one process (i.e., apoptosis or necrosis) lead to another? It also remains to be determined whether SE-induced PCD – in any of the above models – results from activation of a single pathway or (more likely) by activation of multiple simultaneous and inter-related pathways. Despite the many questions that remain to be answered, it is clear that SE-induced cell death manifests significant age-, cell type-, region-, and model-related differences. And that apoptosis is an important mode of neuronal injury in the developing brain.

See also: **Cell Damage/Excitotoxicity**: Molecular Mechanisms of Cell Death After Seizures; Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Epileptogenesis**: Specific Gene Expression Before and After Seizure in Epileptic EL Mice; **Pediatric Epilepsy**: Developmental Aspects of Seizures.

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COGNITIVE DYSFUNCTION AND OTHER COMORBIDITIES

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Attention Deficit Disorder and Epilepsy in Children

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Introduction and Background

Epilepsy and attention deficit disorder with and without hyperactivity (ADHD/ADD) are highly prevalent in children and adolescents. The CDC estimates that 4.4 million youths aged 4–17 have been diagnosed with ADHD by a healthcare professional, and as of 2003, 2.5 million youth aged 4–17 were receiving medication treatment for the disorder. The current prevalence of ADHD in the United States is about 5%. The association between ADHD/ADD and epilepsy has been well established since 1955, when Ounsted first reported an elevated incidence of children with hyperactivity in his population of 830 children with epilepsy. Behavior problems of all kinds are 4.8 times more common in children with epilepsy than in the general population, and are still about 2.5 times more common in children with recurrent seizures than in children with non-neurological chronic illnesses such as asthma. The incidence of ADHD/ADD is elevated in children with epilepsy not only when compared with the general population, but also when compared with children with other chronic neurological disorders, as first reported in Rutter's Isle of Wight study. Since then, numerous publications have documented prevalence rates of ADHD/ADD of about 30–40% in children with epilepsy. One recent study noted that ADHD was the most common comorbidity of children with intractable epilepsy referred for presurgical evaluation. The majority of studies have also shown an increased incidence of ADHD/ADD in children with newly diagnosed idiopathic epilepsy. Interestingly, the association between new-onset epilepsy and ADHD is limited to the predominantly inattentive type of ADHD, not the hyperactive or combined types. The diagnosis of ADD predates the diagnosis of epilepsy in most cases. Seizure type, etiology, sex, or seizure frequency at diagnosis do not affect this association, but attentional problems become more frequent over time in children with uncontrolled seizures and persistent interictal abnormalities. Several authors have postulated that ADHD is the most frequent behavioral disorder in children with epilepsy. This association has far-reaching consequences, and has a great impact on social and academic functioning. Children with ADHD and epilepsy have lower academic outcome, lower adaptive scores, and a three-times greater likelihood of lowered health related quality of life than children with epilepsy but without attentional or behavioral problems.

Methods

The diagnosis of ADHD is still made clinically, predominantly by using observer behavior rating scales. Supportive information can be provided by formal neuropsychological testing and continuous performance tests. PET and functional MRI (fMRI) are utilized as research tools. A wide variety of behavior rating scales are available. Commonly used scales include Conner's rating scales, Vanderbilt scales, SNAP-IV, Aschenbach scales, and Child Behavior Checklist. These scales screen for core symptoms of inattention,

hyperactivity, and impulsivity. Most also provide information on oppositional defiant behaviors and symptoms of conduct disorder, as well as an overview of classroom academic performance. Assessments via rating scales are typically made by parents and teachers, although adolescent and adult self-rating scales are also utilized. Shorter follow-up rating scales are used to monitor treatment response. Additional observer and self-rating scales are sometimes used to screen for co-morbid disorders, such as anxiety, depression, and mood dysregulation.

Continuous performance tests are computerized tests of attention. Typically, a series of varying shapes, pictures or letters flash on the screen, and the test subject has to click a mouse button whenever the correct figure appears. The tests typically measure errors of omission (not clicking at the appropriate stimulus) – which correlate with inattention, and errors of commission (clicking when you shouldn't click) – which correlate with impulsivity. Time needed to click and variability of response time are also measured. These measures are typically increased in individuals with ADHD. Formal neuropsychological testing provides additional evidence of frontal lobe dysfunction by demonstrating deficits of working memory, planning, sorting, and shifting attention between sensory modalities.

Summary of Current Research Results

Epidemiology

The prevalence of ADHD in epilepsy populations is quite high. Further, compared with ADHD in the general population, ADHD in epilepsy populations shows some interesting differences. As noted earlier, the purely inattentive type of the attention deficit disorder is much more common in children with both chronic and newly diagnosed epilepsy than in children without epilepsy. Although in the general population ADHD is 4 times more common in boys than in girls, there is no gender predilection in children with epilepsy – perhaps reflecting the much higher proportion of the predominantly inattentive type in epilepsy, which is more common in girls than in boys.

Diagnosis

Continuous video-EEG monitoring has become the gold standard for the diagnosis of epilepsy and characterization of seizure type. Unfortunately, no similarly reliable tool exists for the diagnosis of ADHD, which is generally made based on behavior rating scales. These scales are sometimes augmented by more detailed neuropsychological testing and continuous performance tests, but this type of testing is time-consuming and expensive and difficult to perform in children with developmental disabilities. Symptoms of ADHD and epilepsy can overlap. For instance, daydreaming could be due to absence or partial complex seizures, postictal impairment or inattention. In children with confirmed epilepsy, continuous video EEG-monitoring may be necessary to determine whether a particular clinical event is due to breakthrough seizures or untreated ADHD. Children with ADHD but without epilepsy have a higher incidence of interictal spikes than seen in the general population, leading to further diagnostic confusion. In spite of the high prevalence of ADHD in children with epilepsy, it is advisable to proceed with caution and to re-evaluate the diagnosis repeatedly over time, particularly if the response to treatment is poor.

Pathophysiology

The pathophysiology of ADHD in the general population is thought to be related to dysfunction of dopaminergic and noradrenergic circuits in the prefrontal cortex and subcortical areas. Dopaminergic pathways are involved in inhibition and motor control, while noradrenergic pathways are involved in arousal and maintenance of attention. Causes for dysfunction in these systems are usually genetic, although environmental factors may contribute. To be sure, behavioral disturbances of all kinds, in individuals with epilepsy, are due to a complex interaction between biological and psychosocial factors. Biological factors include neuronal cell loss owing to frequent seizures, disruption of vital brain circuitry at critical periods of development, medication side effects, sequelae of frontal lobe epilepsy surgery, and postictal depression of brain activity. Biological factors can be permanent, such as congenital brain malformations, consequences of head trauma or perinatal asphyxia, etc. Biological contributions may also be transient (i.e., 'state dependent'), such as postictal impairment, sleep disruption from seizures, medication side effects, or postictal impairments. Psychosocial factors include family dynamics and dysfunction, disruption of family life due to the seizures, psychosocial stigmatization, and restriction of activities due to seizures. It is a major challenge of research in this area to try to evaluate how, and to what extent, each of these factors are responsible for a particular child's impairment. A meta-analysis of 46 studies from 1988 to 2003 showed that while behavioral problems of all kinds were significantly increased in children with epilepsy, attentional, thought, and social problems were specifically related to epilepsy rather than family factors or the presence of chronic illness. The fact that ADHD/ADD diagnosis precedes the diagnosis of epilepsy in a significant number of cases suggests that common biological factors cause both disorders, at least in some cases.

Role of Interictal Discharges

The effect of interictal discharges on cognition and attention has come under increasing scrutiny in the past 20 years. Impairment of cognitive functioning was first demonstrated

in childhood absence epilepsy. In the 1980's, transient cognitive impairment (TCI) was demonstrated to occur during both generalized and focal interictal discharges. Focal spikes specifically impaired cognitive functioning in the area(s) involved in spike generation. Interictal spiking of all kinds can also impair attention indirectly, by lowering quality and quantity of sleep. Some studies suggested that these effects can be reversed with anticonvulsant therapy, but those results have been inconclusive. Children with ADHD (but no diagnosed epilepsy) have an increase in epileptiform abnormalities on interictal EEGs when compared to the general population, particularly rolandic spikes. The incidence of interictal spikes may be as high as 30% in this population, compared to an incidence of 3.5% in typically developing school age children. This observation raises the question of whether treatment of these abnormalities can improve neurodevelopmental outcome. One study of a small number of children with interictal spikes, but no clinical seizures and various neurodevelopmental disorders including ADHD, found an 80% improvement in developmental outcome with chronic anticonvulsant therapy. Many anecdotal reports are consistent with these results, but there are no controlled studies with large numbers of children.

Some recent studies suggest that prolonged focal epileptic activity during sleep may permanently impair learning. Frontal lobe executive functioning, which is also impaired in ADHD/ADD, appears to be particularly vulnerable to disruption by interictal spiking during childhood – i.e., during the period of greatest neuronal plasticity. Even temporal lobe epilepsy may permanently affect frontal lobe functioning in children, by disrupting temporal lobe input to the frontal lobes during a critical period of neuronal maturation.

Research on the Interaction of the Type of Epilepsy and ADHD

Benign childhood epilepsy with centrotemporal spikes (BCECT)

Numerous studies have shown a very high prevalence of cognitive and behavioral dysfunction in benign childhood epilepsy with centrotemporal spikes (BCECT). This association is sufficiently well-established so that BCECT is no longer thought of as a 'benign' epileptic syndrome. Furthermore, BCECT probably represents one of a spectrum of rolandic epilepsies, ranging from benign to severe. Typical BCECTS represents the mildest form of this disorder, atypical benign partial epilepsy or pseudo-Lennox syndrome is in the intermediate part of the spectrum, and Landau–Kleffner syndrome is at the severe end. The presence of rolandic discharges is associated with increased impulsivity in children with ADHD/ADD. Several detailed studies of neuropsychological performance in children with rolandic discharges have shown that cognitive and behavioral dysfunction is related to duration and anatomical distribution of interictal spiking rather than to number or duration of clinical seizures. Transient impairments of attention and learning have been demonstrated in direct relationship to frequency of interictal spiking in children with BCECTS. One study suggested that attention is impaired with children with right-sided or bilateral rolandic discharges, as opposed to children with purely left-sided focal discharges. Other studies have shown that morphology of the epileptic dipole is related to cognitive and behavioral problems. Specifically, discharges with a frontal-temporal dipole had fewer cognitive problems than children with focal central, temporal or centrotemporal discharges. Studies of small numbers of children suggest that these effects are reversible with anticonvulsant therapy, but results have been conflicting and have not been sufficiently striking to cause a change in treatment guidelines for children with BCECT. Permanent cognitive dysfunction may be related to the significant increase in interictal spiking during non-REM sleep, particularly if this occurs during critical periods of development of fronto-temporal circuits. BCECT and related epilepsy syndromes may be associated with electrical spike-wave during sleep (ESES); persistence of ESES for more than two years is associated with permanent cognitive and behavioral changes, even after remission of the epilepsy and normalization of the EEG.

Temporal lobe epilepsy

Temporal lobe epilepsy (TLE) has primarily been associated with an impairment of memory, which is generally already present at the time of diagnosis. Neuropsychological studies have shown impairments of frontal lobe executive functions in children with TLE, most likely due to interruption of fronto-temporal circuits.

Frontal lobe epilepsy

Frontal lobe epilepsy has not been studied as extensively as BCECT, but the literature that exists suggests the presence of significant attentional problems. One retrospective study of 21 children with non-lesional frontal lobe epilepsy demonstrated ADHD/ADD in 2/3 of the population, while half of the children had cognitive impairments. Another study evaluated 16 children with frontal lobe epilepsy via an extensive neuropsychological test battery and continuous performance testing. The authors found significant impairments of attention and inhibition when compared to children with temporal lobe and childhood absence epilepsy. Frontal lobe seizures can also present with ictal behaviors which are difficult to distinguish from ADHD, such as screaming, kicking and flailing of the extremities (so-called 'hyperkinetic seizures'). Video EEG-monitoring may be necessary to arrive at the correct diagnosis.

Occipital lobe epilepsy

Idiopathic occipital lobe epilepsies are associated with visuoperceptual problems, lowered IQ, and behavioral

problem such as anxiety and depression, but not with ADHD/ADD.

Idiopathic generalized epilepsies

Childhood absence epilepsy very often presents as school failure, and causes significant impairment of attention if untreated. However, deficits of attention have been demonstrated even in children with well-controlled absence and generalized tonic clonic seizures. Adolescents with juvenile myoclonic epilepsy (JME) may have personality changes and even personality disorders, although the literature has been conflicting on this point. These patients have been described as impressionable, easily influenced and unreliable – features that have been attributed to subtle frontal lobe dysfunction. ADHD/ADD has not been specifically associated with JME, although one recent study of 50 patients with juvenile myoclonic epilepsy demonstrated widespread dysfunction on a detailed neuropsychological test battery, which included tests of attention and executive function.

Severe epilepsies of childhood

Severe epilepsies of childhood have long been associated with poor cognitive outcome and behavioral problems, typically following a developmental regression at the time of onset of seizures. Hyperactivity and attentional problems are particularly common in infantile spasms, severe myoclonic epilepsy of infancy, Lennox Gastaut syndrome and Landau Kleffner syndrome. These patients usually are treated with polytherapy, which may further contribute to behavioral and attentional impairments. The etiology of these impairments is uncertain, and does not appear to be related to specific seizure frequency or EEG patterns. The behavioral problems improve over time when the seizures remit, but permanent impairment is the rule. It has been shown that early initiation of treatment improves cognitive outcome in infantile spasms. Generally, poor behavioral and cognitive outcome correlates with early seizure onset, particularly if seizures start in the first year of life.

Effect of Anticonvulsants on ADHD

Anticonvulsants can affect behavior and attention in children with epilepsy, although this effect may be subtle and may be overshadowed by the positive effects due to reduced seizure-frequency. Increased rates of hyperactivity and distractibility were initially demonstrated for the older anticonvulsants, particularly phenobarbital. One study of 109 children with febrile seizures, who were treated with phenobarbital and had therapeutic anticonvulsant levels, showed a significant increase in hyperactivity. However, subsequent controlled studies in individuals with partial complex seizures only showed subtle effects on attention and concentration, once results were controlled for seizure frequency and anticonvulsant levels. Among the newer anticonvulsants, cognitive complaints–including impairment of attention–are particularly common with treatment with topiramate, although slow titration reduces this symptom considerably. Levetiracetam has behavioral side effects as high as 10%, including increase in aggression and irritability, but has not been shown to significantly affect attention. Lamotrigine has been shown to have no adverse effect on attention and may have favorable effects on mood and behavior. However, lamotrigine, gabapentin and tiagabine have been reported to cause absence status, which can imitate the inattentive type of ADHD. Gabapentin may cause somnolence and has been reported to cause increased aggression in children with developmental disabilities. Oxcarbazepine does not appear to cause any impairment of attention or behavior when compared to children treated with carbamazepine or valproate.

Behavioral side effects of anticonvulsants are generally thought to be more common with polytherapy than with monotherapy, regardless of the anticonvulsant used, although one recent study didn't find any differences in behavioral scores of children treated with 0, 1, 2 or 3 anticonvulsants. Negative effects on attention and behavior can be diminished by a slow titration schedule and decreases in dosage. Drug effects on behavior must be distinguished from the so-called 'release phenomenon,' which refers to the appearance of hyperactivity in children that were previously oversedated due to frequent, uncontrolled seizures.

Does ADHD resolve when epilepsy is treated? State-dependent biological factors such as postictal impairment, medication side effects, and postictal psychosis contribute significantly to the attentional problems in children with epilepsy. Studies have shown progression of these effects over time, and raises the question of whether attentional problems and associated executive dysfunction may be reversible–if the underlying epilepsy is successfully treated, particularly if complete seizure control is achieved quickly. Similarly, impairment of family dynamics due to the child's chronic illness would also be expected to improve when the seizures are controlled. So far, this result has not been documented in the medication literature. The consensus is that ADHD should be treated separately, with appropriate medications.

Treatment of ADHD in Children with Epilepsy

There are no treatment guidelines for children with ADHD/ADD and epilepsy, but treatment of ADHD symptoms is clearly justified considering that ADHD/ADD contributes significantly to the morbidity of epilepsy in children. A stepwise approach seems warranted in view of the complexity of ADHD/ADD in this population. Children with behavioral and attentional problems should

be evaluated further for degree of seizure control, the presence of nocturnal interictal discharges, and anticonvulsant side effects. They also should be evaluated for proper educational placement (as is the case for children with ADHD/ADD without seizures). Behavioral measures (e.g., reward systems, timers, and other devices to improve focusing, homework trackers, and so on) should be implemented when these evaluations indicate their appropriateness. Medications used to treat ADHD in children with epilepsy are the same as those used in children with ADHD alone, including stimulants such as methylphenidate and dextroamphetamine, and the non-stimulant medication atomoxetine. Controlled trials have proven the efficacy of stimulants, in particular methylphenidate, for children with ADHD. Methylphenidate has also repeatedly been shown to be effective and safe in many studies (of small numbers) of children with epilepsy and of children with interictal abnormalities but without clinical seizures. It should be noted, however, that some investigators have found that methylphenidate may increase the risk of breakthrough seizures in children with uncontrolled epilepsy (although the numbers of children studied were very small). One recent review of the atomoxetine clinical trials database and the atomoxetine postmarketing spontaneous adverse event database did not show an increased incidence of seizures in children who were treated with atomoxetine versus methylphenidate versus placebo. There are no controlled trials of the use of atomoxetine in children with epilepsy. There is very little literature on amphetamine products, but there is no evidence to suggest that these medications increase the risk of epilepsy when used in therapeutic doses. Only buproprion has been associated with an increased risk of epileptic seizures.

Challenges for the Future

Assessment of ADHD in Epilepsy Populations

There are many problems involved in assessing ADHD/ADD in children with epilepsy. The lack of objective measures is a major issue. Behavior rating scales and their cutoff scores haven't been validated in epileptic populations and are prone to observer biases. Continuous performance tests are an attempt at objective measurement of behavior and attention, but they only measure one type of attention (generally sustained attention) and represent only a snapshot of the individual's behavior and attention. They also require at least some cooperation from the child, making them difficult to use in developmentally disabled populations. These measures are unable to adjust for marked fluctuations of behavior and attention, such as can occur owing to postictal state, interictal discharges, medication side effects, and even motivation or lack of sleep. The ideal assessment tool would provide a reliable, objective and quantifiable measure of attention and behavior over time. Results should correlate with educational and social outcome, as recorded on quality of life studies and reviews of academic achievement. Modern imaging tools, which assess state-dependent changes in cerebral blood flow, metabolism, oxygenation, and neurotransmitter status, might be able to fulfill this role.

Role of Interictal Activity

The assessment of the role of subclinical seizures, and interictal spikes without clinical seizures, is another major challenge. There is increasing evidence that treatment of interictal spikes may improve long-term developmental outcome, but there are to date no well-controlled studies of large numbers of children. We need guidelines to determine when to initiate treatment, which treatment is beneficial, when to stop treatment, and how to determine that treatment has been successful or has failed. Should children with interictal abnormalities be treated, regardless of the presence or absence of developmental impairments? Should only nocturnal spikes be treated or only specific focal interictal spikes? Should treatment be continued until the EEG normalizes? How long would one monitor these patients to determine that treatment has been successful?

Other Questions

Other questions which need to be answered include:

1. Which biological factors contribute most significantly to the high incidence of ADHD/ADD in children with epilepsy?
2. Is the mechanism of ADHD in epilepsy different from ADHD in non-epileptic patients? If so, should different treatment approaches be used?
3. Is ADHD in children with epilepsy reversible if treated early?
4. Does treatment of epilepsy improve attentional problems in selected populations?
5. Finally, can we develop standardized assessment and treatment guidelines for children with ADHD/ADD and epilepsy?

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Behavioral and Cognitive Comorbidities

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Introduction

To get a better understanding of how a chronic illness involving the brain impacts the development of children's behavior, a study has been made on psychopathology in children with epilepsy. Given the association of cognitive difficulties with behavioral and emotional problems in the general population of children and the high rate of behavioral problems in children with mental retardation, we were interested in determining if children with epilepsy with average intelligence have specific behavior problems related to their epilepsy, comorbid cognitive problems, and effects of seizure variables.

In the first stage, these mainly phenomenological studies addressed three main questions. First, do children with epilepsy have a wide range of behavior problems similar to children in the general population and children with chronic illness involving the brain, such as head trauma or are certain psychiatric disorders overrepresented in these children? Second, what role does IQ play in the psychopathology of children with epilepsy? Third, how do seizure variables contribute to both behavioral and cognitive comorbidities in pediatric epilepsy?

To answer these questions, it has also been necessary to conduct studies that address methodological issues, such as the quality of the behavioral information derived by using different diagnostic tools and informants, as well as how to assess the effects of seizure variables when they are interrelated and also associated with cognition. Most recently I have examined the relationship of psychopathology and subtle cognitive impairments with structural abnormalities using magnetic resonance imaging (MRI) in children with epilepsy.

Background

In 1970, Rutter and colleagues' pivotal Isle of Wight epidemiological study demonstrated increased psychopathology in children with epilepsy, a wide range of psychiatric diagnoses, and an association between type of epilepsy and type of psychiatric diagnosis. Thus, children with temporal lobe epilepsy were described as hyperactive, antisocial, and aggressive, particularly if they had left sided foci. Children with childhood absence epilepsy (CAE) were thought to have neurotic traits, but no other behavioral disturbances.

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In terms of seizure variables, most early studies reported a relationship between poor seizure control and behavioral problems in children with epilepsy. Yet, several of these studies included children with mental retardation and difficult to control early onset epilepsy. Furthermore, follow-up studies on surgically treated and medically treated children have not confirmed that seizure control was related to behavior problems. In addition, a cross-sectional study found no difference in parent reports of behavior problems in children with generalized epilepsy and complex partial seizures (CPS).

Regarding cognition, Camfield *et al.* (1984) and Rutter *et al.* (1970) found that the behavioral deficits of children with epilepsy were associated with the severity of their neuropsychological deficits. Nevertheless, subsequent studies have not separated out the effect of IQ, as well as its relationship with both seizure variables and psychopathology. Our studies, therefore, focused on ascertaining the role of this variable and its possible interaction with seizure variables in the behavioral problems of these children.

From the methodological perspective, studies in child psychiatry have identified differences in the behavioral information obtained using different informants (e.g., children, parents, and teachers) and instruments (e.g., structured psychiatric interviews, self-report instruments, parent, or teacher questionnaires). In terms of the informant, children and adolescents frequently do not share 'internalizing' symptoms, such as depression, anxiety, fears, suicidal ideation, hopelessness, helplessness, hallucinations, and delusions with their parents. Alternatively, parents are a better source when measuring acting out, irritable, and aggressive behavior with poor judgment in the child or adolescent. The informant's emotional state, age, and gender also can affect the type of information provided. For example, parents with a history of depression tend to overdiagnose depression in their children. Although anxious parents are more sensitive to the child's emotions and fears, they might also worry about behaviors in their children that would otherwise be considered normal. Adolescents are more likely to report depression than children, particularly if they are females.

Some informant differences are specifically pertinent to epilepsy. Some parents of children with epilepsy misinterpret behavioral phenomena as seizures while others might mistake manifestations of seizures as behavior problems. For example, parents might report what might appear to be unprovoked irritability as a child being 'out of it' and having a seizure. Alternatively, parents might interpret repeated brief loss of consciousness in CAE as the child being distractible and having attention deficit hyperactivity disorder (ADHD).

Regarding diagnostic instruments, structured psychiatric interviews, and some questionnaires yield DSM-IV diagnoses, whereas most self-report, parent, and teacher questionnaires provide information on behavioral/emotional symptoms. Concerned that parent questionnaires might not identify all the children who have psychiatric diagnoses, we, therefore, compared psychopathology findings with parent questionnaires to those of structured psychiatric interviews.

Additional methodological concerns relate to the effects of seizures on behavior. Thus, some studies have examined individual variables, such as type of epilepsy, seizure control, seizure frequency, duration of illness, antiepileptic drugs (AEDs), and their relationship to behavioral measures, and others have devised a severity measure. However, early onset of epilepsy is confounded by poor seizure control, increased seizure frequency, AED polytherapy rather than monotherapy, longer duration of illness, and prolonged seizures, as well as lower IQ score. Ascertaining which aspects of seizures are related to behavior, therefore, necessitates conducting studies on large population of children and use of statistical techniques, such as principal component analysis to identify independent variables.

Finally, the rationale for our imaging studies was based on increasing evidence for structural and functional abnormalities in children with ADHD, depression, anxiety disorders, and psychosis who do not have epilepsy. We applied these techniques to identify the neural circuits and substrates associated with psychopathology in children with epilepsy, and to determine if abnormalities in these circuits reflect effects of the underlying neuropathology, seizures, and IQ.

Methods

Subjects

These studies included 103 CPS and 80 CAE children, aged 5.1–16.3 years with IQ scores of 70 and higher and 101 normal children. CPS subjects had to have cryptogenic epilepsy and CPS, as defined by the International Classification of Epilepsy, and at least one seizure during the year prior to the child's participation in the study. Children with a clinical history of CPS, but no EEG evidence for focal epileptic activity were also included in the study sample. The study inclusion criteria for each CAE subject were a diagnosis of CAE, absence seizures induced by hyperventilation, at least one seizure in the year prior to participation in the study, and EEG evidence of 3 Hz spike and wave. We excluded patients with a mixed seizure disorder, previous epilepsy surgery, atypical spike and wave complexes, juvenile myoclonic epilepsy, generalized tonic–clonic seizures, a structural MRI abnormality but not those with mesial temporal sclerosis, a neurological illness other than epilepsy, chronic medical illness, imaging evidence for structural brain abnormalities, a metabolic disorder, a hearing disorder, mental retardation based on school/classroom placement, and bilingual

speakers of American English who did not attend English speaking schools or speak English at home.

To include control children from a wide range of ethnic and socioeconomic status backgrounds similar to that of the CPS and CAE groups, we recruited the normal control subjects from four public and two private schools in the Los Angeles community. We excluded children with diagnoses of neurological, psychiatric, language, and hearing disorders in the past from the study.

Procedures

Psychopathology: We determined the presence/absence and type of DSM-IV diagnoses using the *Schedule for Affective Disorders and Schizophrenia for School-Age Children, Epidemiologic Version,* and the *Present and Lifetime Version*, administered separately to each child and parent. Parents completed the *Child Behavior Checklist (CBCL)* and children filled out the *Child Depression Inventory (CDI)* and the *Multidemnsional Anxiety Scale (MASC) for Children.*

Cognition: The *Wechsler Intelligence Scale for Children-Revised (WISC-R)* and the *Wechsler Intelligence Scale for Children-3rd edition (WISC-III)*, administered to children generated Full Scale, Verbal, and Performance IQ scores.

Imaging: All subjects underwent brain MRI scans at 1.5 T. Tissue was segmented and total brain, frontal lobe, frontal parcellation and temporal lobe volumes, hippocampus volumes, and amygdala volumes were computed.

Results

Range and Type of Behavior Problems in Children with Epilepsy

Using a structured psychiatric interview administered separately to the child and parents, we found high rates of psychiatric diagnoses in children with CPS and children with CAE, children with epilepsy recruited from community and from epilepsy centers compared to normal children (Table 1). In both CPAS and CAE the most frequent diagnosis was ADHD followed by anxiety/mood disorders and then both ADHD and anxiety/depression. Among the children with anxiety/depression, 62% had anxiety disorders, 24% depression, and 14% both anxiety disorder and depression diagnoses. We also found high rates of suicidal ideation in 20% and suicidal plans in 37% of our sample, particularly in children with both ADHD and anxiety/depression diagnoses.

Unlike other psychiatric diagnoses, we diagnosed psychosis in children with CPS not CAE, and made this diagnosis in less than 10% of these children. However, a long-term follow-up study of children following hemispherectomy for early onset intractable epilepsy, most of whom had mental retardation, demonstrated psychosis in 33%. We also diagnosed autism and autism spectrum disorders infrequently in the CPS and CAE groups with average IQ in contrast to the high rate of these disorders in children with epilepsy with mental retardation.

Table 1 Psychiatric Diagnoses in CPS, CAE, and Normal Children

	CPS	*CAE*	*Normal*
N	103	80	101
Psychiatric Diagnosis	56%	57%	15%
ADD*	17%	23%	6%
Affective/Anxiety	14%	18%	7%
ADD + Affective/Anxiety	23%	12%	2%
Other	2%	4%	0%
No diagnosis	56%	43%	85%
CBCL T > 60			
Total	40%	35%	12%
Internalizing	42%	35%	16%
Externalizing	19%	26%	7%

*Attention deficit disorder.

Associated Variables

A principal components analysis of seizure variables in a large sample of 101 children with CPS demonstrated no significant relationship between four epilepsy components (e.g., age of onset/duration, seizure frequency/number of AEDs, history of prolonged seizures or febrile convulsions, localization, and lateralization of epileptic activity on EEG) and psychiatric diagnosis. Although the prolonged seizure/febrile convulsions and the seizure frequency/AEDs components of the principal components analysis were significantly related to IQ in CPS children, Verbal IQ was a robust predictor of psychopathology above and beyond the seizure effects. With both epilepsy and IQ variables in the model, significantly more CPS children with lower Verbal IQ scores had a psychiatric diagnosis and CBCL scores in the clinical/borderline range.

In contrast, in the CAE group, duration of illness and seizure frequency predicted the presence of a psychiatric diagnosis, as well as the type of psychiatric diagnosis, ADHD, and anxiety disorder diagnosis. Being on AED treatment predicted parent total and internalizing CBCL scores in the clinical/borderline range whereas longer duration of illness was related to higher CBCL externalizing scores. Unlike the CPS group, increased duration of illness was significantly related to lower Verbal IQ scores, and AED treatment, whether monotherapy or polytherapy, was associated with lower Full Scale, Verbal, and Performance IQ scores.

Our findings are remarkable as they demonstrate similar rates and types of psychopathology, as well as IQ in children with a focal and a generalized type of epilepsy but different profiles of associated variables. These different profiles imply that despite similar end points, different mechanisms underlie the psychopathology and

IQ of children with CPS and CAE who have average intelligence.

In addition, the robust association of Verbal IQ with psychopathology in CPS and between emotional maladaptation and school performance in children with epilepsy and in the general population underscore the importance of ruling out subtle learning disorders in CPS children with behavioral disturbances. Subtle difficulties with verbal cognition might also reflect poor use of language to problem solve and manage emotional and behavioral responses. Furthermore, these findings point to a possible role for the underlying neuropathology in both the Verbal IQ and psychopathology of children with CPS.

The frontal lobe, thalamus, and impaired 5HT metabolism are implicated in both absence seizures and anxiety disorders. The high rate of anxiety disorder diagnoses in CAE might reflect prominent involvement of the frontal lobe in the neuropathology underlying CAE, response of this brain region to effects of ongoing seizures, and the child's feeling of lack of control and increased anxiety with repeated episodes of loss of consciousness over a prolonged period. Similarly, the frontal lobe has been implicated in both ADHD with and without epilepsy and probably plays a role in this frequent CAE comorbidity.

Longer duration of illness and AED treatment in CAE children with cognitive comorbidities also imply possible adverse effects of continued uncontrolled CAE on children's cognitive skills. These findings might also reflect the developmental effects of CAE on maturation of the frontal lobe and thalamus, brain regions involved in the neuropathology of CAE, and in cognition.

Instruments, Informants

Parent CBCL scores identified behavioral problems in only 40% of CPS and CAE children who had a psychiatric diagnosis. However, having a K-SADS diagnosis significantly predicted clinically relevant CBCL scores. Our findings suggested that parent based CBCL scores appear to be more helpful identifying children who are more behaviorally deviant but not children whose behavioral/emotional difficulties are less obvious.

Comparison of the sensitivity (i.e., correct classification of children with a diagnosis) and specificity (i.e., correct classification of children without a diagnosis) of the parent CBCL with the CDI and MASC child self-report questionnaires in identifying children with structured interview based anxiety disorder and depression diagnoses revealed good sensitivity for the MASC (84.2%) but low sensitivity of the CDI (50%), CBCL internalizing scores (45%), and CBCL anxiety/depression scores (40%). These instruments, other than the CBCL anxiety/depression scores (91.7%), had low specificity (60%–74%). The combined MASC, CDI, and anxiety depression factor score of the CBCL provides a 87.5% sensitivity and the CBCL anxiety depression factor score a 91.7% sensitivity. Screening children with epilepsy for mood and anxiety disorder diagnoses should, therefore, involve a combination of instruments to avoid false negative (i.e., missing children with anxiety/depression diagnoses) and false positives (i.e., making these diagnoses in children who do not have them).

Unmet Mental Health Needs

These high rates of psychiatric diagnoses together with low treatment rates in only 33% of these children highlight the unmet mental health needs of children with epilepsy. Absence of mental health care was associated with younger age of the children, less parental education, limited number of AEDs (i.e., one or none), and higher verbal IQ. Children with CAE and a single psychiatric diagnosis were also less likely to have mental health treatment. We concluded that these findings highlighted the need to educate parents and clinicians about the undiagnosed behavioral comorbidities of pediatric epilepsy, particularly if children are younger, less severely ill, and have CAE, traditionally considered a 'benign' form of epilepsy.

Psychopathology and Brain Volumes

Children with CPS have significantly larger white matter volumes of the temporal lobe, smaller gray matter volumes of the orbital frontal gyrus, no difference in amygdala volumes, and smaller hippocampal volumes compared to age and gender matched normal children. In terms of the relationship of these volumes with psychopathology, CPS children with a psychiatric diagnosis have significantly smaller white matter volumes of the inferior frontal gyrus controlling for effects of seizure variables and IQ. These inferior frontal gyrus findings are interesting given our prior findings that Verbal IQ is a robust predictor of psychopathology in these children, the central role of this brain region in syntax, semantics, phonology, and higher-level linguistic functions, and recent clinical evidence for higher level linguistic deficits, disruptive disorder diagnoses, and externalizing behaviors in children with CPS. Together these findings implicate the inferior frontal gyrus in the behavioral and cognitive comorbidities of pediatric CPS. Of note, Hermann and colleagues recently demonstrated increased frontal lobe gray matter volumes in a large population of children with new onset epilepsy that was related to ADHD diagnoses and not seizure variables.

We had predicted that total and frontotemporal brain volumes in CPS children would be related to IQ. However, we found this relationship in the normal children not in those with CPS. Age (not IQ) was associated with these volumes in the CPS group both with and without seizure variables in the model. Although cross-sectional, this

finding implies that brain development in CPS might alter the normal association between IQ and cortical volumes. Given the small sample size, we were unable to examine if these volumes were associated with an interaction effect of psychopathology and IQ.

Similar to findings in children with depression and anxiety disorders without epilepsy and adults with temporal lobe epilepsy and depression, the CPS children with anxiety disorder/depression diagnoses had significantly larger left amygdala volumes than those without these diagnoses. Seizure variables were unrelated to these findings. However, despite evidence for involvement of the hippocampus in pediatric depression unrelated to epilepsy, we found no relationship of these volumes with either the presence of a psychiatric diagnosis or IQ.

Conclusions and Future Directions

The association of psychopathology with both subtle cognitive deficits involving verbal skills and smaller white matter volumes of the inferior frontal gyrus, a brain region involved in integrating language and cognition, together with the abnormal lack of a relationship between IQ and fronto-temporal volumes in CPS attests to the integral involvement of the underlying neuropathology in the behavioral problems of these children. These findings also imply that behavioral and cognitive comorbidities are part and parcel of pediatric CPS. Increased left amygdala volumes in the children with anxiety and depression diagnoses further supports this conclusion.

In contrast, the relationship of seizure variables (i.e., frequency, duration, number of AEDs) with the psychopathology of the CAE group implies that in this disorder ongoing seizures might contribute to behavioral problems. However, prominent involvement of the frontal lobe in this disorder underscores the importance of our ongoing neuroimaging studies of this brain region to advance our understanding of how the underlying neuropathology might be associated with the comorbid behavior problems in pediatric CAE.

Our future directions include prospective studies in CPS and CAE examining age-related changes in psychopathology and their relationship with changes in seizure variables, cognitive/academic performance, and multimodal imaging findings (e.g., functional magnetic resonance imaging, magnetic resonance spectroscopy, diffusion tensor imaging, morphometry). To ascertain if the psychopathology and cognitive comorbidities of children with epilepsy reflect 'familial/genetic' factors, we are comparing psychopathology, cognition, and brain structure of the siblings of children with CPS and CAE to those of the probands and normal children. Finally, we are also examining possible barriers to mental health care in children with epilepsy.

See also: **Cognitive Dysfunction and Other Comorbidities**: Attention Deficit Disorder and Epilepsy in Children; Behavioral Comorbidities in Children with Epilepsy; Cognitive Effects of Seizures in Animal Models; Language and Communication Disorders; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Behavioral Comorbidities in Children with Epilepsy

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Introduction

Focus of Our Research and Why It is Important

The focus of our research program is to identify factors that account for the high rates of behavior problems in children with epilepsy. These research goals are important for three major reasons: First, children with epilepsy are almost five times more likely to have behavior problems than children from the general population and over two times more likely than children with other chronic physical conditions. Second, these behavioral comorbidities in children with epilepsy are stressful for parents and disruptive to families. In interviews with parents of children with new-onset seizures, parents reported that dealing with their children's behavior problems was a major source of worry and an area for which they were in need of professional help. In recent studies, parenting problems were related to stress arising from the parents' attempt to manage the behavior of their child with epilepsy. Third, these behavioral difficulties in children with epilepsy are enduring and result in significant social morbidity with aging. As children with epilepsy pass through adolescence and into young adulthood, they are more likely to leave school early, be either unemployed or underemployed, and be unmarried. In the very few available longitudinal studies, investigators found that adults with childhood-onset epilepsy had more psychiatric difficulties, more unemployment, and lower rates of marriage than the general population. In summary, behavior problems in children with epilepsy are common, disruptive to the family, and lead to later social problems.

What Kinds of Problems Do Children with Epilepsy Have?

Findings from descriptive studies consistently show higher rates of internalizing behavior problems (i.e., symptoms of anxiety, depression, social withdrawal) than externalizing behavior problems (i.e., acting out, conduct problems). A recent meta-analytic study provided the most definitive statement on the nature of the behavior problems that are found in children with epilepsy. In this meta-analysis of 46 studies, behavior problems in children with epilepsy were contrasted to those in the general population, in healthy siblings, and in children with other chronic childhood conditions. Children with epilepsy were found to differ more from healthy controls than either children with other chronic conditions or their healthy siblings. Common problem areas for both children with epilepsy and those with other chronic physical conditions were: social withdrawal, somatic complaints, anxiety/depression, delinquency, and aggression. Interestingly, many of these problems fall under the rubric of internalizing problems, suggesting that having a chronic illness leads to more social withdrawal, worry, and symptoms of depression. The similarity in problems between these two groups suggests that some of the problems that children with epilepsy experience might be related to having a chronic physical condition. Areas in which children with epilepsy had more difficulties than the other groups were attention problems, thought problems, and social problems. Notably, none of these problem areas is a type of internalizing problem. Overall, these findings suggest that having epilepsy affects behavior beyond that associated with living with a chronic illness.

Background

The major goal of our research program has been to identify factors that might explain the high rates of behavior problems in children with epilepsy. We propose that understanding which factors are associated with the problem can provide important information on which to base interventions to prevent or reduce these problems. We have identified four broad categories of variables (see Table 1) as potential causes of behavior problems: (a) neurological dysfunction; (b) seizure condition variables; (c) neuropsychological deficits; and (d) poor child and family response to the condition. Each of the potential causes of problems is briefly reviewed below.

Neurological Dysfunction

Neurological dysfunction is a known risk factor for behavioral difficulties. Children with mental retardation or intellectual disability have very high rates of behavior problems. Epidemiological studies showing that children with neurological conditions such as epilepsy have higher rates of behavioral problems than children without central nervous system involvement; such studies provide indirect evidence that neurological dysfunction is a potential cause of behavioral problems. Additional support for the relative importance of underlying neurological problems comes from

Table 1 Risk factors for behavioral comorbidity in children with epilepsy

I. Neurological dysfunction
A. Central nervous system abnormality
B. Neurological disorder in addition to epilepsy
II. Seizure condition variables
A. Age of onset and duration of epilepsy
B. Seizure type and epileptic syndrome
C. Seizure control/frequency
D. Epileptiform discharges on EEG
E. Antiepileptic drug side effect
III. Neuropsychological deficits
A. Intellectual disabilities
B. Learning disorders
C. Cognitive deficits
IV. Child and family variables
A. Parental psychopathology
B. Family dysfunction
C. Child attitude toward illness
D. Child social skills

studies of new-onset epilepsy. For example, in recent studies by our research team and others, children were found to display behavioral problems *before* the first seizure was recognized – in other words, prior to other potential causes such as side effects of antiepilepsy drugs (AEDs) or poor psychosocial responses to dealing with epilepsy. It is important to study neurological dysfunction in order to increase our understanding of why children with epilepsy have behavior problems.

Seizure Condition Variables

Because seizures are the defining characteristic of epilepsy, a large number of studies have investigated different seizure variables (e.g., age of onset, seizure frequency, seizure type, and epilepsy syndrome) for relationships with behavior problems in children with epilepsy. Findings have varied greatly, with some authors finding support for greater seizure frequency being associated with more behavior problems but other authors showing no association. A major conclusion from a recent review of the literature was that seizure variables were not consistently related to behavior problems (across the studies surveyed), regardless of study design and sample. One possible explanation for the mixed findings is that seizure variables such as time of onset, seizure type, and epilepsy syndrome are interrelated and confounded. As a result, findings could vary if only one seizure variable was studied (e.g., age of onset), especially in a small sample.

Nevertheless, there is evidence that children with symptomatic syndromes experience more cognitive deficits and behavioral disruptions than children without such symptomatic syndromes. Because neurological dysfunction is strongly associated with symptomatic syndromes, neurological dysfunction might account for the relationship between syndrome and behavior problems.

An important seizure-related variable that has received much attention is AED treatment. Prospective studies have not found support for a relationship between AEDs and behavior problems. For example, in a study comparing behavior from before and after stopping AED treatment, only a decrease in tiredness was found. A major conclusion from this study was that AED monotherapy does not have an impact on behavior.

To summarize, although studies do not show consistent relationships between seizure variables and behavior problems, the mixed findings might be explained by study design and variables studied. Prospective studies tend to show fewer associations between seizure variables and behavioral comorbidities than do cross-sectional studies. Moreover, some seizure variables are related to neurological dysfunction, making it difficult to clearly identify the relevant causal mechanism.

Neuropsychological Deficits

As previously mentioned, behavioral problems are most common in children with epilepsy who also have intellectual deficits. In one of the very few studies on the occurrence of psychiatric disorders in children with both epilepsy and intellectual disability, ~60% were found to have at least one psychiatric diagnosis. These exceedingly high rates of psychiatric diagnoses provide indirect empirical support for a relationship between cognitive functioning and behavior problems. Moreover, this study is consistent with the epidemiological studies showing the highest rates of behavioral comorbidities in children with both epilepsy and other neurological deficits. In addition, studies exploring predictors of behavior problems in children with epilepsy showed that those with intelligence in the low normal range had more problems than those in the average and high normal intelligence ranges.

Children with epilepsy have more cognitive deficits than their healthy siblings or the general population. In a study of children with complex partial epilepsy and their siblings, children with epilepsy performed significantly worse across seven cognitive domains (verbal memory, nonverbal memory, language, academic achievement, problem solving, mental efficiency, and motor skills). Only a few studies have explored the relationship between cognitive deficits and mental health problems in children with epilepsy. When global neuropsychological functioning was explored for relationships with mental heath problems, a moderate correlation was found, with poorer cognitive functioning being associated with more mental health problems. In studies in which children with epilepsy were placed into high or low cognitive functioning groups, children in the low functioning group had significantly more behavior problems. Finally, in the few

studies in which specific areas of cognitive functioning were explored for relationships with mental health comorbidities, low verbal IQ was related to greater behavioral disruption. In summary, although there are relatively few studies in this area, findings consistently show a moderate positive relationship between neuropsychological deficits and behavior problems in children with epilepsy.

Child and Family Variables

Research on child and family variables as they relate to child behavior problems has increased substantially in the last decade. Most of the studies have focused on family variables, however, and only a few have explored child variables. One probable reason is that there are fewer epilepsy-specific instruments available to measure child variables. Interestingly, almost all of the family studies that have included both seizure variables and family variables as predictors have found family variables to be more strongly related to behavior problems than were seizure variables. However, most of these studies were cross-sectional in nature and focused on children with chronic epilepsy, so it is almost impossible to know whether problems in the family preceded or followed problems in the children.

Two approaches have primarily been used to explore relationships between family variables and child behavior problems, group comparison studies, and within-group correlational studies. When comparisons were made to control families or families of children with other physical conditions, families of children with epilepsy showed more family dysfunction, disturbed parent–child interactions, and maternal depression. In the within-epilepsy sample of correlational studies, a number of variables have been associated with behavioral comorbidities, including greater family stress, fewer family resources, disturbed parent–child interactions, and poor family adjustment to the epilepsy. In one of the few prospective correlational studies that explored parenting variables, our research team found that an increase in parent emotional support to the child (over the first 24 months after the first seizure) predicted less child anxiety and fewer depression symptoms at 24 months after seizure onset.

Our research team is one of the few groups that has focused on how child perceptions related to epilepsy are related to child mental health functioning. We found that fewer negative beliefs about having epilepsy were associated with more positive self-concepts and fewer symptoms of depression. In a study of symptoms of depression in adolescents with epilepsy, we found negative attitudes toward having epilepsy and beliefs in an unknown or external locus of control (i.e., that events are controlled by luck or external forces and not the actions of the affected individual) had the strongest associations with depressive symptoms.

Methodological Approaches

How have Behavior Problems been Measured?

Child behavior problems have primarily been measured using parent-completed questionnaires. Although a few studies have used structured interviews with children to measure psychopathology, most have relied on either parent or teacher report of the child's behavior. The most commonly used instrument is the Child Behavior Checklist (CBCL). The CBCL contains 118 items on which parents rate their children's behavior on 3-point scales: 0 (Not True), 1 (Somewhat or Sometimes True), and 2 (Very True or Often True). A number of versions of the scale are available for use with different ages (e.g., preschool, school age, young adult) and for other raters such as teachers. The CBCL provides a total score, two broad factor scores, and eight syndrome scores. The internalizing behavior problems factor is composed of three syndromes: anxious/depressed, somatic complaints, and withdrawn. The externalizing behavior problems factor is composed of two syndromes: delinquent behavior and aggressive behavior. The three syndromes scores that are not included in either of these two broad factors are social problems, thought problems, and attention problems. All CBCL instruments are well-validated and have had extensive research carried out to develop norms for age and gender for each of the scores. In addition, CBCL instruments have been translated into other languages. Because the CBCL is commonly used in several countries, it has facilitated comparisons across studies internationally. Despite the many advantages of the scale for assessing behavior in children with seizure disorders, some of the individual items on this instrument reflect behaviors that might be directly related to seizures (i.e., more of a reflection of acute seizure-associated behavioral changes). To address this potential weakness, our research team has used structured interviews and asked parents not to rate any behavior that might be related to their child's seizures or that occurs in close proximity to a seizure (i.e., behavior that occurred directly before, during, or directly after a seizure). Another potential weakness of this instrument is that the CBCL measures children's problem behaviors, but does little to assess areas of positive child adaptation.

What are the Most Common Designs?

Because both the frequency and the nature of behavior problems have been well-described, cross-sectional descriptive studies of behavior problems have been decreasing in number and are no longer needed. Likewise, cross-sectional studies that compare behavior problems in

children with epilepsy with other illness groups are not needed, because it is well established that children with epilepsy fare worse in specific areas. In contrast, studies are needed that delineate specific factors associated with or predict behavior problems in children with epilepsy. Early studies in this area had cross-sectional designs and focused on children who had had epilepsy for a number of years. Although these studies showed associations of neurological, seizure, cognitive, and family variables to child behavior problems (as described earlier), their designs limited their ability to determine the temporal order of the relationships (i.e., when the behavioral problem arose relative to the onset of seizure activity). In the past two decades, more studies have been conducted that had longitudinal or prospective designs. Once predictors of behavior problems are identified from these studies with strong designs, interventions to reduce or prevent problems can be developed and tested in randomized clinical trials.

Important Recent Results

In the past decade, prospective studies investigating a comprehensive set of predictors in large samples of children with new-onset seizures have been carried out and have yielded new and unexpected findings. In particular, a number of studies have demonstrated that behavior problems actually precede the onset of seizures and the diagnosis of epilepsy. In a prospective study of children who had a first recognized seizure, our research team collected parent ratings of their children's behavior in the 6 months prior to the first seizure and found that 32% of children already had behavior problems. Other investigators have found that children with a diagnosis of Attention Deficit Hyperactivity Disorder (ADHD) are at greater risk for developing seizures. Findings from these studies have led to a sea change in the conceptualization of the causes of behavior problems. If behavioral comorbidities precede seizure onset, it is unlikely that side effects of medications and poor family adjustment to epilepsy are responsible for these behavior problems.

Future Research Challenges

Although descriptive studies have categorized the types and frequency of behavioral comorbidities in children with epilepsy, many questions remain to be answered if we are to develop interventions to reduce behavioral comorbidities. We need to delineate further the underlying mechanisms for behavioral comorbidities. Although neurological dysfunction is a known risk factor, little is known about neurological function changes over time – and how such changes might be related to changes in behavior problems. In this regard, recent imaging studies are revealing evidence of progressive changes that may be important for cognitive and behavioral functioning. Studies are needed to understand which specific domains of neuropsychological function are related to behavior problems. Although some studies of change in intelligence have been carried out, more comprehensive batteries that test multiple domains of neuropsychological functioning over time will provide a more complete picture of the relationships between cognitive functioning and behavioral comorbidities. Once specific domains have been identified, interventions can be developed to address specific cognitive deficits. Newer antiepileptic drugs appear to have fewer cognitive and behavioral side effects than do traditional AEDs; however, adequate methodological studies of the positive and negative cognitive and behavioral effects of these AEDs are still needed. In addition, pharmacogenetics studies have the potential to provide a better understanding of which patients are at risk for adverse behavioral effects from particular medications. Studies are also needed to clarify the physiological mechanisms through which depression and ADHD increase the risk of seizures (as well as epilepsy). Finally, studies are needed in the area of child and family factors, including investigations that identify risk and protective factors as well as those that test interventions aimed at reducing behavioral comorbidities.

See also: **Cognitive Dysfunction and Other Comorbidities**: Attention Deficit Disorder and Epilepsy in Children; Behavioral and Cognitive Comorbidities; Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; Cognitive Function in Temporal Lobe Epilepsy; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model

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Introduction

Numerous clinical studies have identified cognitive and behavioral effects following seizures. These seizure consequences include deficits in intelligence, learning and memory, heightened anxiety, and altered emotional and affective responses. Seizures in infants and young children had previously been thought to cause fewer and less-significant cognitive sequelae than equally severe seizures in the adult brain, but it is now appreciated that seizures at any age can lead to persistent adverse effects on the brain, including increased susceptibility to subsequent seizures and deficits in cognition and behavioral function. Even children with 'benign' childhood epilepsies have a significantly higher incidence of memory, attention, and intellectual dysfunction than those without epilepsy. Factors that influence cognitive outcome include the age of seizure onset, the number and timing of seizures, seizure frequency and severity, and seizure type, syndrome, or etiology. Cognitive assessment in children with epilepsy can be complicated by preexisting deficits in cognition and behavior, making it difficult to discern whether seizures or their treatment are primary or secondary causes of the dysfunction.

Seizures during development present a paradox. Seizure threshold is low in the young brain, with seizures occurring in response to much smaller convulsant stimuli than in adults. At the same time, seizure-induced behavioral and cognitive consequences, though measurable, are less profound in the immature brain than those seen after seizures in mature animals. However, careful consideration of the effects of seizures on the developing brain have revealed that this paradox is more apparent than real; i.e., the young nervous system actually does sustain seizure-induced cognitive deficits in distinct and significant ways. The sensitivity of the brain at all ages to the deleterious effects of seizures necessitates a concerted effort to avoid, or at least minimize, those adverse effects.

Animal models are useful in addressing some of the questions that evade experimental analysis in patients. Many of the seizure variables discussed earlier can be controlled in animal models. For example, seizure etiology plays a major role in outcome, but it is difficult in humans to separate the effects of seizures from the consequences of the brain pathology that caused the seizures. The creation of etiology-specific neonatal seizure models is necessary to increase our ability to compare animal models with human data. However, increasing evidence suggests that seizures can engender adverse effects on the developing brain, regardless of etiology or model. Further, while prolonged seizures unequivocally result in adverse long-term cognitive consequences, the effects of frequent, brief seizures are also associated with deficits (not discussed in this article). Recognition of cognitive and behavioral comorbidities and their relationship to various seizure types and ages of occurrence poses both challenges and opportunities for early intervention.

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Background

This article focuses on contributions of the kainic acid (KA) model in studying cognitive and behavioral consequences of seizures early in life. In this chapter, 'early life' refers to the first 2–3 weeks of life in rodents, which correlates with several parameters of brain development during the first few months to about 3 years of age in humans.

Various models of developmental epilepsy have contributed to our understanding of the consequences of seizures during this vulnerable time period. For example, seizures caused by convulsant stimuli as diverse as pilocarpine, tetanus toxin, hypoxia, and hyperthermia have been demonstrated to lead to deficits in spatial learning, memory, and other cognitive functions. Seizures induced by hyperthermia (resembling human febrile seizures) or by deprivation of oxygen (mimicking hypoxia ischemia) are examples of acquired brain pathology causing epilepsy. Other epilepsy models involve genetic lesions or focal cortical dysplasia. In comparison, KA is an exogenous convulsant agent that, though working via a specific glutamate receptor subtype (KA receptors), is not a naturally occurring stimulus. KA is a glutamate analog that has been used as an experimental convulsant since the 1970s. The main advantage of KA as a developmental epilepsy model is not that it mimics a specific clinical scenario, but rather, that it causes – via overactivation of a physiologically relevant glutamate receptor subtype – exquisitely age-dependent neuronal dysfunction at the cellular, synaptic, network, and behavioral levels. It is worth emphasizing that none of the developmental epilepsy models cause epilepsy or its adverse consequences by killing neurons, implying that any long-term impact is due to developmentally-specific alteration of cell proliferation or migration, ion channel function, receptor formation and subunit composition, synapse formation, circuit connectivity, or other cellular or subcellular pathology.

Age-related effects of KA seizures are summarized in Table 1. In adult rats, parenteral administration of KA results in a well-defined sequence of changes leading to epileptogenesis, i.e., the creation of the epileptic state. Three phases in this process are recognized: initial status epilepticus (SE) lasting hours, a latent period of days to weeks, and finally the development of spontaneous recurrent seizures (SRS). Each phase is characterized by specific cellular and morphological changes. SE is defined as seizure activity for 30 min or longer. KA administration initially causes limbic seizures, beginning with immobility and staring, leading to unilateral limb clonus, and eventually culminating in generalized tonic–clonic convulsions. After recovery from SE, adult rats develop spontaneous brief limbic seizures over the next few weeks to months, defining a chronic epileptic state. Physiological and cellular changes underlying KA-induced epileptogenesis, not all of which are fully defined, include altered synaptic inhibition, increased excitability due to effects on ion channel and neurotransmitter function, new recurrent excitatory circuits (sprouting of hippocampal mossy fibers), and neuronal death in vulnerable hippocampal regions (dentate hilus, subfield CA3). The pattern of neuronal loss resembles that in human mesial temporal sclerosis, the pathological basis of temporal lobe epilepsy (TLE). As such, the KA model in adult rats mimics human TLE and has been used to study several aspects of the human syndrome. Of relevance here, rats treated with KA as adults develop profound cognitive and behavioral impairments in spatial learning and memory, exploration and activity level, and emotional responses to handling with heightened aggressiveness.

In contrast to the adult syndrome, KA administered to developing rats (under 3 weeks of age) causes SE but seizure-induced cell death is minimal, axonal reorganization involves the CA3 region but not mossy-fiber sprouting, and neurogenesis is decreased. While some differences in seizure semiology are seen at different ages (e.g., tonic–clonic convulsions preceded by repetitive scratching under P20, and wet dog shakes over P20), equally intense electrographic seizure activity is seen

Table 1 Comparison of kainic acid seizures in developing and adult rats

	Immature ($p < 20$)	*Adult ($p > 60$)*
Status epilepticus features	Immobility, staring → repetitive scratching → tonic–clonic activity	Immobility, staring → wet dog shakes → forelimb clonus → tonic–clonic activity with rearing, falls
Spontaneous recurrent seizures	No	Yes
Neuronal death	None	Dentate hilus, CA3, CA1
Axonal reorganization – DGL (mossy fibers), CA3 IPL	Some	Significant
Dentate granule-cell neurogenesis	Variable results: Both increases and decreases reported	Increased
Increased susceptibility to subsequent seizures	Some	Significant
Cognitive deficits	See Table 2	Significant

DGL, dentate granule layer; IPL, infrapyramidal layer.

across ages. Under 2–3 weeks of age, KA seizures do not lead to SRS, and cognitive sequelae of KA-induced SE in immature rats are not nearly as dramatic as in adult rats. Yet, these cognitive impairments, though subtle, certainly occur and are strongly age dependent. Therefore, the cognitive impairments in immature rats occur in the absence of structural or morphological changes, suggesting that channels, receptors, or neurogenesis alter network excitability enough to engender long-lasting functional changes. As mentioned earlier, other developmental epilepsy models share this feature of minimal cell death secondary to seizures.

Early studies showed that KA seizures were associated with cognitive impairments only when occurring after 3 weeks of age, about the time that excitatory glutamate neurotransmission matures. However, recent studies necessitate a revision of that concept. Not only do seizures very early in life lead to cognitive deficits, they also interrupt the ongoing processes necessary for the development of normal excitability and neurologic function.

Recently, Cornejo *et al.*, showed that even a single seizure episode induced by KA in the neonatal period (P7) caused subsequent lifelong cognitive impairment by altering synaptic plasticity. Rats undergoing KA-induced seizures on P7, and later tested on P60 in a hippocampal-dependent radial-arm water maze task, had decreased working and episodic memory, without any mossy-fiber sprouting or change in dendritic spine density. In the CA1 region, long-term potentiation was decreased and long-term depression was increased. The authors attributed the cognitive deficits to alteration in the expression of NMDA receptor subunits (NR2A) and a synaptic scaffolding protein (PSD95). These dramatic results, if confirmed, have profound implications on the mechanisms of early-life seizure consequences.

Clinically, at least 30–40% of children who experience a seizure will have another seizure. Animal models facilitate investigations of whether a prior seizure 'sensitizes' the brain to make it easier to generate a subsequent seizure (and hence, lead to cumulative cognitive risks). In one such a 2-hit model reported by Koh *et al.*, rats underwent KA seizure on both P15 and P45, or on P45 only. When tested as adults, rats that had an initial KA seizure at a young age (P15) exhibited more severe cognitive deficits in spatial learning and memory than rats undergoing a KA seizure only on P45. Presumably, the initial seizure altered network excitability significantly such that the second insult (the KA-induced seizure on P45) occurred in an already compromised brain.

Methodology

We have used the KA model to mimic partial complex seizures with secondary generalization. The consequences of KA-induced SE in adult rodents include damage to hippocampal neurons in a similar distribution as seen in TLE, the occurrence of SRS with limbic features, and distinct learning and memory deficits, especially involving hippocampal-based functions such as special learning and memory. In developing animals, KA elicits all of those consequences but they are less severe than those seen after seizures in adulthood. Therefore, research is focused on the mechanisms by which KA induces damage at each age, why glutamate is less toxic to the immature brain, and why the young brain shows some resistance to the deleterious effects of KA seizures.

The overall protocol employed by several studies using the KA model entails administration of age-specific doses of KA intraperiteonally (i.p.) to rats of various ages. Initial SE is assessed in terms of severity and duration. Later, spontaneous seizure frequency is monitored, either by visual observation or using a variety of video-EEG recording techniques. Subsequently, parameters of the SE and subsequent spontaneous seizures can be correlated with behavioral and histological analyses.

Behavior and cognition can be assessed using a variety of tests of cognition and memory, including the Morris water maze and radial-arm maze (both assess hippocampal-based spatial learning and memory), open field (measures exploratory behavior in a novel environment), handling (assesses stress responses), elevated plus maze (assesses anxiety level), etc. Each of these tests is designed to address specific questions, and is essential to apply each test appropriately.

The Morris water maze (and its variations) deserves special mention because of its common usage in laboratories studying cognitive effects of seizures. This test involves the ability of an animal to use visual cues in its environment to locate and escape onto a small platform in a pool of water. The time and swim path taken by the animal over repeated trials is recorded and compared among experimental groups. Importantly, the acquisition portion of the water maze (when a hidden platform is present) measures spatial learning and memory; the 'probe' test (with the platform removed and the rat's search pattern analyzed) measures retention of that memory. Numerous variations of the water maze have been devised to address specific cognitive questions. For example, in the recent study by Cornejo *et al.*, performance of control rats and KA-seizure rats was similar on the standard water maze and 4-trial radial-arm water maze. The authors then explored cognitive abilities of the two groups in further detail, using a variation called the 2-trial radial-arm water maze. In this version of the test, rats learn the location of a hidden platform on one trial and then are tested to see if they remember its location 4 h later in a single additional trial; the test measures episodic-like memory for a single event. In the Cornejo *et al.*, study, rats with neonatal seizures made significantly more errors in finding the platform (showing defective episodic-like memory); they also took longer to find the platform and

more often re-entered an incorrect arm (both measures of working memory). Therefore, a single episode of neonatal seizure activity was sufficient to produce robust, lifelong deficiencies in episodic and working memory, but these deficits only became apparent when a specialized testing paradigm was employed.

It is important to recognize that the water maze is not a test of general intelligence or cognitive ability, but rather, a specific measure of spatial memory and learning, which is dependent upon integrity of the hippocampus. Other tests mentioned earlier often have their own anatomical substrates. To conclude that a treatment or intervention has an effect on cognition, the investigator must consider the coordinated appraisal of multiple behavioral tests.

Results

In our recent experiments (Table 2), rats experienced an episode of KA-induced SE on various postnatal (P) days from P1 to P24, and were then tested as adults (P90–100) on several behavioral tests. On a radial-arm maze test designed to assess short-term spatial learning and memory, all groups of rats exhibited impaired performance in terms of latency to achieve criteria (learning bait location) over repeated trials (compared with age-matched controls that received saline rather than KA and did not undergo seizures). Radial-arm maze testing showed an increased number of reference errors in KA-treated rats, suggesting impaired short-term spatial memory. Similarly, rats receiving KA at the early ages and later tested as adults in a standard version of the Morris water maze took significantly longer to find the hidden platform (acquisition test) and spent less time swimming in the target quadrant (probe test) than did non-KA-treated controls. Deficits varied inversely with age of SE (worst in P24-treated rats). On the elevated plus maze, a measure of anxiety, rats with KA seizures were significantly more anxious, preferring to remain in closed arms of the maze. Finally, the open field test was used to assess locomotion and exploratory behavior; there was no difference in open field behavior between controls and KA-treated rats (KA at any age), suggesting that the differences in anxiety in the plus maze were not secondary to differences in motor activity. Therefore, these results provide additional support for the concept that seizures early in life can produce long-term impairment in certain cognitive and behavioral functions. Recent physiological studies (Sayin and Sutula, unpublished) suggest that reduced inhibition in the dentate gyrus, as measured by paired-pulse inhibition, correlates with these cognitive effects. Similarly, Lynch *et al.*, previously showed that lifelong cognitive deficits in rats treated with KA between P1 and P24 correlated with enhanced dentate gyrus paired-pulse inhibition as well as with decreased long-term potentiation, suggesting a permanent seizure-induced alteration of neuronal plasticity.

Table 2 Some cognitive deficits reported after early-life KA seizures in rats

- Impaired long-term spatial memory in Morris water maze
- Impaired short-term spatial memory in radial-arm maze
- Impaired working and episodic memory in 2-trial radial-arm water maze
- Increased aggressiveness on handling test
- Increased anxiety in elevated plus maze
- No difference in exploratory activity in open field test

Future Goals

The long-term goal of research in this field is to elucidate the mechanisms by which seizures at various ages induce long-term cognitive and behavioral damage, in order to intervene and hopefully prevent such damage. In approaching this daunting task, it is important for investigators to acknowledge the differences between seizure-induction methods and experimental protocols across laboratories, and hopefully work out a means for examining those differences. Further, the presence of critical periods during brain development and stage-specific maturational factors are clearly important and need to be elucidated. The KA model is but one of numerous seizure-induction techniques that can yield useful information about the mechanisms and consequences of seizures during development.

One area of research that could provide practical benefit in the developmental epilepsies is environmental enrichment. This idea is based on the concept that the quality and quantity of external stimulation (availability of novel, interesting objects for exploration, presence of other animals for socialization, etc.) can prevent or even reverse expected seizure-induced cognitive impairment. Enriched environments have been associated with reduced seizure damage and fewer deficits in visual spatial learning. Mechanisms for such neuroplasticity need to be determined, and could involve genetic or cellular signaling pathways in different brain regions. It will be important to establish which aspects of environmental enrichment are sufficient and which aspects are necessary for adaptive neuroplasticity. The effects of neuroprotective factors such as brain-derived neurotrophic factor (BDNF), changes in synaptic efficacy, and stabilization of neuronal structure could all be critical factors, as could alteration of specific early genes or transcription factors that can be up-regulated or down-regulated after seizures.

Limitations to the use of animal models, and of the KA model in particular, need to be acknowledged. The absence of SRS after KA is a major impediment for its usefulness as an epilepsy model in the developing brain, but this disadvantage also holds true for all the other convulsant techniques used in developing rodents. Any

single model is insufficient to explain the diversity and rich variability of developmental epilepsy. Yet, each model offers the opportunity to address specific questions, and together they can increase our understanding of the complex relationship between seizures and behavior. For example, we obviously cannot model language in animal models – even though language is one of most sensitive measures of cognitive impairment in children with epilepsy. Is there a surrogate function testable in rats?

The opportunity to prevent seizure-induced cognitive damage has widespread implications for the lives of children. The comorbidities in children with epilepsy include numerous psychological, physical, and educational impairments. We need to understand the effects of seizures on already established cognition as well as seizure-induced alteration of new learning and memory formation. It is important to recognize and address these comorbidities in addition to the seizures themselves, and to devise innovative ways in which the lives of these children can be improved. The goal of basic research is to figure out how to exploit the natural resiliency of the brain's immature neural circuitry to avoid epileptogenesis and its adverse consequences.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Cognitive Effects of Seizures in Animal Models; Cognitive Function in Temporal Lobe Epilepsy; **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood; **Psychiatric Symptoms**: Interictal Behavioral Comorbidities in a Model of Epilepsy.

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Cognitive Effects of Seizures in Animal Models

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Introduction

Epidemiological studies indicate that there is a high rate of mental retardation and learning disabilities in individuals with epilepsy. In many cases, both the epilepsy and the cognitive impairment will have a common cause, such as a metabolic disorder, congenital brain malformation, or brain trauma. However, there is also evidence that in some individuals, the epilepsy itself may cause either temporary or permanent interictal (i.e., between seizures) learning problems.

It can be argued that memory is one of the most essential higher brain functions, since it provides continuity in time, personal history, and awareness. Memory is often impaired in patients with epilepsy. Cross-sectional and longitudinal analyses of memory performance in a large series of patients with epilepsy have shown a high risk of episodic memory impairment.

The mechanisms responsible for the high incidence of memory impairment in individuals with epilepsy are not entirely clear. As with other cognitive problems, the memory impairment may be secondary to the underlying pathology responsible for the seizures. However, the temporal lobe, and particularly structures of the mesial temporal lobe, is highly susceptible to seizure-induced cell loss, and patients with temporal lobe epilepsy appear to be at particularly high risk for memory deficits. There is also evidence that in some patients, recurrent seizures can lead to progressive changes in cognitive function, most notably memory.

While seizure-induced cell loss in the hippocampus may account for some of the cognitive impairment in patients with temporal lobe epilepsy, there are indications that seizures may cause functional impairment without necessarily causing cell loss. For example, patients with idiopathic generalized epilepsy perform worse than controls on a range of memory tests, including face recognition, word recognition, verbal recall, and complex figure recall. This observation supports the contention that memory deficits in epilepsy may be due to neuronal dysfunction secondary to epileptic activity rather than due to macroscopic lesions in the temporal lobes.

Understanding the pathophysiological basis of seizure-induced cognitive deficits is critical to the development of therapies that will prevent the deficits. While the human condition clearly differs from that occurring in animals, human studies are often difficult to interpret because of the large number of variables associated with human epilepsy. Neuropsychological outcome in individuals with epilepsy may be influenced by factors such as etiology; age of onset; duration of the disorder; seizure type, frequency, and severity; concomitant disorders; genetic background; and treatment. Studies on animal models allow investigators to control many of these variables, such as age, seizure type, frequency, and antiepileptic drug therapy. Indeed, the widespread use of animal models has led to many fundamental insights into seizure-induced changes in the brain.

Background

Because of costs and convenience most investigators have used rodent models. While these models provide considerable insight into pathophysiological mechanisms, results from these studies must be interpreted cautiously for their relevance to the human condition. Subtle cognitive and behavioral changes are difficult to measure, and higher cortical function such as language and abstract thinking cannot be studied in rodents.

A large number of animal models have been employed in epilepsy research. Most animals models have been used to induce a single seizure (usually prolonged – e.g., status epilepticus) or recurrent repetitive seizures. Chemoconvulsants and electrical stimulation have been widely used in adult rodents, although recently a larger number of genetic models have been employed. Genetic models often are complex, since the animals may have structural, functional, or developmental issues beyond the seizure phenotype. In addition, there may be considerable differences among strains with regard to seizure susceptibility and outcome.

Methods

Status Epilepticus (SE) Model

Induction of seizures

SE is induced in our laboratory with the lithium–pilocarpine. Lithium chloride is given intraperitoneally 18 h before an intraperitoneal injection of pilocarpine ($34\,mg\,kg^{-1}$) to induce SE. Systemic injection of pilocarpine produces severe seizures within 30 min, progressing into SE in almost all rats. Two hours after the onset of SE, pentobarbital ($30\,mg\,kg^{-1}$) is given intraperitonally to stop the seizures. Following recovery from the SE, rats are returned to the

animal care facility for a minimum of one month before they are implanted with electrodes or undergoing behavioral testing.

Water maze

While a variety of tests can be used to assess learning and memory, one of the most popular is the Morris water maze, a measure of visually guided spatial memory. In the water maze, the animal uses visual clues to learn the location of a platform submerged below the water line in a tank of water. The primary outcome measure is the time it takes for the rat to find the platform. Although rats swim well, they would prefer to be out of the water and will seek the platform to avoid swimming.

As used in our laboratory, test animals are placed in a 2 m in diameter tank filled with water (Figure 1). Four points on the rim of the pool are designated north (N), south (S), east (E), and west (W), thus dividing the pool into four quadrants (NW, NE, SE, SW). An 8×8 cm plexiglass platform, onto which the rat can escape, is positioned in the center of one of the quadrants, 1 cm below the water surface.

Following a day of habituation where the rats are placed in the swimming tank without a platform present, rats are trained for 24 trials (six trials a day over 4 days) to locate and escape onto the submerged platform. The latency from immersion into the pool to escape onto the platform is recorded for each trial, and the observer also manually records the route taken by the rat to reach the platform. One day after completion of the last latency trial, the platform is removed and animals are placed in the water maze in the quadrant opposite to where the platform had previously been located. The path and time spent in the quadrant where the platform had been previously placed is recorded. In this part of the water maze, the probe test, normal animals typically spend more time in the quadrant where the platform had been previously located than in the other quadrants.

The Morris water maze is a test of hippocampal-dependent spatial memory, which parallels episodic memory in humans. While intact hippocampus function plays a major role in the test, lesions in other brain regions, including neocortex, retrosplenial cortex, striatum, basal forebrain and cerebellum, can alter water maze performance. The testing procedure used during the 4 days of locating the hidden platform provides a measure of both working memory and reference memory. The animal uses working memory to know which areas of the tank have been visited while trying to find the platform, and uses reference memory to know where the platform was previously placed.

Place cell recordings

In addition to behavioral tests, pathophysiological mechanisms responsible for seizure-induced memory impairment in epilepsy can be assessed using electrophysiological measures. Hippocampal place cells fire selectively in specific and restricted locations (place fields) as rodents move through open environments. Field location, size, and shape are specific to each cell and each environment, and fields cover the surface of the environment homogeneously. In normal rats, for a given environment, the place field for a given cells remains unchanged, evenbetween exposures separated by months. Hippocampal cells 'learn' to encode the salient features of experience through *N*-Methyl-D-Aspartate (NMDA) receptor-dependent synaptic plasticity mechanisms, and this rapid and persistent neuronal encoding is a crucial step toward the formation of long-term spatial memory. Since there is a strong relationship between place cells activity and the ongoing navigational behavior of rats, it is believed that such signals provide the animal with a spatial representation in order to navigate efficiently within the environment. Place cells therefore serve as powerful surrogate markers for assessing spatial memory.

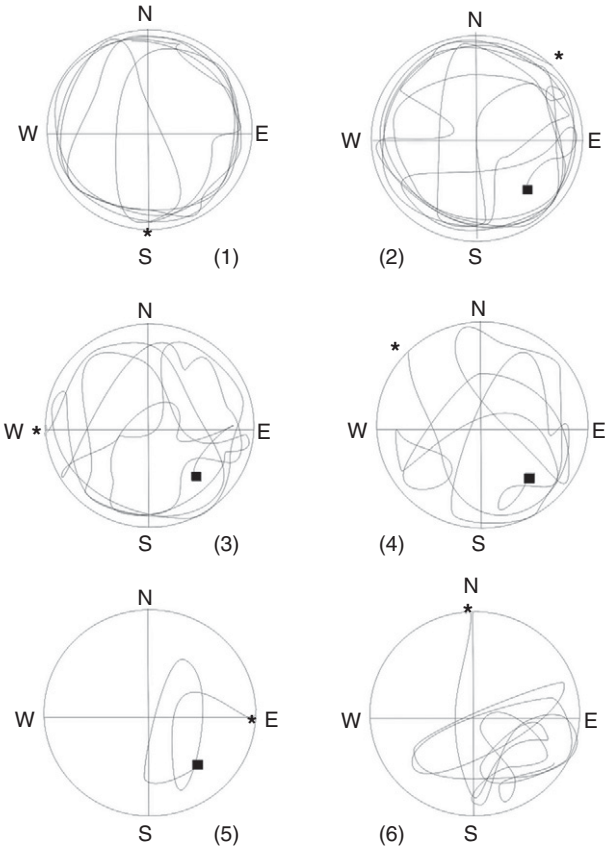


Figure 1 Example of swimming path during water maze testing. During the first day the rat is placed in the water maze without the platform present to habituate the rat to the environment. On days 2–5 the rats are placed in randomized starting spots in the water maze and the time and path taken to find the submerged platform is recorded. On the sixth day the platform is removed and the time the rat spends in the quadrant where the platform had previously been located is recorded.

We use special microdrives to place eight tetrodes (a group of four 25 µm nichrome electrode wires twisted together) in the CA1 region of hippocampus. Tetrodes allow excellent signal detection and cell discrimination, and also allow recording from several cells simultaneously. The signal coming from the headstage preamplifiers is differentially amplified 10 000 times and the acquired waveforms are sorted off-line using a computer program.

For these place cells experiments, rats are fitted with a light-emitting diode (LED) on its head, which enables rat movement (and direction) to be tracked with an overhead TV camera. Action potentials from individual cells are recorded as the animal chases food pellets randomly distributed on the floor of a cylinder 76 cm in diameter (**Figure 2**). The floor area is divided into 'pixels.' A sheet of white cardboard occupies 90° of inside arc of the cylinder and is the only polarizing stimulus. The total time the LED is detected in each pixel and the number of action potentials (spikes) fired in each pixel is accumulated. A time-averaged firing rate distribution is calculated by dividing the number of spikes in each pixel by the dwell time in that pixel. Color-coded firing rate maps are then used to visualize positional firing distributions. Pixel rates are coded in the sequence: yellow, orange, red, green, blue, and purple, in order from lowest to highest firing frequency. The firing rate is exactly zero for yellow pixels.

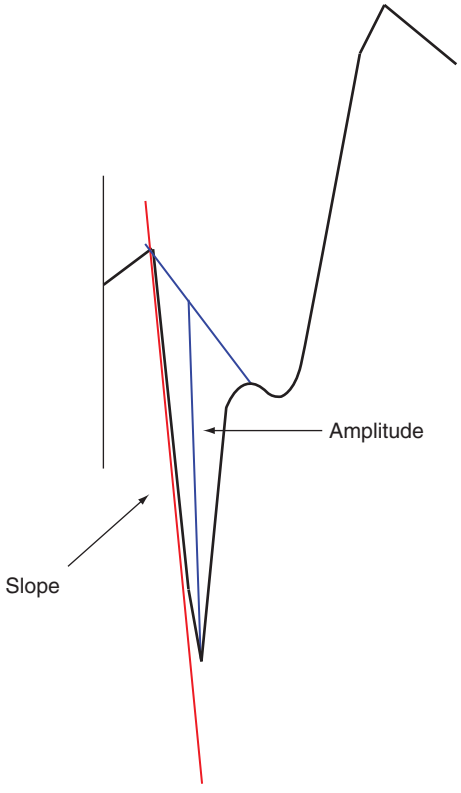


Figure 2 Example of an evoked fEPSP in CA1 following stimulation from the contralateral CA1. The slope and amplitude measurements of the initial negative deflection are shown.

For the pyramidal neurons that show location-related firing of spikes, we assess the following characteristics: (1) Coherence: This measure is a two-dimensional nearest-neighbor autocorrelation, calculated by listing the firing rate in each pixel and the average firing rate in its eight nearest neighbors. Coherence is the z-transform of the correlation between these lists and estimates the local smoothness of the positional firing pattern; (2) Firing rate: The number of spikes firing per second; (3) Information content: Information content is a measure of the amount of information (in bits) conveyed about spatial location by a single action potential emitted by a single cell; (4) Field size: Size of the place cell field in pixels; (5) AP rate: firing frequency of action potentials; and (6) Center rate: Rate of spikes firing in the center of the firing field.

Serial Seizures Model

Seizure induction

Serial seizures are induced by flurothyl, which readily induces generalized seizures following inhalation. Rats are placed in a plastic container (length = 28 cm, width = 18 cm, height = 26 cm) and liquid flurothyl (2,2,2-trifluoroethyl ether) is delivered through a plastic tube and dripped onto filter paper in the center of the chamber. Experimental rats are exposed to flurothyl until tonic extension of both the forelimbs and hindlimbs is observed. Rats are then quickly removed from the chamber and allowed to recover in room air until the animals return to their baseline activity level. After recovery, they are returned to their cages. Rats are allowed at least 2 h of rest between induced seizures. Rats received flurothyl-induced seizures at the rate of two times per day for 5 days.

Long-term potentiation

Another electrophysiological method useful in studying seizure-induced changes in memory is long-term potentiation (LTP). Since enduring spatial memory and stable place fields each depend upon synaptic plasticity mechanisms that normally rely on the same NMDA-receptor mediated events, LTP appears to be a useful measure of seizure-induced changes in plasticity.

Rats are implanted with bipolar 125 µm wires twisted together in the dorsal hippocampus. The recording electrode is implanted in the CA1 region on the left and the stimulating electrode in the homotopic region in the right stratum oriens of CA1. The stimulus and recording electrodes are positioned so that stimulation produces an extracellular field excitatory post-synaptic potential (fEPSP) which has an initial negative deflection in the recording electrode (**Figure 3**).

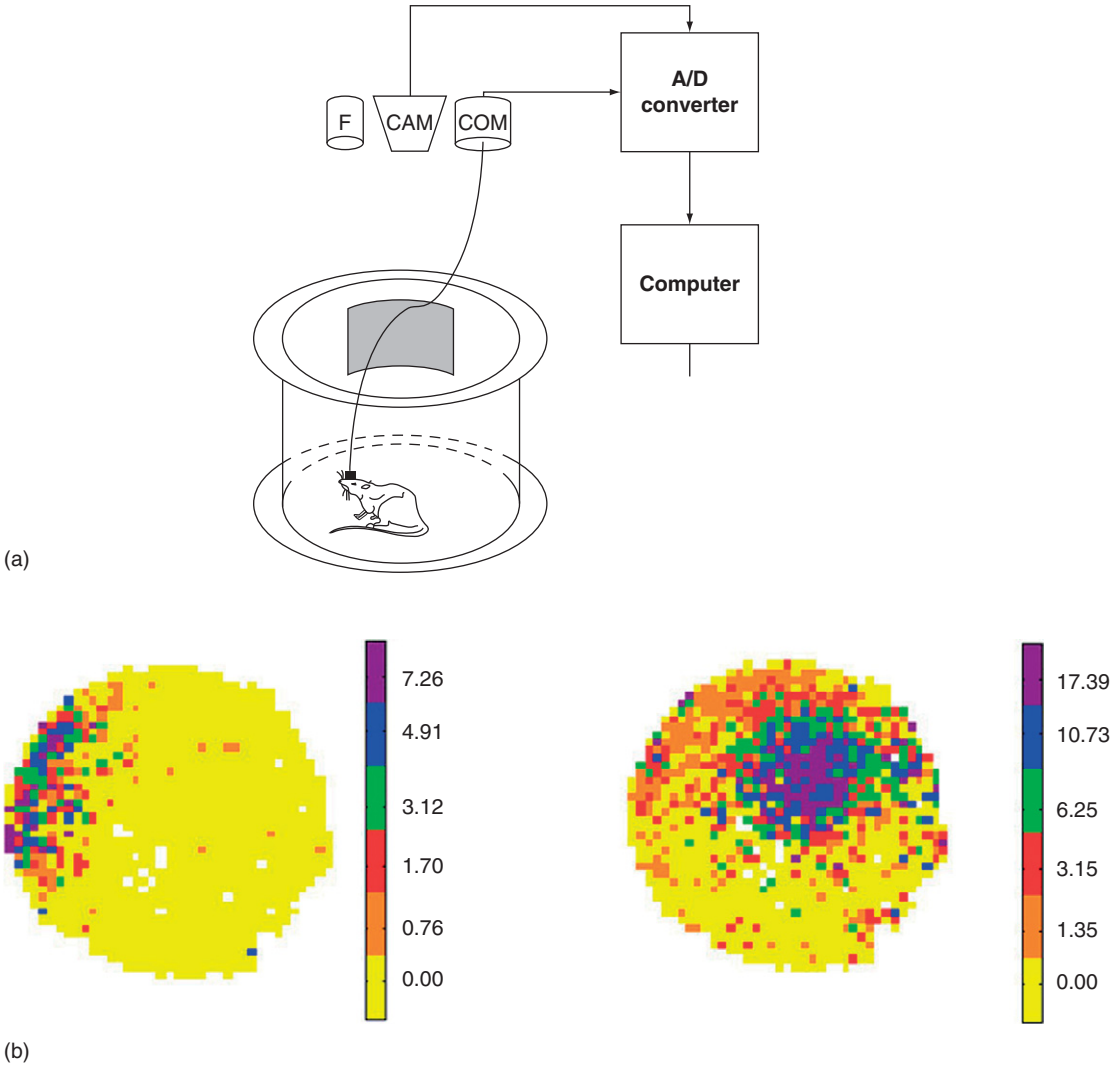


Figure 3 Example of place cell recording chamber and two place cells. (a) Rat has diode on head which allows tracking of position. An orienting card is on the wall of the cylinder. The intracranial electrodes are attached to a cable with preamplifier in place. A food pellet dispenser is attached on the ceiling along with the camera and commutator. The recording system amplifies and digitizes the analog signal and allows the recording of single cell action potentials while also recording position. Food pellets are dispensed randomly about the cylinder which causes the rat to visit all locations in the cylinder. (b) Color-coded firing rate maps were used to visualize firing distributions. Pixel rates were coded in the sequence: yellow, orange, red, green, blue and purple. The firing rate was exactly zero for yellow pixels. Unvisited pixels in the cylinder and pixels outside the cylinder were coded white. In the left hand figure a place cell at approximately 11:00 is shown. In the right hand figure a place cell in the middle of the field was seen.

One week after surgery to implant electrodes, recordings of evoked fEPSPs are initiated. Stimuli consist of 0.2 ms monopolar pulses with an intensity starting at 40–50 μA; intensity is gradually increased until an evoked response is obtained. The threshold intensity is then defined as the lowest stimulus intensity that can evoke a detectable and reproducible excitatory post-synaptic response. An input/output curve is developed to show the change in the output of the neuronal population (EPSP amplitude and slope) over a range of input intensities. Prior to LTP, rats are stimulated every 20 s at an intensity 1.5X greater than threshold, for a minimum of 30 min or until a stable response is obtained. LTP is then induced with a 200-Hz high frequency train (pulsewidth of 0.2 ms, 1 s train duration, stimulus intensity of 1.2X the single-pulse threshold). Following the LTP-inducing stimulus train, responses to single stimuli (delivered using the same parameters as used prior to LTP) are obtained at 5, 10, 20, 30, 60, 90, 120, and 180 min. Synaptic transmission efficacy is assessed by measuring the fEPSP slope and population spikes (PS) amplitude from averaged waveforms before and after the tetanic stimulation (Figure 3). An average of all of the slope and amplitude measurements recorded before the tetanic stimulation is used as the baseline value.

Recent Results

Status Epilepticus Model

Water maze

As found in other studies, rats subjected to SE had significantly impaired performance in the water maze. Both reference and working memory are impaired in these studies. The increased latency to the escape platform was not related to motor deficits or swimming speed.

Place cell recordings

The SE group differed strikingly according to 'coherence' which measures the local smoothness of spatial firing pattern. Numerically, the mean coherence for control place cells of 0.81 ± 0.04 was reliably higher than the mean of 0.50 ± 0.06 for the place cells from the SE rats. Thus, firing field organization is weaker in adult rats subjected to SE earlier in development (in prepubescence). Furthermore, this weaker organization of the spatial firing pattern is not secondary to other firing pattern defects (such as decreased rates or major changes in the positional distribution of place cell discharge) as measured by field area or information content. On the assumption that place cell activity is essential to normal navigational behavior, the decreased coherence helps account for the poorer water maze performance shown by SE rats.

We also asked if the positional firing patterns of the place cells from the SE rats were stable over time. To this end, we ran four recording sessions (within a 7-h period) for each control cell and for each cell from SE rats. Place cells in the control rats appeared stable; their fields remained in approximately the same position and retained their shapes across the four recording sessions. In marked contrast, the positional firing patterns of SE rats often changed considerably over time. These findings indicate that the positional firing patterns for the place cells from the SE rats drift away from their initial states in a time-dependent fashion. The reduced stability of the place cells from the SE rats at short intervals, and the progressive decay of stability at longer intervals, provide additional bases for explaining observed deficits in complex spatial tasks. Thus, adult rats that experienced SE during pubescence had defective place cells.

Serial Seizures Model

LTP

Following 10 flurothyl-induced seizures in our rats, LTP could not be elicited. The impairment in LTP appeared to be 'dose-dependent'; following two flurothyl-induced seizures, the rats had normal LTP.

Water maze

The rats undergoing serial seizures had increased latencies to the platform compared with the controls. The probe test also showed a significant difference between groups, with the serial seizure rats showing less swimming time in the target quadrant than the non-seizure controls.

Place cell recordings

Place cells were recorded 1 day prior to flurothyl-induced seizures, 24 h following the tenth seizure, and 72–128 h following the last flurothyl-induced seizure. All of the rats were behaving normally when they were studied. There were significant decreases in coherence, information content, and center firing rate. When the rats were studied 72 h after the last flurothyl seizure, there was a return of place cell firing patterns to baseline values, except for reduced coherence values. Stability was assessed numerically by computing change in the location of place field center with serial recordings. Stability was substantially impaired following the tenth flurothyl seizure. These changes persisted when the animals were studied >72 h following the last seizures.

Future Goals

Our major goal in understanding the role of seizures in cognitive dysfunction is to determine the precise reasons by which seizures cause cognitive impairment. Clearly, seizures can result in cognitive impairment as measured behaviorally and electrophysiologically (e.g., as seen in single cell place cell recordings and field recordings). While these studies demonstrate that seizures can impair brain function, the remaining challenge is to determine why such impairment occurs. A myriad of changes occur in the brain following seizures. Determining which of these changes result in seizure-induced cognitive impairment is the next step.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; **Pediatric Epilepsy**: Developmental Aspects of Seizures; **Plasticity**: Seizure-Induced Alterations in Motor and Sensory Maps.

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Cognitive Function in Temporal Lobe Epilepsy

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Introduction

Of the epilepsies, complex partial seizures are particularly common, with the majority originating in the temporal lobes. There is special interest in this patient group, as they represent a large cohort of persons with surgically curable epilepsies. As such, they have been very carefully studied with advances made in characterizing the neurobehavioral characteristics of localization-related syndromes of temporal lobe origin. The most prominent syndrome is *mesial temporal lobe epilepsy* (MTLE). This disorder is among the most common form of epilepsy and among the most refractory to medical treatment (often intractable by adolescence). Its defining features include an early age of onset (primarily in the first decade), underlying hippocampal sclerosis reflected on magnetic resonance imaging (MRI) as unilateral hippocampal atrophy, and a history not uncommonly including complicated febrile convulsions or other early etiological insults. Other defining symptoms (e.g., clinical semiology, interictal/ictal EEG characteristics) associated with MTLE have been described. Two other localization-related epilepsies of temporal lobe origin have also been described, and include so-called idiopathic, cryptogenic, or MRI-negative temporal lobe epilepsy (here called *MRI-negative TLE*), and lesional (e.g., tumor, stroke) epilepsy of temporal lobe origin.

Background

The most prominent cognitive deficit in MTLE occurs in memory function; this is, due to the effects of the primary epileptogenic lesion. However, it has become apparent that cognitive impairments may often be evident in other cognitive domains, abnormalities that cannot be explained by the primary temporal lobe disturbance. The etiology underlying this distributed cognitive effect is an interesting issue. A long-standing literature has shown associations between clinical seizure features and disrupted cognitive function. Seizure-related contributors to cognitive impairment in chronic temporal lobe epilepsy, as well as in other types of epilepsy, include the underlying etiology of the disorder, adverse effects of medications, seizure frequency and severity, medical complications such as status epilepticus, rate and type of EEG abnormalities, and other factors.

However, recent advances in neuroimaging have provided new perspectives. Alterations in brain metabolism demonstrated by FDG-PET often appear in lateral and mesial temporal lobe, and may also extend to other areas including thalamus, basal ganglia, and frontal and parietal lobes. This associated hypometabolism has been linked to abnormalities in cognition and behavior. Specifically, thalamic hypometabolism has been shown to be associated with memory impairment, and frontal hypometabolism associated with impairment in higher order cognitive abilities and mood disorder.

Especially interesting are quantitative MRI findings. Initial quantitative MRI studies identified atrophy of the hippocampus, followed by reports of abnormalities in related structures including the enthorhinal cortex, fornix, parahippocampal gyrus, amygdala, basal ganglia, and thalamus. Additionally, more distant temporal and extratemporal lobe regions have been implicated, including the

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temporal pole, frontal lobe, and cerebellum. Most recently, abnormalities in cerebral white matter have been reported, including the corpus callosum and distributed regions within the temporal, parietal, and frontal regions, both ipsilateral and contralateral to the affected temporal lobe. In the context of these findings, it is not surprising that reduction of whole brain volume has been reported. With few exceptions, most volumetric investigations have examined a single or limited number of neural structures – as opposed to characterizing a broader and more diverse range of regions and their relative degree of atrophy. Preliminary investigations that do make such comparisons have helped to characterize the degree of abnormality that exists both within and outside the epileptogenic hippocampus (**Figure 1**).

Also striking are recent investigations of cortical thickness, demonstrating diffuse thinning of the ipsilateral and contralateral cortical mantle in unilateral temporal lobe epilepsy (**Figure 2**), as well as diffuse abnormalities in cortical complexity in unilateral temporal lobe epilepsy.

In the studies to be reviewed here, we begin by characterizing and comparing the degree of cognitive pathology in patients with temporal lobe epilepsy to healthy controls, in order to determine the nature and range of cognitive dysfunction both within and outside memory function. It was our hypothesis that given a mean cognitive profile of greater than expected neuropsychological impairment in a group of persons with well-defined temporal lobe epilepsy, there might be distinct subgroups of patients with specific patterns of cognitive impairment (and sparing), and that these cognitive phenotypes would have distinct clinical seizure and MRI volumetric correlates, with varying prospective cognitive courses.

Methods

Our research cohort consisted of 96 patients with well-defined chronic temporal lobe epilepsy and 82 healthy controls, aged 18–60. All research participants underwent comprehensive cognitive assessment (intelligence, language, perception, learning and memory, executive function, psychomotor abilities) and quantitative MR volumetrics of total and lobar gray and white matter volumes. Cognitive performance in the epilepsy subjects was compared to that in the healthy controls after adjustment for age, education, and gender. Volumetric measurements were adjusted for age and total intracranial volume, as is the custom. The cognitive data were later subjected to cluster analysis, and identified groups were related to adjusted volumetric measurements of total hippocampal volume and gray and white matter volumes. Finally, subjects were reassessed after 4 years, and test–retest cognitive trajectories in the epilepsy participants were analyzed via standardized regression-based norms for change.

Results

Patients with chronic temporal lobe epilepsy were compared with healthy controls (**Figure 3**). Impairments were evident in memory function, but this deficit occurred in the context of a cognitive profile notable for generalized cognitive dysfunction, with significant differences between the control and temporal lobe epilepsy patients across all cognitive domains – findings that are inconsistent with the neuropsychological consequences of exclusively hippocampal pathology.

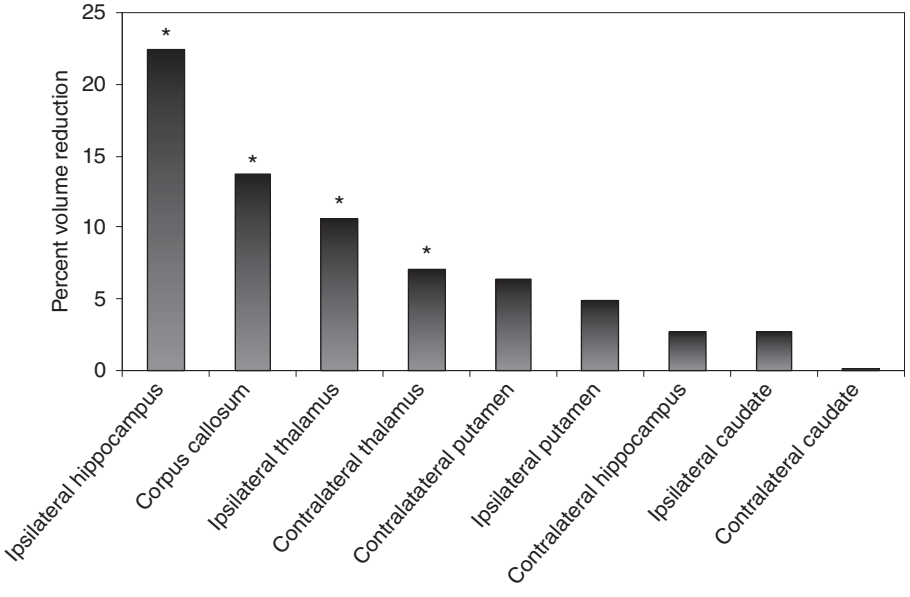


Figure 1 Degree of atrophy across diverse regions both ipsilateral and contralateral to side of temporal lobe seizure onset (Pulsipher *et al.*, 2007).

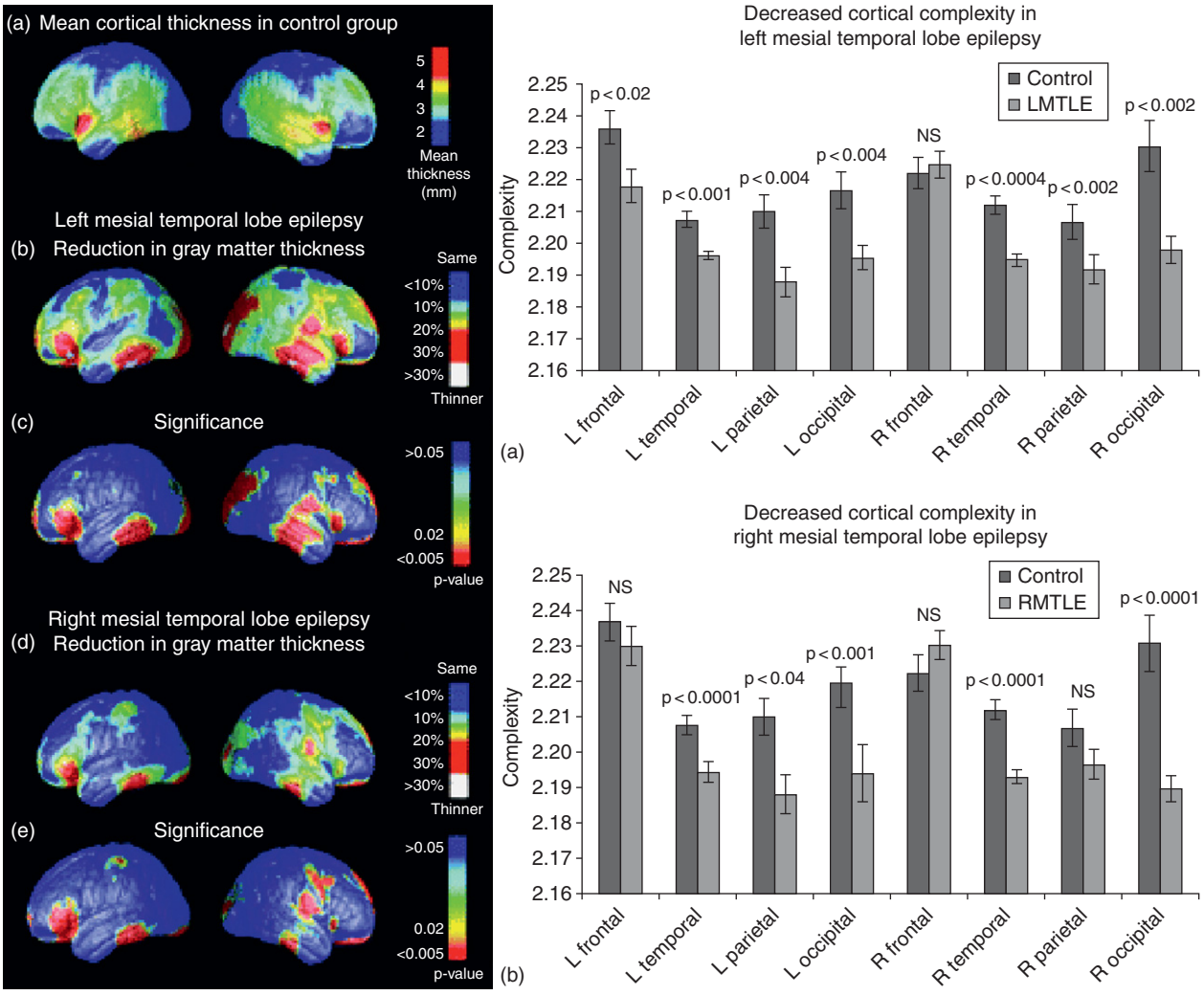


Figure 2 Patterns of cortical thinning in unilateral mesial temporal lobe epilepsy. Lin JJ, Salamon N, Lee AD, *et al*. (2007) Reduced neocortical thickness and complexity mapped in mesial temporal lobe epilepsy with hippocampal sclerosis. *Cerebral Cortex* 17: 2007–2018.

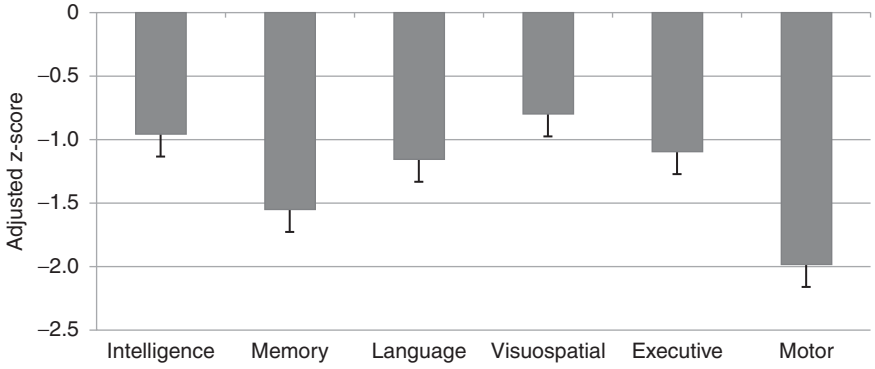


Figure 3 Age, gender, and education corrected *z* scores for patients with temporal lobe epilepsy (solid bars) compared with controls ($z = 0$) (Oyegbile, 2004).

Modal descriptions of patients with epilepsy syndromes are helpful in characterizing general patterns of cognitive performance, but they do not give a sense of the interesting heterogeneity that can be seen within and across epilepsy syndromes. Our experience had been that among patients with ictally confirmed unilateral mesial onset of spontaneous seizures – in whom there was a pathologically confirmed hippocampal sclerosis – there was considerable heterogeneity in individual cognitive profiles (in the context of the above mean cognitive profile). These observations suggested that specific cognitive phenotypes might be represented in such patient groups.

A yet untapped approach to understanding cognitive morbidity in epilepsy is taxonomic in nature. This approach involves addressing the question of whether empirically derived groupings of *patients* with similar profiles of cognitive function can be identified, either within or across epilepsy syndromes. Taxonomies facilitate reliable clustering of individuals into meaningful groups, provide a common language and organizing influence in the field, and set the stage for further investigation of clinical and neurobiological correlates. To date, taxonomic approaches have rarely been used to advance understanding of the neurobehavioral complications of the epilepsies. Toward that goal, rather than grouping patients on the basis of clinical seizure characteristics (e.g., seizure frequency) and examining the relationships of individual clinical seizure characteristics to cognition, one would derive a grouping of patients based solely on their pattern of performance across several cognitive domains and then look for potential electrical or imaging correlates.

We applied cluster analysis to a large sample of patients with temporal lobe epilepsy and identified distinct cognitive subgroups or phenotypes. From a neuropsychological perspective, three distinct cognitive profiles were uncovered (**Figure 4**); Cluster 1 was minimally impaired, Cluster 2 was memory impaired, and Cluster 3 was more diffusely affected – with memory, executive, and psychomotor speed impairments. These distinct groups were then compared across demographic factors, clinical seizure characteristics, quantitative MRI volumetric variables, and cognitive course. A brief description of the cognitive phenotypes is presented here.

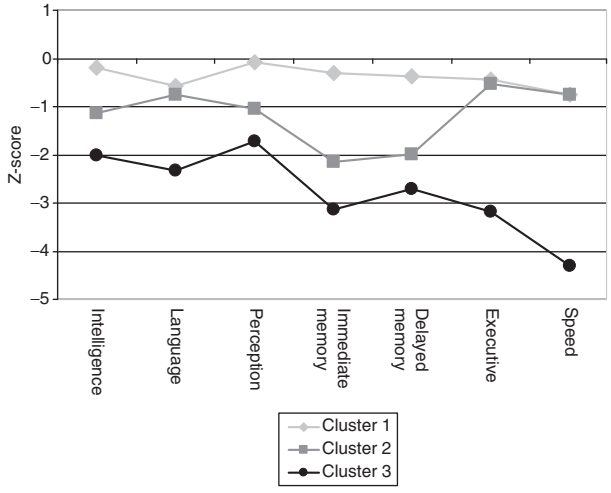


Figure 4 Cognitive profiles in temporal lobe epilepsy. Reproduced from Hermann *et al.*, 2007, with permission of Cambridge University Press.

Cluster 1 (*minimally impaired*) consisted of approximately half (47%) the temporal lobe epilepsy subjects who exhibited the most intact cognition of the three cluster groups. That said, their performance was significantly worse than controls across several cognitive domains, including language, immediate and delayed memory, executive function, and psychomotor speed domains. While statistically significant, the pattern was one of very mild but discernable cognitive dysfunction. These patients were clearly distinct from the other epilepsy subjects, and closest to the healthy controls in terms of cognitive status.

Cluster 2 subjects (*predominantly memory impaired*) consisted of 24% of the patient sample, who exhibited marked impairments in immediate and delayed memory – in the context of significantly poorer performance than controls across all other cognitive domains. That is, memory was the most striking cognitive abnormality, but it occurred in the context of a mild generalized depression of overall cognition compared to controls.

Cluster 3 (*generalized impairment*) consisted of 29% of the temporal lobe epilepsy subjects. They exhibited the poorest cognition across all domains compared with controls, and also demonstrated significantly poorer performance across all cognitive domains compared with both Clusters 1 and 2. The most striking impairments in this group fell in the areas of executive function and cognitive/psychomotor speed. This group exhibited an especially prominent degree of impairment in executive function impairment, which has been an interesting finding in both adults and children with temporal lobe epilepsy.

Thus, an underlying taxonomy characterized by the nature, pattern, and severity of evident cognitive complications can be identified. The clinical correlates of these cognitive phenotypes was then examined by examination of profiles of demographic features (age), clinical seizure features (duration of epilepsy, AED polytherapy), and brain volumetrics (segmented whole brain and lobar tissue volumes, CSF, and hippocampus). In summary, the volumetric findings (**Figure 5**) paralleled the cognitive results. The most intact group (Cluster 1) showed significant hippocampal atrophy with minimal change in other morphometric measurements. As the degree of cognitive impairment increased (Cluster 2–3), there was a pattern of corresponding increase in volumetric abnormality, including greater hippocampal atrophy and culminating in Cluster 3 where there was evidence of widespread volumetric abnormalities. These relationships were examined largely in terms of whole brain volumes (cerebral gray,

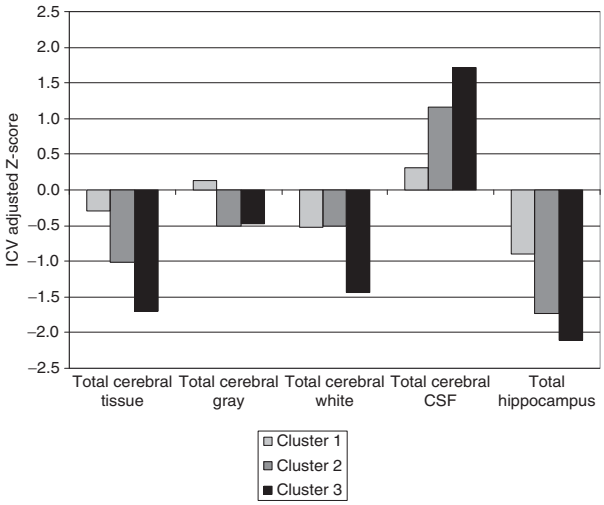


Figure 5 MRI volumetrics in cognitive profile groups. Reproduced from Hermann *et al*., 2007, with permission of Cambridge University Press.

white, and CSF volumes). In the future, we plan to examine more specific relationships, such as those between frontal lobe volumes and tests of executive function.

In addition, the most cognitively impaired group (Cluster 3) was older, had the longest duration of epilepsy, and took more medications than the other groups (especially Cluster 1). There were also meaningful but statistically nonsignificant trends with regard to other clinical seizure features. Cluster 3 had the highest proportions of patients with histories of >50 lifetime generalized tonic–clonic seizures, status epilepticus, and severe initial precipitating injuries. Thus, this appears to be a group that is most likely to have incurred both an earlier neurodevelopmental insult and a more protracted and severe course of epilepsy.

Of considerable interest is the degree to which abnormalities in mental status may progress in the face of an increasingly chronic (longer duration) disorder. The issue of cognitive progression in temporal lobe epilepsy is important, as curative surgical treatments exist but are frequently delayed. Patients with medication-resistant temporal lobe epilepsy often present with considerable cognitive and behavioral handicap when finally referred for surgical consideration – unfortunately, sometimes after decades of unsuccessful medical management.

Prospective cognitive studies of patients with epilepsy date back to the early part of the last century, but these studies are often characterized by rather limited assessment of cognition (often just IQ), the inclusion of mixed seizure types, varying test–retest intervals, lack of control groups, and other methodological shortcomings. More common are cross-sectional studies, which, while informative, suffer from the obvious limitation of providing an indirect evaluation of neuropsychological change over time, cohort effects, and other methodological problems that prevent a clear and unequivocal characterization of the cognitive course of epilepsy. A recent review concluded that progressive cognitive decline does occur in a proportion of patients, and appears to be associated with markers of a difficult epilepsy course (e.g., number of lifetime generalized tonic–clonic seizures).

Progressive changes in cognitive function, especially memory, have been demonstrated in careful prospective investigations by the group in Bonn (Germany). We also found in our sample that over a 4-year prospective period, an abnormal cognitive trajectory was exhibited in approximately 20–25% of patients. A poorer cognitive course was associated with a variety of factors, including demographic features (older age), clinical seizure features (longer duration of epilepsy), MR variables (baseline volumetric abnormalities), and cognitive findings (lower baseline intelligence).

But just as modal cognitive profiles harbor interesting subgroups (as demonstrated in the results of the cluster analysis described earlier), so is the case when considering prospective cognitive change. If we examine prospective cognitive course as a function of our cognitive phenotypes, meaningful differences in their prospective cognitive trajectory can be seen across the cognitive phenotype groups.

As shown in **Figure 6**, the most abnormal cognitive trajectory was observed in Cluster 3 subjects; that is, those who were the most impaired at baseline appeared to be quite vulnerable to ongoing cognitive change. In contrast, the most optimal cognitive course was observed in Cluster 1 subjects – those who were most intact at baseline.

Conclusion and Future Goals

There is a remarkable degree of heterogeneity in the cognitive profiles of patients with a discrete syndrome of epilepsy. This heterogeneity is only just beginning to be characterized and understood. The average cognitive profile of these patients is interesting in that it suggests that despite a relatively focal zone of seizure onset, there can be a remarkably diverse range of cognitive difficulties. These cognitive phenotypes can be identified, and they appear to reflect clear differences in the degree of underlying structural abnormality (as revealed by quantitative volumetrics) as well as clinical seizure features.

Not discussed here, but important, are abnormalities evident in other neuroimaging modalities (voxel-based morphometrry, diffusion tensor imaging and tractography, fMRI), which also demonstrate interesting patterns associated with cognitive abnormality and cognitive reorganization.

Specific aspects of underlying volumetric abnormalites in chronic unilateral temporal lobe epilepsy are likely to contribute to the pattern of generalized cognitive impairment described previously. There is developing evidence

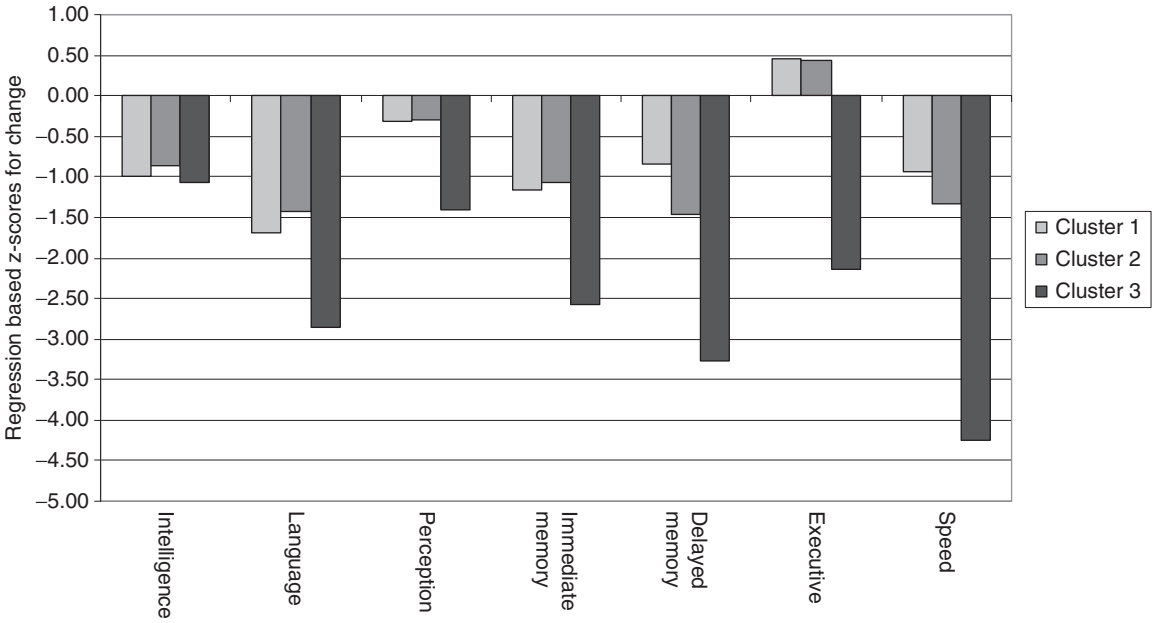


Figure 6 Prospective cognitive trajectories in cognitive phenotype groups. Down going bars represented poorer obtained test performances compared with expected test performances. Reproduced from Hermann *et al*., 2007, with permission of Cambridge University Press.

of specific structure–function relationships linking abnormalities in cognitive processing speed with reduction of total cerebral white matter volume, abnormal procedural memory (classical eyeblink conditioning) with cerebellar atrophy, abnormal confrontation naming with hippocampal atrophy, and impaired aspects of semantic memory with atrophy of the temporal pole. Overall, the image of this *focal* brain disorder is changing, with linkages between distributed abnormalities in brain structure and cognition just beginning to be understood.

In the future, similar approaches could be taken with other forms of localization-related (frontal lobe epilepsy) or idiopathic generalized epilepsies (absence epilepsy). It would prove interesting to identify both shared features (e.g., generally intact cognition) as well as syndrome-specific cognitive patterns (e.g., memory impairment in TLE, executive impairment in frontal lobe epilepsy, attentional disruption in absence epilepsy) and their relative distributions across epilepsy syndromes. These findings would facilitate genotype–phenotype investigations as applied to higher cognitive function and brain structure, as has been undertaken in other disorders.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Language and Communication Disorders; **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; **Plasticity**: Seizure-Induced Alterations in Motor and Sensory Maps; **Surgery**: Approaches for Individually Optimized Surgical Intervention.

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Epilepsy and Autism in Tuberous Sclerosis

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Introduction

Autism spectrum disorders are a heterogeneous group of neurodevelopmental disorders characterized by impairment in three domains: deficits in verbal and nonverbal communication, impairments in reciprocal social interaction, and the presence of restricted, repetitive behaviors or interests. The term 'autism' is used throughout this chapter to refer to the broader spectrum of autistic disorders, including Asperger's syndrome and atypical autism, with classic autism lying at the more severe end of the spectrum. Autism is associated with significant long-term impairment for affected individuals, and prevalence rates have increased with better recognition of the wider spectrum of more subtle symptoms. Approximately, 10–15% of cases of autism are associated with known medical conditions and 5–10% of those with autism have genetic and chromosomal disorders. The remaining cases are idiopathic with unknown etiology. However, genetic factors appear to be of significant importance, with 3–7% recurrence risk in families of individuals with autism and with twin data strongly suggesting the involvement of multiple interacting genes.

While current evidence robustly points at a genetic etiology for autism, there is also emerging evidence that nongenetic factors may play an equally important etiological role. Autism is clearly recognized as a neurobiological disorder, in part due to early recognition of the

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While current evidence robustly points at a genetic etiology for autism, there is also emerging evidence that nongenetic factors may play an equally important etiological role. Autism is clearly recognized as a neurobiological disorder, in part due to early recognition of the

association with clinical seizures and abnormalities in the EEG. Epilepsy is the most highly prevalent medical comorbidity with autism. Depending on the population studied, rates of epilepsy in autism range from 11% to 39%, with the highest prevalence being reported by studies of adolescents and young adults, those with moderate to severe learning disabilities, and those with severe receptive language disorders. The likelihood of developing epilepsy in autism increases with age, with a peak incidence before 5 years and in adolescence. Children with autism seem more likely to have partial onset epilepsy than primary generalized epilepsy, and the multiple seizure types seen in association with autism can often be treatment-refractory. The frequency of abnormal (epileptiform) discharges on EEG has been found to be almost twice as high as the frequency of clinical epilepsy, with a wide range of rates reported from 15% to as high as 68%. The abnormalities most reported are focal or multifocal spiking, over the temporal region or more commonly over the frontal areas. Although there are relatively few data on the rates of autism in children with epilepsy, studies suggest that up to one-third of children with epilepsy are at risk of having autism, with the risk highest amongst those with early onset of seizures.

Despite this strong association, the relationship between epilepsy or epileptiform EEG abnormalities and autism is poorly understood. It is possible that epilepsy and autism are separate manifestations of a dysfunctional brain, most likely stemming from genetic vulnerability. It may also be that epileptiform abnormalities interfere with early brain development, thus potentially contributing to cognitive, language, and behavioral dysfunction of an autistic type. The association of both conditions with mental retardation makes the relationship even more complex. There are a number of genetic disorders associated with both autism and epilepsy, including Tuberous Sclerosis, fragile X syndrome, and Rett syndrome. However, it is tuberous sclerosis complex (TSC) that, due to the high rates of mental retardation, autism and epilepsy with which it is associated, offers a unique neurogenetic model for investigating the relationship and testing theories about the brain basis of autism.

Background

TSC is a multisystem autosomal dominant genetic disorder, characterized by widespread tumor-like malformations (hamartomas) of many organs, including the brain, skin, kidney, eyes, and heart. Hamartomas are benign 'tumors' that resemble the tissue that is ordinarily found in the location; however, the tissue is disorganized and malformed and consists of poorly differentiated and immature cells. Mutations which give rise to the disorder occur in the TSC1 gene in 20% of cases and in the TSC2 gene in 80% of cases. The mutations are spontaneous in ~60% of cases while the remaining cases are familial. Individuals with TSC2 mutations may have a more severe form of tuberous sclerosis and more adverse developmental outcomes than individuals with TSC1 mutations. The TSC1 gene is located on chromosome 9q34 and codes for the protein hamartin. The TSC2 gene is on Ch 16p13.3 and codes for the protein tuberin. The complex formed by these proteins acts in the insulin signaling pathway to regulate mTOR and hence S6 Kinase; mutations in TSC1 or TSC2 lead to abnormalities of the tuberin/hamartin complex, dysregulation of mTOR, and possibly other pathways with the final effect being abnormalities in cell growth, differentiation, and migration.

The prevalence of TSC in children is around 1 in 10 000–20 000. Autistic behaviors were first described in TSC in 1932, and recent epidemiological studies of children with TSC have found rates of autism between 43% and 86%, thus demonstrating that not all children with TSC develop autism. There is some evidence that the prevalence of autism may be higher in those with TSC2 mutations compared with individuals with TSC1 mutations. This difference may reflect the increased severity of TSC in those with TSC2 mutations. More severe intellectual impairment also appears to occur in children with TSC2 mutations. The distribution of intelligence quotient (IQ) seems to be bimodal with one peak in the severely impaired range and another in the low average range. The risk of mental retardation is increased in children with early seizure onset, intractable seizures, and infantile spasms. Mental retardation is a correlated feature with autism in TSC, although some individuals of normal intelligence may develop autism or Asperger's syndrome. Approximately, 1–4% of children with autism have TSC.

Neuropathological manifestations of TSC include cortical tubers, subependymal nodules, and in ~10% of cases, subependymal giant cell astrocytomas. Cortical tubers form during fetal life and vary in number and location, but with most individuals having several. They appear to act as foci for epileptic discharges, producing multiple types of difficult to control, often multifocal, seizures.

The majority of patients with TSC will present in childhood and usually with seizures. Epilepsy is a common feature in TSC, with rates of 60–90%. However, not all individuals with TSC develop epilepsy or come to medical attention. Therefore, the true incidence of epilepsy in TSC is unknown. Of those who do develop epilepsy, the most frequent seizure types are infantile spasms and/or partial seizures. Earlier seizure onset is correlated with increasing numbers of cortical tubers. Up to 69% of patients with TSC present with infantile spasms, often in the first year of life and in most cases

between 4 and 5 months of age. TSC is associated with 25% of all cases of infantile spasms. Infantile spasms and associated EEG abnormalities may be atypical in TSC. They are often preceded by partial seizures, although the latter can be missed in the first few months. The majority of children with TSC who have infantile spasms at onset eventually develop partial seizures (partial motor, complex partial) with secondary generalization. Focal or multifocal epileptiform abnormalities on EEG may be found before the onset of seizures and between seizures. Hypsarrthymia on EEG (often with focal features) may also develop. The treatment of epilepsy in TSC is often difficult, with seizures frequently being intractable despite treatment with many different antiepileptic medications. Poor prognostic signs include multiple seizure types, seizure onset before 1 year, and multifocal EEG abnormalities. Children with TSC and treatment-refractory epilepsy have undergone resective surgery of cortical tubers with variable outcomes.

Methodological Considerations

Although the early studies have provided many important leads and produced some fundamental insights, various methodological challenges and shortcomings have led to some inconsistencies and uncertainties. For example, due to the rarity of TSC, studies have almost inevitably involved small sample sizes, with all the concomitant limitations that stem from this point. Moreover, most of the samples have been based on clinic populations, where possible ascertainment biases may be operating. In addition, data have frequently been collected retrospectively and therefore may be subject to various biases. As such, data suitable for rigorous multivariate analysis, aimed at delineating developmental causal pathways from genetic mutation to neurological, cognitive and behavioral outcomes, are lacking or only just emerging.

Several challenges are also evident in terms of the need to develop suitable standardized measures and protocols for various important manifestations of the disease. For example, the recording and categorization of seizures and the type of epilepsy represents one challenge; similar difficulties can arise when attempting to standardize and interpret EEG recordings. Many similar considerations apply to the identification and measurement of the cerebral changes seen in TSC. New MR imaging techniques that are optimized for tuber identification and mapping are emerging, but there are as yet no agreed 'gold standard' protocols. To fully exploit the potential of any prospective population based studies, there is a need to develop agreed protocols, classification systems, and minimum standards for the collection of high quality epilepsy data, EEG recordings, autism assessments, psychometrics, and neuroimaging.

Results – The Unique Relationship between Autism and Epilepsy in TSC

Autism may develop in individuals with TSC1 or TSC2 mutations, although the risk in individuals with TSC1 mutations appears to be lower. It may be that the risk is also dependent on the nature of the mutation, although this possibility has yet to be established. There is also evidence for nongenetic determinants of development outcomes in TSC. Such evidence, for example, has come from case reports of monozygotic twins in whom quite different developmental outcomes were found despite their identical genetic abnormalities and background. The nongenetic influences may reflect unique environmental effects and/or the random nature of second hit events that give rise to the hamartomatous lesions/cortical tubers.

Different explanations for the pathogenesis of autism in TSC have been postulated. It may be that abnormalities in a specific key brain system (e.g., the social brain network or the mirror neuron system) may give rise to the risk for autism. Alternatively, abnormalities within several different brain systems may be required (the multiple primary deficit hypothesis). According to this latter hypothesis, abnormalities in the social brain system might give rise to social impairments, whereas abnormalities in the frontal regions could underlie the stereotyped and repetitive behaviors. Abnormalities in these brain systems could derive from tuber-related anatomical abnormalities or from the adverse effects of epilepsy on brain development. Lastly, widespread abnormalities in 'connectivity' stemming from the effects of seizures on synaptic development and dendritic arborization may create the increased risk.

Due to the presence of easily identifiable cortical lesions in TSC, investigators have carried out studies in patients with autism and TSC to try to correlate the neurodevelopmental disorder with the location of tubers. One of the first clinical series of cases investigating the distribution of cortical tubers in TSC reported a strong association between the presence of temporal lobe tubers and the development of autism. In this study of 18 TSC patients, the number of tubers in the group overall were greatest in the frontal lobes, followed by the parietal, temporal, cerebellar, and occipital lobes. However eight of the nine patients with autism had temporal lobe tubers, whereas none of those without autism had tubers in this region. In the autistic patients, the lateralization of the temporal tubers was on the left for three patients, on the right for four, with bilateral temporal tubers for the remaining two. There was a history of epilepsy in eight of the autistic and in seven of the nonautistic participants. Overall, the number of tubers was significantly greater in patients with mental retardation. This study provided the first clear evidence between localized, gross structural abnormalities in the brain and the development of autism.

Testable hypotheses for similar studies could be offered by a number of neuropsychological theories of autism. One of these more influential theories was evaluated in a retrospective case study investigating neuroepileptic risk factors of autism in individuals with TSC. The theory proposes that autism is a specific cognitive disorder of 'mind-blindness,' whereby the social impairments associated with autism are due to an inability to read other people's minds. The study hypothesized that such impairment is the end result of a dysfunctional neural network that involved ventral frontal cortex, the amygdala, and temporal lobes. The 'mind-blindness' theory proposes that the inability to follow another person's eye gaze is a core deficit of autism. The processing of information from faces – such as recognition of faces, facial expressions of emotion, and the direction of another person's eye gaze – are recognized to be key functions of the temporal lobes and amygdala within this neural network. Further, the function of detecting another's direction of gaze has been shown to be related to the superior temporal gyrus.

Results from this retrospective case study confirmed that a diagnosis of autism was associated with the presence of cortical tubers in the temporal but not other lobes of the brain. The presence of cortical tubers appeared to be a necessary factor for the development of autism in TSC. However, it was noted that not all individuals with temporal lobe tubers developed autism; therefore, temporal lobe tubers might be necessary but not sufficient for the development of the neurobiological disorder. Contrary to the hypothesis that tubers located in the temporal superior gyrus would be associated with autism, neither the number nor the location of temporal lobe tubers was significantly associated with the development of autism spectrum disorder in this study. Therefore, it was proposed that epileptiform activity in the temporal lobes, rather than the structural abnormalities represented by the tubers, may be the risk factor for the development of autism. Further analysis of the retrospective case study data revealed that outcome was associated with various indices of epileptic activity, including evidence of temporal lobe epileptiform discharges on EEG, the age of seizure onset in the first 3 years of life, and a history of infantile spasms. The study concluded that children with tuberous sclerosis are, therefore, more likely to develop autism when temporal lobe tubers are present and associated with temporal lobe epileptiform discharges as well as early-onset, persistent spasm-like seizures.

These findings support the hypothesis that the developmental timing of seizures influences outcome. In particular, temporal lobe epileptiform activity occurring early in life, at an early critical period, may perturb the normal development of key social cognitive functions within the temporal lobes and finally leads to autism. The key risk period was identified in the first year of life when social behaviors begin to emerge and develop. The risk of developing autism was decreased if seizures began in the second year of life, consistent with the evidence that epilepsy syndromes which begin after 18–24 months are not associated with an increased risk of autism. Idiopathic autism rarely occurs after 36 months. Although a history of infantile spasms was found to be associated with autism, not all children with spasm-like seizures developed autism and not all children with autism suffer infantile spasms. Therefore, infantile spasms appear to be a marker for liability rather than a risk factor. Further analysis in the retrospective case study showed that the risk of developing autism associated with infantile spasms was no longer evident when age to seizure onset and temporal lobe epileptic foci were accounted for.

There is evidence indicating a temporal association between epilepsy and regression of development. Similar evidence has been reported in cases studies of autistic regression in patients with TSC and with either seizures or EEG abnormalities. One such report described a male with TSC at 6 months of age. Before the onset of seizures but after detection of an abnormal EEG, the child was treated with vigabatrin (at 6 months of age) to prevent the onset of seizures. In order to avoid possible visual field defects, vigabatrin was discontinued at 13 months of age. At 21 months, the child developed partial seizures with secondary generalization, as well as infantile spasms. Cognitive and social development were normal until 21 months of age and the onset of seizures, but following the onset of seizures regressed developmentally. The child met the criteria for autism and mental retardation when assessed at 24 months. Such case studies indicate that the onset of epilepsy during an early stage in brain development can be associated with autistic regression and pervasive developmental disorder.

A temporal association between onset of epilepsy and regression in language has also been found in children with regression after a period or normal or near – normal development. In Landau–Kleffner syndrome (rapid loss or previously normal language skills in association with either seizures or epileptiform abnormalities on EEG), some investigators have found that successful treatment of EEG abnormalities may improves the language deficits. An earlier age of onset of seizures has the worst outcome. Recent work has demonstrated some favorable long-term effects of vigabatrin on overall cognitive function, epilepsy, and autistic behavior in children with infantile spasms, with and without TSC. The cessation of spasms with vigabatrin was associated with significant improvement of cognition and behavior in children with TSC; controlling infantile spasms seemed to be a key factor for mental development. Similar results have been reported in children without TSC. Recently, however, vigabatrin has been linked with visual field defects which may limit its use.

Taken together, these lines of evidence support the notion that temporal lobe epileptiform discharges are most strongly associated with autism in TSC when occurring during an early critical period of social development in the brain. However, a causative relationship of epilepsy to autism is yet to be established and the findings are inconsistent, with data from some studies being interpreted as supporting the multiple primary deficit hypothesis or as suggesting abnormalities within the cerebellum as primary. Methodological differences probably underlie some of the inconsistencies in findings.

Future Directions

Most of the evidence to date stems from retrospective studies of clinic populations of children with autism, with or without TSC. As such, findings may have been biased in one way or another. Prospective, longitudinal studies are therefore needed to find out whether early seizures occurring during a critical time of development, specifically increases the risk of developing autism in children. There is also a need to understand whether the risk for developing autism depends on the type and severity of seizures as well as on treatment response. Such an investigation may lead to a better understanding of clinical prognostic indicators and of the neurobiological pathways leading to autism.

To address the above questions, a prospective longitudinal study of children with TSC was planned and has commenced. The study (TS2000 Study) sample comprises a population-based sample of newly diagnosed individuals with TSC, diagnosed in the UK between 2001 and 2006. Systematic longitudinal data are being collected on the natural history, prognostic indicators, and the influence of genotype on developmental outcome. Following diagnosis and recruitment children are evaluated using standardized instruments and their development is monitored and assessed at regular intervals. The aims of the TS2000 Study include determining to what extent the natural history (in terms of intellectual, cognitive, behavioral, and neurological impairments) is related to genotype, gender, mode of presentation; the number, distribution, and type of brain lesions; and the type, severity, and course of seizures in the early years, including response to treatment. Prospective studies that follow infants who are diagnosed with TSC before the onset of seizures are also underway and will be particularly informative. Such longitudinal studies with repeated testing at regular intervals should contribute towards identifying the sequence of unfolding events, thereby clarifying the causal chain.

Treatment studies and trials of antiepileptic medications in TSC could potentially be very informative. The few medication trials to date are small pilot studies highlighting the need for large-scale better randomized control designs. It will be important to identify whether treatment efficacy is associated with the development of autism – which could lead to a change in prescribing guidelines and a better outcome for affected children. Intervention research studies will also be required to elucidate the value of neurosurgical interventions.

Future directions also include further clarification of genotype and phenotype correlates, in relation to the genotype/phenotype analysis of autistic behaviors in TSC. Studies to date have demonstrated that mental retardation is more highly associated with TSC2 mutations. More studies are required to identify whether this association also applies to autism and therefore whether less severe presentations of autistic spectrum disorders might be associated with TSC1 mutations.

Molecular studies of TSC have identified a cascade whereby hamartin and tuberin form a protein complex which inhibits mTOR (mammalian target of rapamycin); abnormalities in this pathway, resulting in higher mTOR activity, are thought to lead to increased cell proliferation and growth. The identification of this pathway has lead to early clinical trials of mTOR inhibitors (e.g., rapamycin) in the treatment of TSC. Early evidence suggests that some of the brain lesions may shrink with drug treatment, and these initial studies are now being followed up with randomized controlled clinical trials – the results of which are eagerly awaited, to determine whether these drugs can improve other outcomes such as seizure severity, intellectual disability, and autistic behavior.

Finally, the high rates of autism in TSC highlight the importance of evaluating these TSC children for autistic behaviors. In fact, higher rates have been reported for autism than for the cardiac or renal abnormalities (for which children with TSC are routinely screened). In order to identify children with autism and the associated long-term impairment, improved assessment procedures for this unique population are required.

See also: **Cognitive Dysfunction and Other Comorbidities**: The Relationship of Epilepsy to Other Conditions/Disorders; **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis.

Further Reading

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Language and Communication Disorders

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Introduction

Language, a fundamental component of social behavior, is a tool for communicating thoughts and feelings. The development of children's linguistic skills extends from the toddler period through adolescence. In parallel, there is a protracted maturation of brain regions involved in language. The extensive nature of this period increases the vulnerability of this developmental process. In addition, the development of language parallels the prolonged, age-related changes in children's behavior, cognition, and social skills. Language, therefore, provides a gateway to studying illness effects not only on children's language itself, but also the child's intelligence, social skills, emotions, behavior, and brain development. As such, it is essential to understand the functional implications of a developmental disorder like epilepsy on language development.

My laboratory has studied how the development of children's use of language, to organize and formulate their thoughts, goes awry in pediatric neurobehavioral disorders such as epilepsy. To this end, we have used clinical psychiatric measures of 'thought disorder.' These higher level linguistic or discourse deficits involve impaired integration of language, cognition, and social behavior. Studies of thought disorder in pediatric epilepsy are important for three main reasons. First, from the neurological perspective, they provide information on how ongoing seizures and the underlying neuropathology impact the development of children's higher level linguistic skills. Second, from the psychiatric perspective, they offer an understanding of the functional implications of higher level linguistic impairments on behavior and emotions in children with epilepsy. Third, from the theoretical perspective, use of a developmental model and neuroimaging techniques in these studies advance our understanding of how epilepsy impacts development.

Background

Thought disorder involves impairment in the form or manner in which a speaker presents thoughts to a listener and is characterized by clinical signs such as illogical thinking, loose associations, incoherence, poverty of content of speech, tangentiality, circumstantiality, and others. Thought disorder represents abnormal organization, control, and processing of thinking; improper use of semantic and relational aspects of speech; and poor use of linguistic context to process and produce speech. Although regarded as a hallmark of schizophrenia, thought disorder (a normal developmental phenomenon in young children) is also found in pediatric neurobehavioral disorders including attention deficit hyperactivity disorder (ADHD), high functioning autism, complex partial seizures (CPS), and childhood absence epilepsy (CAE).

Prior evidence for schizophrenia-like psychosis and thought disorder in adults with temporal lobe epilepsy (particularly left temporal lobe epilepsy) led us to study thought disorder in children with temporal lobe epilepsy. The study was begun to identify brain substrates involved in pediatric thought disorder, such as those seen in childhood schizophrenia. Being conducted on children with cryptogenic epilepsy with CPS and focal EEG findings in the temporal and frontal regions, the study included control groups of children with CAE and normal children. We initially predicted that we would find thought disorder in

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Language and Communication Disorders

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Introduction

Language, a fundamental component of social behavior, is a tool for communicating thoughts and feelings. The development of children's linguistic skills extends from the toddler period through adolescence. In parallel, there is a protracted maturation of brain regions involved in language. The extensive nature of this period increases the vulnerability of this developmental process. In addition, the development of language parallels the prolonged, age-related changes in children's behavior, cognition, and social skills. Language, therefore, provides a gateway to studying illness effects not only on children's language itself, but also the child's intelligence, social skills, emotions, behavior, and brain development. As such, it is essential to understand the functional implications of a developmental disorder like epilepsy on language development.

My laboratory has studied how the development of children's use of language, to organize and formulate their thoughts, goes awry in pediatric neurobehavioral disorders such as epilepsy. To this end, we have used clinical psychiatric measures of 'thought disorder.' These higher level linguistic or discourse deficits involve impaired integration of language, cognition, and social behavior. Studies of thought disorder in pediatric epilepsy are important for three main reasons. First, from the neurological perspective, they provide information on how ongoing seizures and the underlying neuropathology impact the development of children's higher level linguistic skills. Second, from the psychiatric perspective, they offer an understanding of the functional implications of higher level linguistic impairments on behavior and emotions in children with epilepsy. Third, from the theoretical perspective, use of a developmental model and neuroimaging techniques in these studies advance our understanding of how epilepsy impacts development.

Background

Thought disorder involves impairment in the form or manner in which a speaker presents thoughts to a listener and is characterized by clinical signs such as illogical thinking, loose associations, incoherence, poverty of content of speech, tangentiality, circumstantiality, and others. Thought disorder represents abnormal organization, control, and processing of thinking; improper use of semantic and relational aspects of speech; and poor use of linguistic context to process and produce speech. Although regarded as a hallmark of schizophrenia, thought disorder (a normal developmental phenomenon in young children) is also found in pediatric neurobehavioral disorders including attention deficit hyperactivity disorder (ADHD), high functioning autism, complex partial seizures (CPS), and childhood absence epilepsy (CAE).

Prior evidence for schizophrenia-like psychosis and thought disorder in adults with temporal lobe epilepsy (particularly left temporal lobe epilepsy) led us to study thought disorder in children with temporal lobe epilepsy. The study was begun to identify brain substrates involved in pediatric thought disorder, such as those seen in childhood schizophrenia. Being conducted on children with cryptogenic epilepsy with CPS and focal EEG findings in the temporal and frontal regions, the study included control groups of children with CAE and normal children. We initially predicted that we would find thought disorder in

the CPS children with left temporal involvement and/or psychotic symptoms (such as hallucinations and delusions) and not in the control groups. However, this study and subsequent studies on larger samples of children demonstrated that, in contrast to our predictions, both children with CPS and those with CAE had thought disorder, irrespective of their psychiatric diagnosis, compared to normal children. In fact, there were no significant differences in the thought disorder of the CPS and CAE groups. Furthermore, evidence for thought disorder in young normal children, and a significantly greater degree of thought disorder in younger CPS, CAE, schizophrenic, ADHD, and high functioning autistic children than in older children with these diagnoses, implied that onset of these abnormalities during active development of children's higher level discourse skills might impair the acquisition of these skills.

These findings underscored the importance of using a developmental model to determine if thought disorder in children with epilepsy reflected effects of on-going seizures, the underlying neuropathology, or both – on the development of children's discourse skills. From the clinical perspective, our results also highlighted the need to determine the functional implications of discourse deficits in children with epilepsy. More specifically, how did these impairments impact or reflect every day life – in terms of the cognitive/academic, behavioral/emotional, and social skills of these children?

Methods

Subjects

These studies included 93 CPS and 56 CAE children, aged 5.1–16.3 years with IQ scores of 70 and higher; 117 normal children were also studied. CPS subjects were diagnosed with cryptogenic epilepsy (as defined by the International Classification of Epilepsy) and had experienced at least one seizure during the year prior to the child's participation in the study. Children with a clinical history of CPS, but no EEG evidence for focal epileptic activity, were also included in the study sample. The study inclusion criteria for each CAE subject were: a diagnosis of CAE, absence seizures induced by hyperventilation, at least one seizure in the year prior to participation in the study, and EEG evidence of 3 Hz spike and wave. We excluded patients with a mixed seizure disorder, previous epilepsy surgery, atypical spike and wave complexes, juvenile myoclonic epilepsy, generalized tonic clonic seizures, a structural MRI abnormality (but not those with mesial temporal sclerosis), a neurological illness other than epilepsy, chronic medical illness, imaging evidence for structural brain abnormalities, a metabolic disorder, a hearing disorder, mental retardation based on school/classroom placement, and bilingual speakers of American English who did not attend English speaking schools or speak English at home.

To include control children from a wide range of ethnic and socioeconomic status backgrounds similar to those of the CPS and CAE groups, we recruited the normal control subjects from four public and two private schools in the Los Angeles community. We excluded children with current or past diagnoses of neurologic, psychiatric, language, and hearing disorders.

Procedures

Thought Disorder

We assessed the four types of thought disorder measures described in children with schizophrenia and presented in Figure 1. The first type, formal thought disorder, represents impaired organization of thoughts and includes illogical thinking (e.g., failure to present the listener with appropriate reasoning in causal and noncausal utterances or contradiction) and loose associations (e.g., unpredictable change in the topic of conversation without preparing the listener for the topic change). The second type, cohesive deficits, involves poor use of linguistic ties, such as conjunctions and referential cohesion that link ideas across sentences. The third and fourth types, deficits in self-initiated repair and revision, respectively, involve impaired use of strategies to correct on-line errors during conversation that occur at the level of the organization of ideas or of the linguistic ties across sentences, word choice, or syntax. As a result of poor organization of ideas, impaired use of cohesive devices, and poor on-line correction of communication breakdown, the listener has difficulty following the reasoning, topic maintenance, ideational contiguity between sentences, and to whom or what the child is referring.

Two raters with no knowledge of the child's neurological diagnoses code videotapes of the Story Game with the Kiddie Formal Thought Disorder Rating Scale (K-FTDS) as we described in detail in previous publications (see Further Readings). During the Story Game, the child hears an audiotaped story about a ghost or an ostracized boy and is presented with structured open-ended questions following each story. The child also chooses to tell a story about a good child, a bad child, the Incredible Hulk, or a witch. The K-FTDS scores are frequency counts of illogical thinking and loose association ratings divided by the number of sentences (clauses) made by the child. Kappa for illogical thinking and the generalizability coefficient for loose associations were 0.75 (SD = 0.15) and 0.66 (SD = 0.01), respectively, in a subsample of 39 epilepsy and healthy subjects.

The cohesion scores, measuring the frequency with which the child used linguistic ties that connect ideas and referents across sentences, include conjunctions,

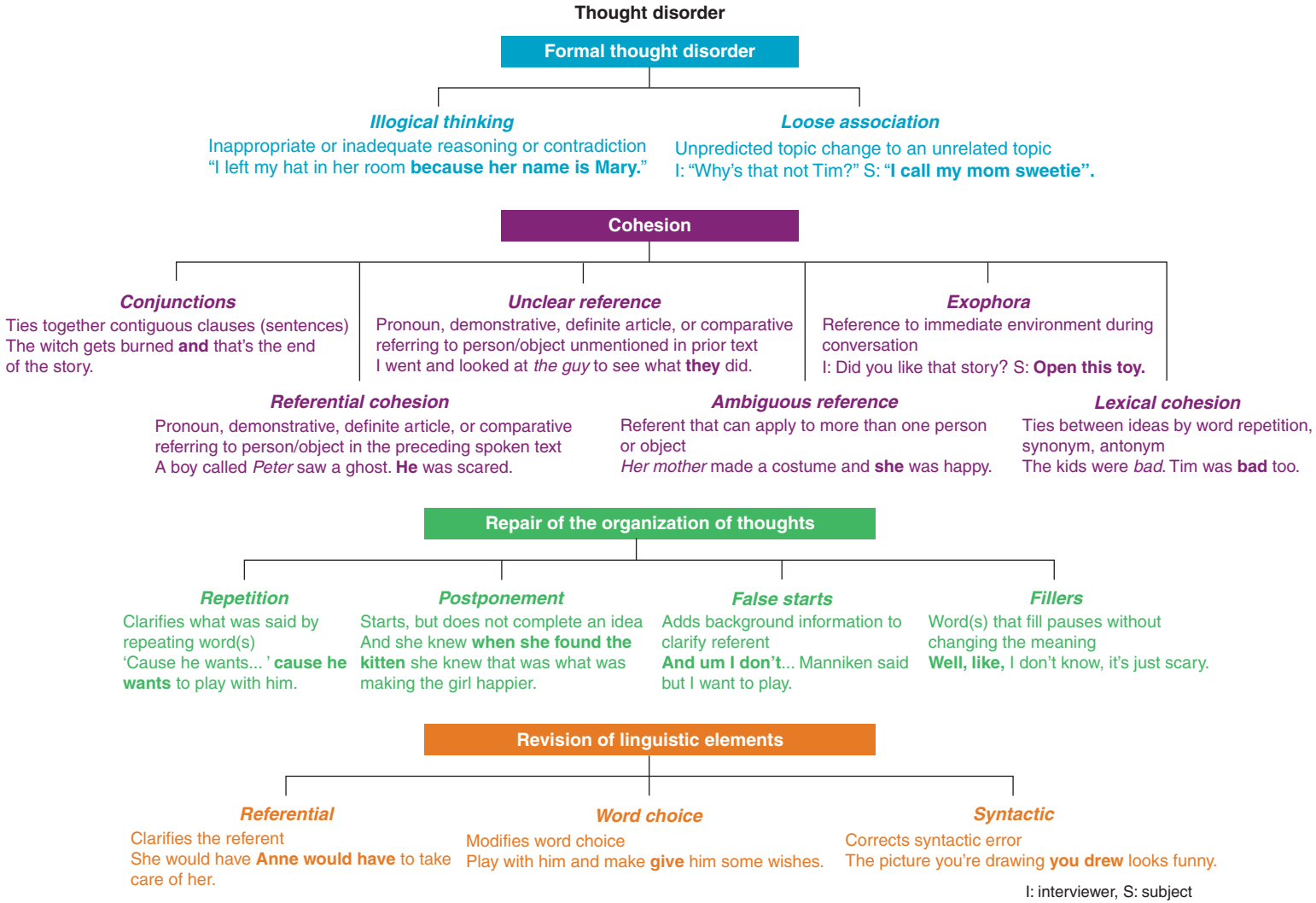


Figure 1 Definition and examples of thought disorder.

referential cohesion, lexical cohesion, ellipsis, unclear reference, ambiguous reference, and exophora (i.e., reference to the immediate environment during conversation). The inter-rater agreement (intraclass correlation) of these scores among a subsample of 31 children was 0.97 for conjunction, 0.96 for referential cohesion, 0.97 for lexical cohesion, 0.99 for ellipsis, 0.99 for unclear/ambiguous reference, and 0.99 for exophora.

The repair scores are frequency measures of how often the child corrects errors in the organization of thoughts (e.g., fillers, false starts, repetition) or linguistic errors (e.g., syntax, semantics, reference) while speaking. Inter-rater reliability (intraclass correlation) for the repair scores was 0.92 in a subsample of 45 children.

Psychopathology

A structured psychiatric interview, the *Schedule for Affective Disorders and Schizophrenia for School-Age Children, Epidemiologic Version (K-SADS-E)* and the *Present and Lifetime Version* (K-SADS-PL*)* was administered separately to each child and parent to determine the presence/absence and type of DSM-IV diagnoses, as detailed previously (see Further Readings). Parents completed the 118 behavior problem items of the *Child Behavior Checklist* (CBCL).

Cognition

The *Wechsler Intelligence Scale for Children – Revised* (WISC-R), and the *Wechsler Intelligence Scale for Children – 3rd edition* (WISC-III), were administered to children to generate Full Scale, Verbal, and Performance IQ scores (see Further Readings).

Imaging

All subjects underwent brain magnetic resonance imaging (MRI) scans in a 1.5-T magnet. Tissue was segmented and the total brain, frontal lobe parcellation (i.e., subregions), and temporal lobe volumes were computed. Using a discourse coherence activation paradigm, previously validated in both normal adults and normal children, children listened to short question–answer dialogs and indicated by key presses whether the answers made sense. Unknown to the subjects, features critical to making this decision were varied so as to challenge either reasoning or topic maintenance. Data were acquired on a Siemens 1.5-T Allegra scanner using an EPI gradient echo sequence (TR = 3000 ms, TE = 25 ms, flip angle = 90°, matrix size 64 × 64, FOV = 20 cm).

Results

The thought disorder in children with CPS and CAE with average intelligence includes: illogical thinking; under-use of cohesive devices that link ideas, people, events, and objects across sentences; and infrequent online monitoring and self repair of errors in the organization of thinking and in the use of syntax, word choice, and reference (**Figure 1**). The younger CPS and CAE children have significantly more thought disorders than the older children with these neurological diagnoses. However, earlier onset of CPS, a history of prolonged or febrile seizures, as well as localization and lateralization of epileptic activity are all related to thought disorder in CPS children. In contrast, the thought disorder in CAE is associated with age, seizure frequency, Verbal IQ, and the type of antiepileptic drug (e.g., valproic acid > ethosuximide). These findings suggest that although CPS and CAE both affect the normal maturation of children's discourse skills, they might to do so through different mechanisms.

From a functional perspective, thought disorder in the CPS group was related to psychopathology, school problems, decreased academic achievement, and poor peer interaction; in the CAE group, thought disorder was related only to school problems. More specifically, children with CPS, with ADHD, and high CBCL externalizing behavior scores had the highest thought disorder scores, lower IQ scores, and poor social skills. Thus, thought disorder appears to be a developmental disability or comorbidity of pediatric CPS and CAE that involves essential areas of the children's functioning.

In CPS children, the association of thought disorder with ADHD, externalizing behaviors, and poor social skills might have important implications for the 'stigma' associated with epilepsy. Children who have difficulty using language to formulate and organize their thoughts are not competent social communicators. Perceiving them as 'different,' their peers might not want to play or interact with them. Thus, children's thought disorder or social communication deficits might underlie certain aspects of the stigma or peer rejection experienced by children with epilepsy. The presence of externalizing behaviors associated with ADHD as well as poor peer interaction and poor academic achievement might further contribute to this perception and to the social difficulties of these children. Thus, certain aspects of the behavior, social, and academic achievement difficulties of children with epilepsy, particularly those with CPS, might reflect their thought disorder-related social communication deficits.

Recent imaging findings demonstrate that the thought disorder of children with CPS is significantly related to smaller orbital frontal and inferior frontal gray matter volumes, increased Heschl's gyrus gray matter volumes, and smaller superior temporal gyrus white matter volumes. These brain regions are associated with language and, like language, undergo protracted maturation. The association of thought disorder with volumes in these brain regions and the relationship of both thought disorder and these

volumes to the age of seizure onset, history of prolonged seizures, and lateralization of EEG in our studies provide additional support for our conclusion that in CPS, thought disorder might represent a developmental disability involving fronto-temporal regions involved in language.

Our fMRI findings (during a discourse coherence paradigm) show that normal children – compared to CPS children – have significantly increased cortical activity in the left inferior frontal gyrus when they use reasoning to make sense of the on-going conversation and in the right temporal region when they assess topic maintenance. Thus, even though the normal and CPS children are performing the task accurately, and are not told how to determine if the conversation makes sense or not, the CPS group engages the inferior frontal gyrus and the right temporal region significantly less than normal children. It remains to be determined, however, if our structural and functional imaging findings represent the effects of on-going seizures or the underlying neuropathology (or both) on the development of these brain regions.

Future Directions

Given the behavioral, cognitive, academic, and social implications of having thought disorder in children with epilepsy, our future studies will examine the change over time in the thought disorder measures of children with CPS and CAE – and how these changes might be related to changes in seizure variables, IQ, comorbid psychopathology, social competence, and brain volumes. We will also use MRI and diffusion tensor imaging to further our understanding of the association of thought disorder with brain function and the structure of white matter, respectively, to identify the neural substrates of thought disorder in these children.

Due to evidence for thought disorder in the families of schizophrenic patients, we are also examining thought disorder in the siblings of children with CPS and CAE. Finally, treatment studies are needed to ascertain the appropriate timing and method (i.e., speech and language, psychopharmcological, and educational treatment) of intervention for thought disorder and related functional impairments in CPS and CAE.

see also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Cognitive Function in Temporal Lobe Epilepsy; Epilepsy and Autism in Tuberous Sclerosis.

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The Relationship of Epilepsy to Other Conditions/Disorders

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Introduction

Autism is a neurodevelopmental disorder with onset before 3 years of age, characterized by impairments of reciprocal social interactions, verbal and nonverbal communication, and markedly restricted repertoire of activities and interests. The reported seizure frequency in autistic individuals ranges between 5% and 39%, and the reported prevalence of epileptiform electroencephalography (EEG) abnormalities in children with autism range from 8% to 60.8%. The prevalence of autism in children with infantile spasms is also higher compared to the general population. Autistic regression, a pattern of autistic development observed in 25–30% of cases wherein affected children initially appeared to have normal development (including speech) but then lose skills either social or language or both, has been reported in children with epileptic encephalopathies. The high prevalence of epilepsy in autism (and vice versa) suggests the existence of a common mechanistic basis (and perhaps a causal relationship) between the two.

Background

Serotonin in Autism and Epilepsy

The initial interest in the role of serotonin in autism arose from the demonstration of hyperserotonemia in autistic children, a result that has been replicated and extended, with the recognition that blood serotonin is also elevated in the first degree relatives of autistic children. Serotonin, in addition to functioning as a neurotransmitter, has an important role in brain development. Serotonergic fibers innervate Cajal–Retzius cells (CRC), which are necessary for cortical column development. Furthermore, in experimental studies, investigators found that treatment with the serotonin agonist 5-methoxytryptamine during fetal development led to altered brain reelin levels and abnormal presubicular cortical column development. This observation is consistent with the findings of abnormalities of reelin levels and cortical minicolumns in human autism autopsy brain tissue.

The presence of peripheral blood hyperserotonemia in autism led to investigations of the central serotonergic pathway, by measuring cerebrospinal fluid (CSF) 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of serotonin. These studies, however, did not show consistent findings. To further explore the role of the central serotonergic pathway in autism, the serotonin synthesis capacity in autistic children was evaluated in vivo with positron emission tomography (PET), using the tryptophan analog α[^{11}C]methyl-L-tryptophan (AMT) as a tracer. For nonautistic children, serotonin synthesis capacity was >200% of adult values until the age of 5 years, and then declined toward adult values. In autistic children, serotonin synthesis capacity increased gradually between the ages of 2 and 11 years to values 1.5 times the adult normal values. In addition, unilateral alterations in the serotonin synthesis in the dentatothalamocortical pathway were noted. These data suggest that humans undergo a period of high brain serotonin synthesis during early childhood, and this developmental process is disrupted in autistic children, with some brain regions more severely affected than others. In a study involving a larger group of autistic children, it was shown that subgroups of autistic children can be defined by the presence or absence, and side of cortical asymmetry, in AMT abnormalities; these subgroups differed in terms of language and handedness.

Serotonin and Epileptogenesis

The role of serotonin in epileptogenesis has been demonstrated in both animal and human models. Abnormalities in brain serotonin concentration have been reported in genetically epilepsy prone rat (GEPR). Furthermore, serotonin and selective serotonin reuptake inhibitors have anticonvulsant properties. Investigators have demonstrated that monoamine oxidase A (MAO_A) knockout (KO) mice are more susceptible to seizures, but show reduced epileptogenesis. In human epileptic cortex, high levels of serotonin and 5-HIAA has been noted in spiking regions of the temporal neocortex as compared to nonspiking regions. Serotonergic hyperinnervation of dysplastic human epileptogenic cortex has been described. Using AMT PET scanning, investigators demonstrated (in vivo) increased AMT uptake in epileptic foci as indicated by ictal EEG. These results may indicate increased tryptophan metabolism by either the serotonin or kynurenine pathway.

Considering that serotonergic abnormalities are associated with abnormalities of cortical development and epileptogenesis, it may represent one of the common disturbances in autism and epilepsy and hence may

explain the common association of the two disorders. The association between reelin deficiency (as described earlier for autism) with human temporal lobe epilepsy and lissencephaly reinforces this view of potentially common underlying mechanisms. Reelin dysfunction can lead to neuronal migration disorders that are subsequently often associated with epileptic seizures. An inverse correlation between reelin expression and the degree of granule cell dispersion, in human epileptic hippocampal tissue, has been shown. Similarly, neuronal migration defects have been seen in reelin deficient mice.

Methods

Methods for the studies described here are elucidated in papers included in the Further Reading section.

Results

Rett Syndrome

Rett syndrome (RTT) is a neurodevelopmental disorder primarily affecting females. The syndrome consists of autism, dementia, ataxia, loss of purposeful hand use, and acquired microcephaly that presents after a period of apparent normal development of 6–18 months. The majority of cases are caused by mutations in the X-linked gene encoding methyl CpG binding protein, MeCP2. Mutations in another X-linked gene, cyclin-dependent kinsase-like 5 (CDKL5), have been found in female patients characterized by early-onset encephalopathy and early development of seizures. The phenotype of children with CDKL5 gene mutation overlaps with that of the severe form of atypical RTT or the early seizure variant of RTT (Hanefeld variant) which is characterized by early development of seizures, particularly spasms, with EEG findings of hypsarrhythmia and developmental regression. CDKL5 has highest expression in the brain, thymus, and testes, and is expressed early in development. Investigators have demonstrated that CDKL5 expression in the nervous system overlaps with that of MeCP2 during neural maturation and synaptogenesis. The phenotypic overlap between patients carrying MeCP2 and CDKL5 gene mutations imply that the two genes play a role in a common pathogenic pathway.

Epilepsy has been reported in 50–94% of female individuals with RTT. Likewise, EEG epileptiform abnormalities are common. The EEG is abnormal early in the course, and has been correlated with the clinical staging of the disease. Additionally, both cortical reflex myoclonus and giant amplitude somatosensory evoked potentials have been described in RTT and motor cortex stimulation evokes action potentials at low threshold (with short latency and enhanced duration).

A developmental disorder of synaptogenesis

Neuropathological findings in RTT include low brain weight, reduced neuronal size, and reduced dendritic arborization with relatively preserved neuronal number. Immunohistochemical studies have shown severely reduced levels of the cytoskeletal microtubule associated protein-2 (MAP-2), a dendritic protein necessary for neuronal growth cones extension and dendritic growth. MRI studies show brain atrophy with global reduction of gray and white matter volumes, particularly in the frontal, parietal, and insular cortex. One study involving six girls with RTT, using 2-deoxy [^{18}F] fluoro-D-glucose (FDG) PET, reported relative glucose hypermetabolism in the frontal cortex of the younger children, resembling the pattern seen in normal children of less than 1 year of age; the authors correlated the relative increase in the frontal glucose metabolism with the changes in the excitatory neurotransmitters during the early active stage of the disease and to the EEG frontal lobe abnormalities. Studies of postmortem cerebral cortex of girls with RTT have shown differential expression of a number of genes related to synapses. The onset of decelerated head growth and psychomotor regression during infancy in RTT correlate well with the period of rapid synaptogenesis in normal infants.

The role of MeCP2 gene in synaptogenesis and epilepsy

MeCP2 mediates transcriptional repression through interaction with histone deacetylase and the corepressor Sin3A. It regulates gene expression and chromosomal architecture. In the brain, MeCP2 expression is cell- and region-specific, and tends to correlate with brain maturation; early-developing areas, such as the brain stem and thalamus, express the protein before the cerebral cortex and hippocampus do. Using a mouse model, investigators demonstrated that the expression of MeCP2 increases as neuronal migration progresses, suggesting that it is necessary for neuronal maturation and maintenance. MeCP2 plays a role in synaptic development by regulating the expression of brain-derived neurotrophic factor (BDNF) and Dlx5. MeCP2 binds selectively to the BDNF promoter and functions to repress the expression of BDNF gene. BDNF facilitates excitatory glutamenergic transmission that can also inhibit inhibitory transmission (enhances the release of glutamate and inhibits the release of GABA). Dlx5, a DNA-binding homeobox protein expressed in early-embryonic development, is a protein expressed in virtually all GABAergic neurons and controls genes necessary for GABA synthesis. Abnormal regulation of these two proteins (as presumably occurs with MeCP2 mutations) could account for the dysfunction in synaptic development, synaptic neurotransmission, and the consequent neuronal hyperexcitability noted in RTT.

Altered neurotransmission in RTT

Elevated CSF glutamate has been described in individuals with RTT. One study found age-related changes in the *N*-methyl-D-aspartate (NMDA) receptor densities, with the younger patients having higher densities compared to older patients. Compared with controls, the densities of the NMDA and adenosine monophosphate (AMPA) receptors were higher in the younger RTT. However, in older RTT patients, they were lower than in control. A similar age-related distribution of the AMPA receptor has been shown in the basal ganglia of affected patients. MR spectroscopy showed elevations in the glutamate/*N*-acetylaspartate (NAA) peak in the gray matter, and the reduction of the epileptic seizure in patients with RTT treated with anticonvulsant lamotrigine has been speculated to be due to its inhibition of glutamate release. Inconsistent changes in monoamines have been reported, including increased homovanillic acid (HVA) and 5-HIAA, suggesting increased turnover of dopamine and serotonin (as determined in postmortem brain); in contrast, another postmortem brain study demonstrated reductions in HVA and 5-HIAA. Similarly, studies on the CSF of patients with RTT have shown reduction in the metabolites of serotonin and dopamine in one study, but no difference in the concentration of biogenic amines in CSF in other studies. In MeCP2-null mice, investigators measured the concentrations norepinephrine, dopamine and serotonin and their metabolites, and found a defect in the normal developmental increase of the brain biogenic amine concentrations.

Tuberous Sclerosis Complex

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder caused by mutations of TSC1 or TSC2 genes. It is characterized by hamartomas in multiple organs, including the skin, brain, kidneys, heart, and the eyes. Epilepsy is its most common neurological feature, occurring in 80–over 90% of cases, often beginning in the first year of life. Infantile spasms have been reported in up to 69% of patients with TSC. Partial seizures may precede, coexist with, or evolve into infantile spasms. In addition, a very high proportion of patients with TSC experience mild to severe behavioral problems, which include autism spectrum disorders (ASD), attention deficit hyperactivity disorder (ADHD), and mild to severe aggression. There is some evidence that the neurological and behavioral problems are less frequent and less severe in individuals with TSC1 mutations and in those in whom a mutation is not detected, compared to those with TSC2 mutations.

Autism in TSC

Autism occurs in 17–61% of individuals with TSC. The incidence of autism in girls with TSC is equal to that in boys with TSC – which contrasts to the fourfold higher incidence of autism in males for the entire population of autistic individuals. Infantile spasms are common in children with TSC who are subsequently diagnosed with autism. One study found that 57% of the children with TSC who had infantile spasms were later diagnosed as autistic, while only 13% of children with infantile spasms from any etiology were autistic. Children with TSC and infantile spasms also have more severe mental retardation than children with any other form of epilepsy. Approximately 10% of non-TSC patients with infantile spasms show decreased glucose metabolism bilaterally in the temporal lobes (superior temporal gyrus and hippocampus) and meet the DSM-IV criteria for autistic disorder. Thus, there appears to be a considerable difference in the prevalence of autism between TSC (57%) and non-TSC (10–13%) children with infantile spasms. Notably, one study found that successful treatment of infantile spasms in children with TSC (i.e., cessation of spasms) led to an increase in developmental quotient and verbal communication, but did not change stereotypy or hyperactivity.

Several imaging studies have shown that 43–100% of autistic TSC patients have cortical tubers in the temporal lobes, whereas all the nonautistic TSC patients were free from temporal lobe tubers – although there have been reports that nonautistic TSC patients have temporal tubers. PET glucose hypometabolism in the nonlesional lateral temporal cortex has been correlated with communication delays and a higher incidence of subependymal nodules adjacent to the caudate nucleus in the autistic group compared to the nonautistic mentally retarded group. These subependymal nodules exhibit increased uptake of AMT on PET scanning, and were associated with increased AMT uptake in the caudate nuclei as well. This observed increased AMT uptake in the caudate nucleus of autistic TSC children may be important in light of the role of the caudate in stereotyped or repetitive behaviors. These investigators hypothesized that increased tryptophan metabolism by the kynurenine pathway results in increased production of quinolinic acid in the caudate nuclei or adjacent subependymal nodules in autistic TSC children, and that this quinolinic acid production might be one mechanism underlying their stereotyped behavior; indeed, infusion of quinolinic acid into caudate nucleus in rats resulted in motor stereotypies. Cerebellar lesions in children with TSC showed decreased glucose metabolism and increased tryptophan metabolism and were associated with higher autistic symptomatology. Purkinje cells in the cerebellum were reported to be decreased in individuals with idiopathic autism. TSC1 and TSC2 proteins, hamartin and tuberin, are expressed abundantly in neurons and astrocytes in the developing and adult cerebellum. In addition, tuberin predominantly localizes to the Purkinje cells. Abnormal expression of hamartin or tuberin in Purkinje cells might

disrupt Purkinje-mediated inhibition and result in an abnormal pattern of glucose and tryptophan metabolism in the cerebellum.

TSC and epilepsy

The generation sites of seizures in TSC have been postulated to include the cortical tubers, as well as microscopic abnormalities, such as microdysgenesis, heterotopic gray matter, and dyslaminated cortical structures. Neuropathological studies show that cortical tubers contain heterogeneous populations of cellular elements, including giant cells, dysplastic neurons, and excessive astrocytes. The neurons typically show simplified dendritic patterns and aberrant patterns of spine morphology. The cytoarchitecture of the cortex surrounding the tubers is typically normal, suggesting that tubers result from a developmental defect that affects a restricted population of neuronal precursor cells. In vivo interictal FDG PET studies have shown that the cortical tubers typically appear as multifocal nodular areas of glucose hypometabolism; the hypometabolism can presumably be attributed to the decreased neuronal number and simplified dendritic pattern in the cortical tubers. FDG PET imaging also detects small cortical tubers, not typically observed on T2-weighted magnetic resonance imaging (MRI), but which were detected using fluid-attenuated inversion recovery (FLAIR).

A relationship between the localization of persistent EEG foci and the site of the major MRI lesions has been observed. Immunohistochemical studies have demonstrated that the dysmorphic neurons in the tubers express a variety of neuronal markers, neurotransmitter receptor subunits, neuropeptides, and calcium binding proteins suggesting that they may actively participate in the generation of partial seizures. Epileptogenic tubers are characterized by high-tryptophan metabolism, whereas nonepileptogenic tubers are characterized by low-tryptophan metabolism. There is evidence that the high-tryptophan metabolism is primarily associated with metabolism by the kynurenine pathway, resulting in increased quinolinic acid, an NMDA agonist. Although this association was seen in the majority of TSC patients with intractable epilepsy, approximately one-third of patients failed to show any areas of increased AMT uptake despite seizure intractability. There is enhanced expression of glutamate NMDA receptors and reduced expression of GABA receptors in epilepsy surgery specimens from patients with TSC.

Conclusion and Directions for Future Research

We have reviewed a number of studies that show the common mechanistic features between epilepsy and autism, particularly in RTT and TSC. Serotonergic abnormalities exist in both autism and epilepsy MeCP2, the gene mutated in RTT, regulates both brain maturation as well as synaptic neurotransmission through BDNF (which facilitates excitatory glutamenergic transmission) and Dlx5 (which controls GABA synthesis). Therefore, abnormalities in the expression of these two proteins (due to MeCP2 mutation) might be contributing to the frequent occurrence of epilepsy in RTT. In TSC patients with either intractable epilepsy or autism, abnormalities in tryptophan metabolism via the kynurenine pathway have been demonstrated. Using PET scanning, increased AMT uptake, has been noted in epileptogenic tubers. Likewise, abnormally increased AMT uptake has been noted in the caudate nucleus as well as in the subependymal nodules of TSC patients with autism. This observation suggests that dysfunction in kynurenine pathway with abnormal production of quinolinic acid could be one of the mechanistic links between epilepsy and autism in TSC.

A complex interaction of genetics, neurobiological, and developmental factors, that lead to an imbalance in neurotransmission regulation, and abnormalities in synaptic functions and connectivity, likely contribute to the frequent occurrence of epilepsy in these conditions. These abnormalities may, at least partially, explain the coexistence of epilepsy with a number of developmental disorders.

See also: **Cognitive Dysfunction and Other Comorbidities**: Epilepsy and Autism in Tuberous Sclerosis; **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; **Imaging**: Imaging Approaches to Seizure Analysis: PET Analysis.

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DEVELOPMENT/MALFORMATIONS

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Cortical Malformations as a Cause for Epileptiform Activity: The Freeze Lesion Model

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Introduction

The six-layered cerebral cortex is generated during the earliest developmental stages in an inside first–outside last pattern (layer VI first–layer II last). Newly generated glutamatergic excitatory pyramidal neurons migrate radially from the ventricular and subventricular zone into the developing cortex, whereas γ-aminobutyric acid (GABAergic) inhibitory interneurons migrate along a tangential path from the medial ganglionic eminence to their final cortical destinations. In humans, this period of neuronal migration peaks between the third to fifth months of gestation.

The neuronal migration process is controlled by a number of genes, and genetic deficits are often the primary cause of severe neuronal migration disorders. These migration disorders induce various brain malformations, such as polymicrogyria, periventricular heterotopia, or lissencephaly, and are often associated with drug-resistant epilepsy. Beside these genetic neuronal migration disorders, nongenetic disturbances such as hypoxic–ischemic encephalopathy or infections may also cause cortical malformations leading to catastrophic epilepsies. Recent clinical observations obtained with high-resolution imaging techniques have demonstrated that neuronal migration disorders are more common than what was previously estimated and that a large population of patients suffering from pharmaco-resistant epilepsy show obvious cortical neuronal migration disorders.

Two questions are of central interest to understand the mechanisms and consequences of early neuronal migration disorders and the manifestation of severe forms of epilepsy: (1) What are the genetic and molecular mechanisms underlying normal and abnormal neuronal migration during early corticogenesis? (2) What are the cellular and neuronal network mechanisms leading to hyperexcitability and epilepsy in a cortical network showing developmental malformations? This article addresses the second question, and provides insights into the molecular, cellular, and network mechanisms underlying epileptiform activity in an animal model of cortical malformations induced by an early focal freeze lesion.

Background

A large number of different animal models have been developed over the last 30 years to study the process of normal and abnormal neuronal migration in the developing cerebral cortex. In utero injection of methylazoxymethanol

acetate into pregnant rats, prenatal cocaine or ethanol exposure, in utero RNA interference knockdown, and genetic models of cortical malformation (such as the 'tish' rat or the Dreher mouse) have been all very instrumental in understanding the causes and consequences of neuronal migration disorders. In 1977, Dvoral and Feit presented a model to induce a well-defined cortical malformation by exposing the cerebral cortex of a newborn rat for a few seconds to a freezing probe. This experimental manipulation produced a cortical freeze lesion that, in the adult rat, was characterized by a focal four-layered neocortex (microgyrus). Further experimental analyses demonstrated that the duration of this freezing insult determines the size and the type of the lesion (**Figure 1**). The neocortical freeze lesion model very reliably reproduces the pathology of certain types of human neuronal migration disorders (polymicrogyria, focal heterotopia, cortical dysplasia, schizencephaly) and can be easily established.

It is not entirely clear how the cortical malformation develops, after the induction of the focal freeze lesion at the day of the animal's birth. In the newborn rat cerebral cortex, the lesion destroys neurons and glia throughout the cortical plate and occasionally the subplate. According to Rosen et al., the pial membrane is not destroyed, and damaged radial glial fibers regrow. Supragranular neurons migrate in the first postnatal days along radial glia through the damaged area, and the newly formed supragranular layer overlies the cell-free area in the microgyric cortex.

Methods

The cortical freeze lesion model can be easily established and generates a reproducible pathology in the mature cerebral cortex. The mortality of this model is low (<5%). Depending on the experimental protocol, the freeze lesion induces a three- or four-layered cortex (microgyrus), focal

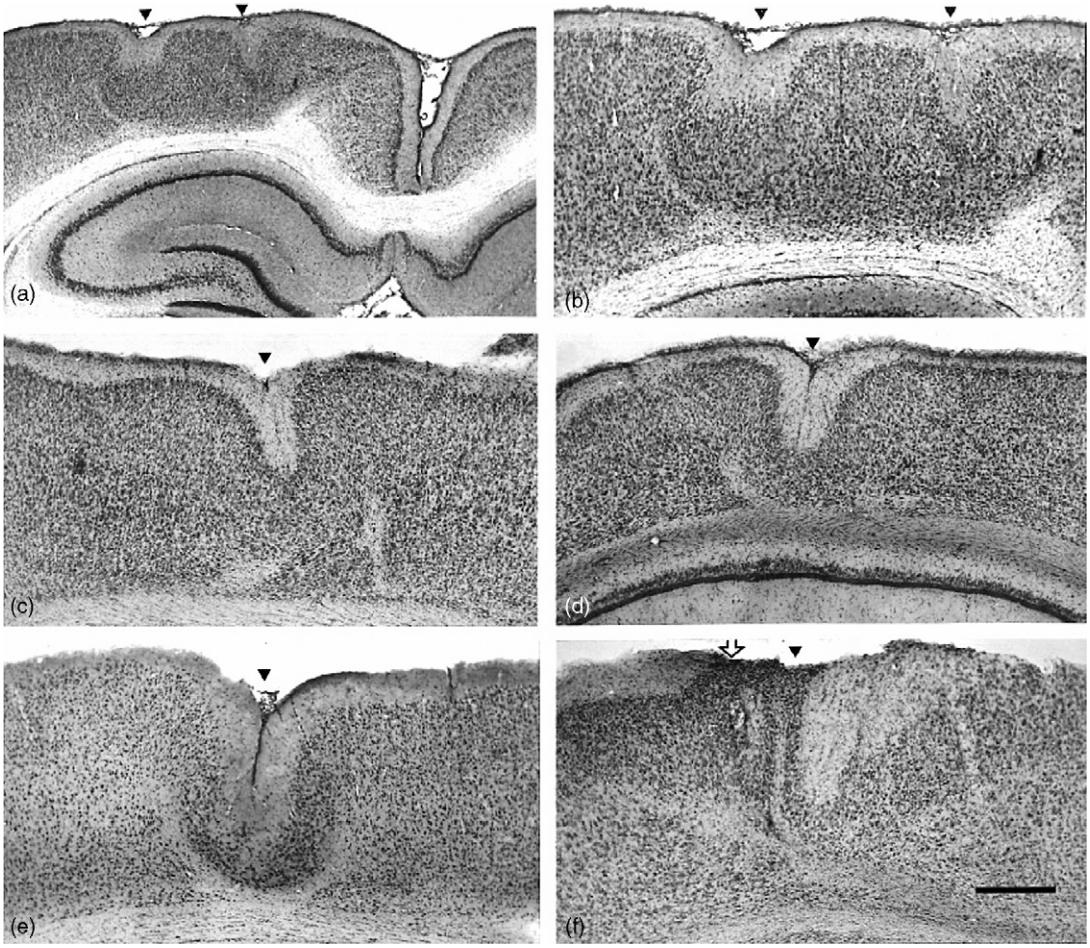


Figure 1 Examples of focal neocortical malformations induced by freeze lesion of the neonatal rat cerebral cortex. Photographs show Nissl-stained coronal sections of the cerebral cortex of adult rats, which received a focal cortical freeze lesion at the day of birth. (a, b) Upper layer cortical dysplasia shown at different magnification. (c–e) Typical examples of microgyrus with sulcus-like malformation. (f) Complete loss of cortical architecture and layer I heterotopia (open arrow). Scale bar in (f) refers to 800 μm in (a) and 400 μm in (b–f). Reproduced from Luhmann HJ, Raabe K, Qü M, and Zilles K (1998) Characterization of neuronal migration disorders in neocortical structures: Extracellular in vitro recordings. *European Journal of Neuroscience* 10: 3085–3094, with permission from Blackwell Publishing.

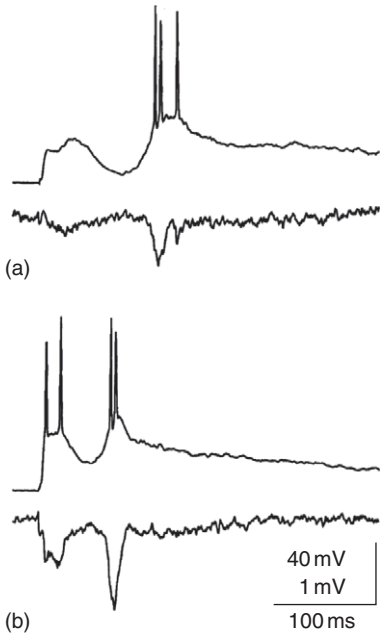


Figure 2 Simultaneous intra- and extracellular recordings of stimulus-evoked epileptiform responses in upper layers of microgyric cortex of an adult rat neocortical slice. Responses to low (a) and high (b) stimulus intensity are shown. Modified from Luhmann HJ, Karpuk N, Qü M, and Zilles K (1998) Characterization of neuronal migration disorders in neocortical structures: II. Intracellular in vitro recordings. *Journal of Neurophysiology* 80: 92–102, with permission from The American Physiological Society.

heterotopia in layer I or a cortical cleft (schizencephaly). This model has been mostly studied in the rat, and to a minor extent also in the mouse. The model has been studied over the last decade by various groups using in vivo and in vitro electrophysiology, in vivo imaging, behavioral tests, immunohistochemistry, receptor autoradiography, molecular biology and neuroanatomical tracing techniques.

The induction of the lesion is relatively easy to perform, and the best reproducible results can be obtained when the lesion is induced during the first 24 h after birth. Cortical migration disorders with a pathology resembling cortical dysplasia, four-layered cerebral cortex, microgyria, or heterotopia in layer I can be obtained when the lesion is induced during the period of neocortical neuronal migration (in rats and mice, approximately up to postnatal day 4). A comprehensive description of the experimental procedure is available in Further Reading.

Recent Results

In vitro electrophysiological studies in different labs have independently demonstrated that the cortical freeze lesion model induces a pronounced hyperexcitability in the developing and mature neocortical network (**Figure 2**). These groups also have provided compelling evidence that the structural lesion does not correlate with the location of the most excitable region, reproducing some

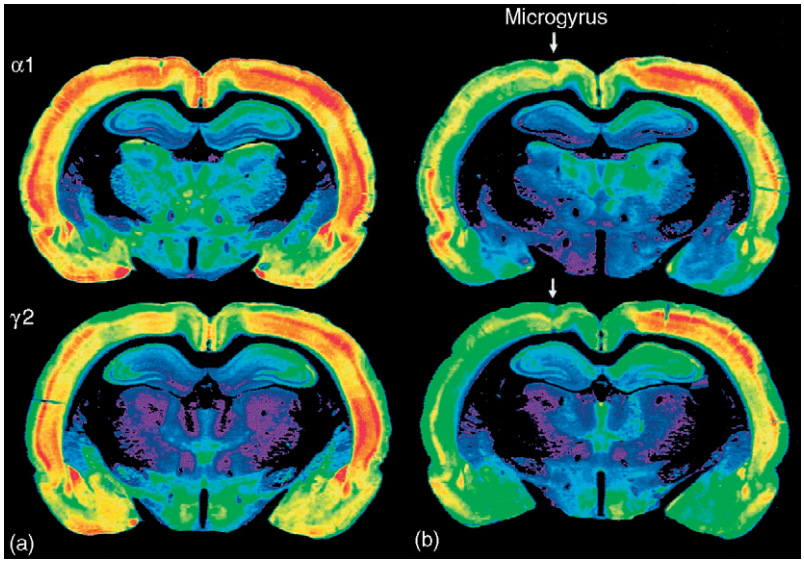


Figure 3 Freeze lesion induced microgyrus and associated widespread down-regulation in α1 and γ2 $GABA_A$ receptor subunits in adult rat cerebral cortex. Color-coded images of whole brain coronal sections immunohistochemically processed for the α1 (upper row) and γ2 (lower row) subunit in sham-operated control rat (a) and adult rat that received a cortical freeze lesion at the day of birth (b). In (b), freeze lesion induced microgyrus is marked by downward arrow. Color code ranges from black for background to violet, blue, green, yellow, and red for most intense signal. Modified from Redecker C, Luhmann HJ, Hagemann G, Fritschy JM, and Witte OW (2000) Differential downregulation of $GABA_A$ receptor subunits in widespread brain regions in the freeze-lesion model of focal cortical malformations. *Journal of Neuroscience* 20: 5045–5053, with permission from Society for Neuroscience.

clinical features of cortical dysplasia associated with pharmaco-resistant epilepsy. In the rat freeze lesion model, epileptiform activity originating from the hyperexcitable region around the lesion propagates (at ~0.06 m s^{-1}) over several millimetres across the neocortex into neighboring cortical areas with a normal histological appearance. Functional modifications in NMDA receptor subunit composition have been described in the rat cortical freeze lesion model and in human cortical dysplastic tissue. Interestingly, the structural lesion is surrounded by a widespread area of GABA and glutamate-receptor reorganization. Quantitative receptor autoradiography demonstrated a widespread and significant reduction in the binding to $GABA_A$ and $GABA_B$ receptors, reflecting a down-regulation in inhibitory receptor number and/or affinity. These observations could be confirmed immunohistochemically by the use of specific antibodies for different $GABA_A$ subunits (**Figure 3**). A widespread regionally differential reduction of the $GABA_A$ receptor subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, and $\gamma 2$ was observed. Furthermore, the down-regulation of $GABA_A$ receptor subunits also involved the ipsilateral hippocampal formation, as well as restricted contralateral neocortical areas, indicating widespread disturbances in the inhibitory neocortical and hippocampal network.

Alterations of glutamatergic receptors in dysplastic cortex are more complex. Quantitative receptor autoradiography demonstrated a significant increase in binding to NMDA, AMPA, and kainate receptors, but immunohistochemically, analyses of four NMDA receptor subunits and two major AMPA- and kainate-receptor complexes did not reveal any remote or widespread changes of glutamate-receptor subunit distribution.

Another controversial issue is the extent to which the widespread down-regulation in $GABA_A$ receptor density and/or affinity and their subunits causes a functional reduction in GABAergic inhibition. Our electrophysiological in vitro recordings from pyramidal neurons in the hyperexcitable region close to the microgyrus have revealed a lack or reduction of stimulus-evoked inhibitory postsynaptic potentials (IPSPs) as compared with the controls (upper row in **Figure 4**), but pharmacologically isolated IPSPs recorded under blockade of ionotropic glutamate receptors (lower row in **Figure 4**) did not reveal a statistically significant difference in IPSP peak

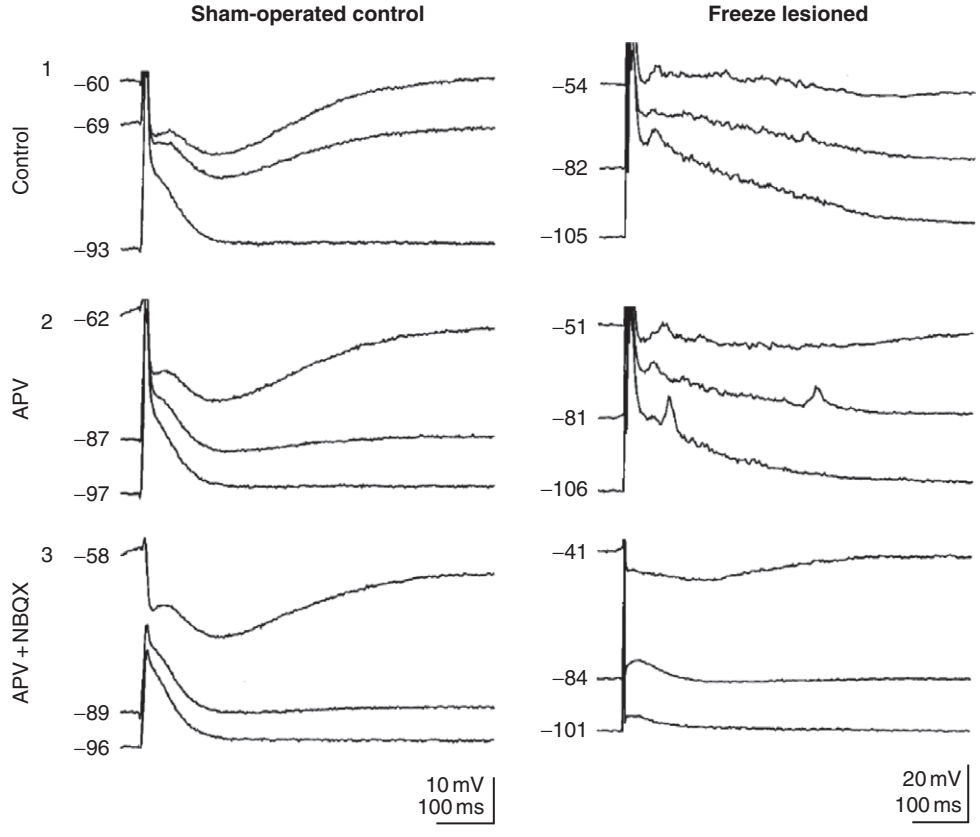


Figure 4 Intracellular recordings from upper layer pyramidal neurons in neocortical slices from sham-operated (left) and freeze-lesioned (right) adult rats. Recordings show stimulus-evoked inhibitory postsynaptic potentials (IPSPs) in normal extracellular solution (upper row), after the addition of NMDA receptor antagonist APV (middle row) and after blocking ionotropic glutamate receptors with APV and NBQX (lower row). Membrane potentials are given for each trace. Modified from Luhmann HJ, Karpuk N, Qü M, and Zilles K (1998) Characterization of neuronal migration disorders in neocortical structures: II. Intracellular in vitro recordings. *Journal of Neurophysiology* 80: 92–102, with permission from The American Physiological Society.

conductances. However, similar experimental analyses by Jacobs and Prince indicate that GABAergic inhibition may be enhanced in the epileptogenic areas. That group has identified, in the paramicrogyral region, an increase in the number of functional excitatory synapses on both interneurons and pyramidal cells, perhaps due to hyperinnervation by cortical afferents originally destined for the microgyrus proper. This disorganization in cortical architecture is associated with an abnormal ingrowth of thalamocortical afferents into layer IV. Rosen *et al.* reported an almost complete lack of thalamocortical or corticothalamic projections between the ventrobasal somatosensory complex in the thalamus and the cortical microgyrus. Furthermore, callosal connections are also disorganized.

A major drawback of the freeze lesion model is the lack of spontaneous epileptic seizures in vivo. Recurrent seizures and status epilepticus have not been described in this model. So far only a lower threshold for hyperthermia-induced seizures and a prolonged ictal manifestation have been reported in freeze-lesioned rats in vivo, suggesting a pathophysiological link between focal cortical dysplasia and atypical febrile seizures.

Future Goals

A major challenge in the future investigation of the cortical freeze lesion model will be a more comprehensive analysis of the EEG or ECoG in freely moving animals that exhibit various extents of cortical malformations. It is currently unclear at which age experimental animals with a cortical freeze lesion might reveal what type of spontaneous seizures or whether seizures can be provoked by systemic application of drugs that lower the seizure threshold. Long-term EEG recordings, preferentially telemetric recordings from freely moving animals, might be most valuable to address this important issue.

It remains to be determined in more detail how the intracortical GABAergic inhibition is modified in the cortical freeze lesion model. The excitatory drive onto certain types of inhibitory interneurons may be enhanced or reduced, the intracellular Cl^- homeostasis might be modified, and the functional consequences of changes in $GABA_A$ receptor subunit composition are also not clear. The differential regulation of the $GABA_A$ receptor subunits is of pivotal interest, since $GABA_A$ receptors mediating conventional synaptic (phasic) inhibition have a different subunit composition as compared with those mediating extrasynaptic tonic inhibition. In many neuronal cell types, tonic inhibition is mediated by δ-subunit containing $GABA_A$ receptors, which are located extrasynaptically. In contrast, phasic inhibition is most likely mediated by synaptic γ-subunit containing $GABA_A$ receptors. These electrophysiological and pharmacological studies would also be valuable in other models of epilepsy.

See also: **Absence Seizures**: Thalamic Neurons and Networks Related to Absence Epilepsy; Thalamocortical Synchronization and Absence Epilepsy; **Development/Malformations**: Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; The Tish Rat: An Animal Model of Cortical Malformation in the Study of Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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Epilepsy-Associated Reelin Dysfunction Induces Granule Cell Dispersion in the Dentate Gyrus

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Introduction

Ammon's horn sclerosis (AHS) is a hallmark of temporal lobe epilepsy. AHS is characterized by neuronal loss in hippocampal regions CA3 and CA1 and in the hilus of the dentate gyrus, and by granule cell dispersion (GCD). The term *sclerosis* describes a glial hypertrophy accompanying the neuronal loss. The neuron loss is likely to represent excitotoxic cell death associated with increased calcium entry into the cells. This mechanism of cell death is supported by studies showing that intracellular application of calcium buffers prevents excitotoxic cell death of hilar neurons. Neurons in hippocampal region CA2, which is located in between CA1 and CA3, are less vulnerable and are particularly rich in calcium-binding proteins.

The factors that induce a broadening of the granule cell layer – i.e., granule cell dispersion – are less clear. Does increased neuronal activity force the granule cells to leave their normally tightly packed layer? Does seizure activity stimulate neurogenesis in the dentate gyrus, leading to an increase in neuron number and thus to a broadening of the granule cell layer? Does glial hypertrophy in AHS play a role in the development of granule cell dispersion?

In this article, we summarize our recent studies that addressed these questions. In these studies, we used tissue samples from temporal lobe epilepsy (TLE) patients subjected to hippocampal resection for therapeutical reasons. In addition, we used tissue from mice that had received a unilateral hippocampal injection of kainate. By comparing our results in human tissue and in experimental animals, we obtained new insights into the processes leading to the development of granule cell dispersion in epilepsy.

Background – Granule Cell Dispersion in Epilepsy – a Migration Defect?

Granule cell dispersion is best described as an abnormal broadening of the granule cell layer, with many granule cells migrating far into the molecular layer (**Figure 1**). GCD is regularly associated with Ammon's horn sclerosis, but there are also cases of Ammon's horn sclerosis lacking a prominent GCD. There is strong evidence that Ammon's horn sclerosis, the degeneration of neurons in hippocampal regions CA1 and CA3 and in the hilus of the dentate gyrus, represents excitotoxic changes resulting from increased neuronal activity. Does this also hold true for GCD? The neurons that migrate out into the molecular layer do not show signs of neuronal degeneration. Alternatively, is GCD a migration defect of the granule cells during development that eventually leads to epilepsy? It would not be too far-fetched to assume a developmental migration defect, as there are mouse mutants that show a structural phenotype reminiscent of GCD and a functional phenotype of seizures/epilepsy.

A more scattered distribution of the granule cells leads to an overlap of the normally segregated input and output sides of the granule cells. In a densely packed granule cell layer, the input side (i.e., the granule cell dendritic arbors in the molecular layer) is normally separated from the output side (the mossy fiber axons invading the hilus). A loss of this clear-cut lamination will allow more superficially located granule cells to contact dendrites of deeply located neurons, resulting in an increased granule cell-to-granule cell connectivity. Would this aberrant circuitry contribute to increased excitability of the granule cells, resulting in epileptic activity?

One mutant that shows a migration defect similar to GCD is the reeler mouse lacking the extracellular matrix protein, reelin. Reelin is known to be synthesized and secreted by Cajal-Retzius cells in the marginal zones of cortex and hippocampus, and there are severe migration defects in these regions in the reeler mutant. Could it be that GCD in epileptic patients is associated with an altered reelin expression?

As a first step towards a better understanding of GCD, we looked at reelin expression in tissue samples from epileptic patients with GCD and in samples from autopsy controls. These control patients did not suffer from epileptic seizures and did not show Ammon's horn sclerosis and GCD. We measured reelin expression by real-time PCR and quantified reelin-expressing cells using in situ hybridization for reelin mRNA. We found a significant decrease in reelin expression in patients with granule cell dispersion with both methods. Moreover, there was an inverse correlation between the number of reelin mRNA-expressing cells

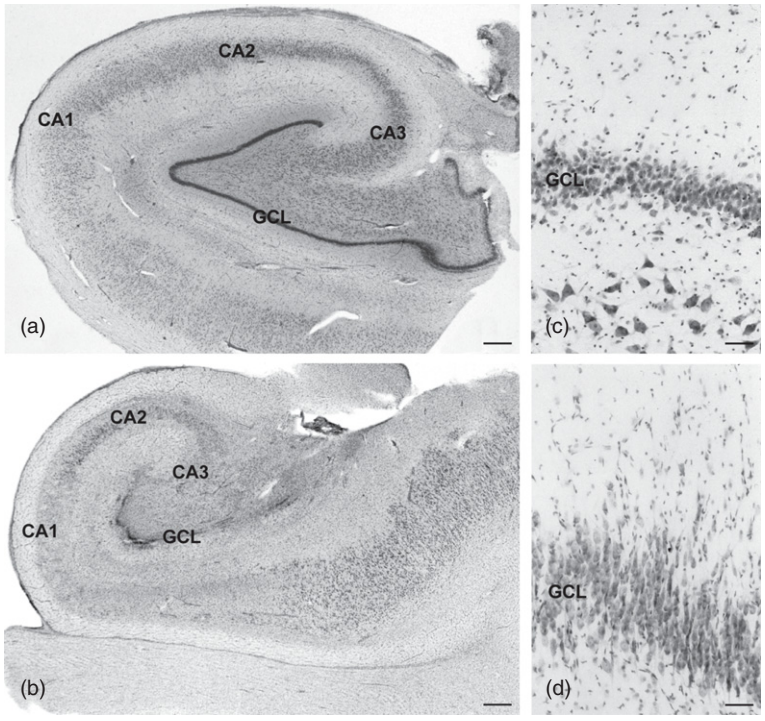


Figure 1 Morphology of normal and epileptic human hippocampus stained with cresyl violet. (a) Human control hippocampus with normal distribution of neurons in hippocampal subfields and in the dentate gyrus, where the granule cells are located in a dense layer. (b) Epileptic human hippocampus with characteristic features of Ammon's horn sclerosis. Selective cell loss is obvious in hippocampal subfields CA1 and CA3. The granule cell layer is dispersed. (c) Granule cell layer of a normal human dentate gyrus. The granule cells are arranged in a densely packed layer. (d) Loss of dense granule cell packing (granule cell dispersion) in temporal lobe epilepsy. Scale bars a, b: 600 μm; c, d: 75 μm. CA1, CA2, CA3, hippocampal subfields; GCL, granule cell layer. Adapted from Haas and Frotscher (2004) copyright; with kind permission of Springer Science and Business Media.

in the dentate gyrus and the extent of granule cell dispersion, quantified by measuring the width of the granule cell layer. Although these findings pointed to a role of reelin in the development of granule cell dispersion, they could not clarify whether decreased reelin expression during development had *caused* granule cell dispersion, which in turn led to epilepsy or, alternatively, epileptic seizures interfered with reelin expression, leading to GCD. For obvious reasons, these questions could not be addressed in studies of tissue samples from patients.

Methods

We used tissue samples from TLE patients and a mouse epilepsy model that recapitulates the main histopathological and electrophysiological features of human TLE. A single unilateral injection of the glutamate agonist kainate (KA) into the hippocampus of adult mice induced spontaneous, focal epileptic seizures and Ammon's horn sclerosis, including GCD. We used cresyl violet staining, immunohistochemistry, in situ hybridization, and real-time PCR analysis to monitor cell death, GCD development, neurogenesis, glial cell proliferation, and reelin expression in human tissue and in tissue obtained from kainate-injected mice.

Recent Results

Unilateral Injection of Kainate, an Agonist of the Excitatory Transmitter Glutamate, Results in the Development of GCD in Mice

Increased neuronal activity is associated with an increased release of the excitatory neurotransmitter, glutamate. Thus, glutamate agonists, such as kainate, are often injected into animals to induce epileptic activity. Unilateral injection of kainate into one hippocampus (in mice) was not only found to induce seizure activity but also to lead to Ammon's horn sclerosis with neuronal degeneration in CA1 and CA3 and granule cell dispersion in the dentate gyrus. Interestingly, these degenerative changes are not found on the contralateral, non-injected side.

After a latency period of 2 weeks, the animals developed seizure activity as monitored by EEG recordings. They also developed a prominent granule cell dispersion on the side of kainate injection but not on the contralateral side, indicating that GCD resulted from the injection

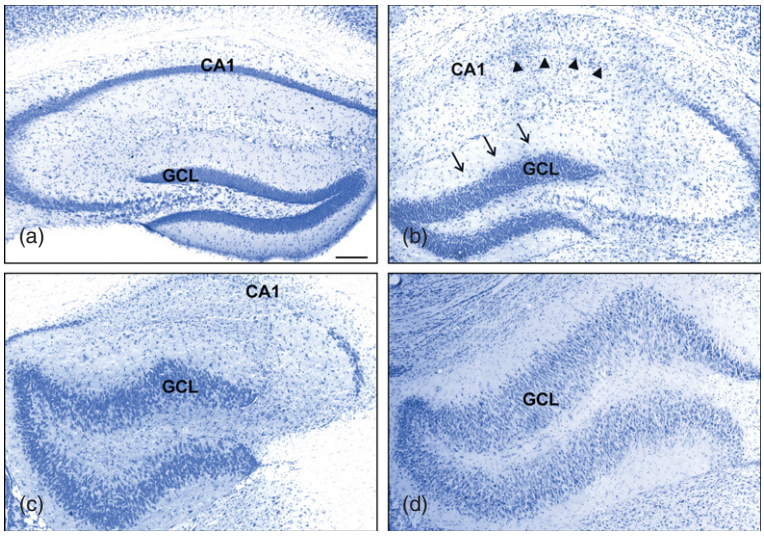


Figure 2 Development of granule cell dispersion induced by unilateral intrahippocampal kainate injection in the adult mouse. Coronal sections of hippocampus were stained with cresyl violet. (a) Contralateral, non-injected hippocampus displaying the normal histology of the hippocampal formation. (b–d) Ipsilateral hippocampus 7 days (b), 21 days (c) and 6 weeks (d) after kainate injection. A progressive dispersion of the granule cells (arrows) is observed. Note neuronal loss in CA1 (arrowheads), CA3, and hilus. Scale bar: 100 μm; GCL, granule cell layer. From Heinrich *et al.*, 2006; copyright by the Society for Neuroscience.

of the excitotoxin. The broadening of the granule cell layer developed progressively; GCD was evident as early as one week post injection and was fully expressed after six weeks (**Figure 2**).

What is the nature of kainate-induced GCD? Is it a migration of fully differentiated granule cells, or does seizure activity increase neurogenesis and aberrant migration of the newly generated granule cells? It is generally accepted that post-migratory neuroblasts that are about to differentiate their dendritic and axonal arbors are no longer able to migrate. How, then, could fully differentiated granule cells migrate out of a densely packed granule cell layer?

In our experimental animals, we were in the position to address these questions by injecting them with bromodeoxyuridine (BrdU), a marker of newly generated cells. We found that neurogenesis was significantly decreased on the side of kainate injection (where GCD occurs) when compared with the non-injected control side (where no GCD was observed). Given the paucity of neurogenesis on the side that shows GCD, we concluded that adult, differentiated granule cells have 'migrated,' and that GCD does not represent an abnormal migration of newly generated granule cells. The cessation of neurogenesis on the side of kainate injection came as a surprise, since studies of other epilepsy models had shown epileptic activity to induce neurogenesis. Further, while neurogenesis had ceased on the side of kainate injection, we noticed an increased gliogenesis, as revealed by double labeling for the astrocytic marker GFAP (glial fibrillary acidic protein) and BrdU. It is reasonable to assume that this increased formation of astrocytes, particularly in the hilar region and in CA1 and CA3, is associated with the excitotoxic neurodegenerative changes observed in these regions. There was also an increase in activated microglial cells, as demonstrated by labeling for the microglial marker Iba1. Studies are in progress to determine the extent to which glial hypertrophy and increased gliogenesis contribute to GCD.

Next, an attempt was made to determine whether or not increased neurogenesis is involved in the formation of GCD in human epileptic patients. To this end we used immunostaining for Ki67, a marker of the cell cycle, in tissue samples from epileptic patients. We were unable to find stained cells among the neurons that had migrated out of the granule cell layer, suggesting that in human epileptic patients, like in kainate-injected mice, granule cell dispersion is not an aberrant migration of young neurons.

Reelin's Role in the Development of GCD

As mentioned earlier, reelin expression was found to be decreased in tissue samples from epileptic patients. This finding pointed to a role of reelin in granule cell lamination, reminiscent of the lamination defects seen in the mouse mutant lacking reelin. However, the dispersed granule cells in the reeler mouse represent a maldevelopment of the granule cell layer, whereas GCD in epileptic patients appears to be a secondary effect associated with seizure activity. Numerous studies have shown that reelin is a molecule important for the normal development of cell and fiber layers in the hippocampus. However, it is

less clear how one might explain a decreased reelin expression in tissue samples of adult epileptic patients.

Animals with unilateral injections of kainate appeared useful in studies of reelin expression associated with GCD. On the kainate-injected side, where GCD developed, we found a dramatic decrease in reelin expression in quantitative real-time RT-PCR studies and by counting reelin mRNA-expressing cells using in situ hybridization (Figure 3). On the contralateral side, where no granule cell dispersion developed, reelin expression was normal when compared with naïve control animals. The findings point to a close relation between reelin expression and the development of GCD, but did not elucidate the underlying mechanisms.

Reelin Keeps Adult Granule Cells in Register

The CR-50 antibody against reelin has proven its usefulness in blocking reelin function in a variety of experimental paradigms. Working with the concept that a decreased reelin expression induces an aberrant migration of granule cells (similar to the aberrant migration of these neurons in reeler mutants), we reasoned that blocking reelin function should lead to granule cell dispersion. We thus used adult naïve mice that received unilateral infusions of CR-50 antibody into one hippocampus via osmotic minipumps for one week. For controls, unspecific IgG was infused. On the side of CR-50 infusion, we observed a significant development of GCD near the infusion site; no development of GCD was observed when unspecific IgG was injected. The results were quantified by measuring the thickness of the granule cell layer and comparing the infused hippocampus with the contralateral one. While no statistically significant differences were observed between the contralateral hippocampus and the ipsilateral hippocampus when unspecific IgG was infused, the thickness of the granule cell layer had significantly increased on the side of

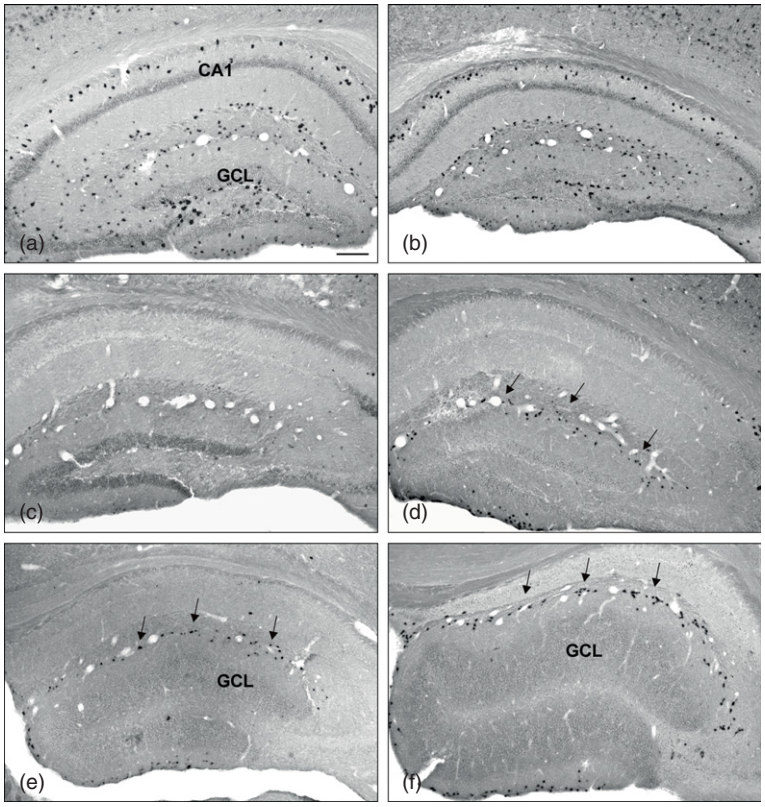


Figure 3 Time course of reelin mRNA expression in the mouse hippocampus after unilateral intrahippocampal kainate injection. Reelin mRNA expressing neurons were localized by *in situ* hybridization histochemistry. (a) Contralateral hippocampus. Many reelin mRNA-positive neurons are present in CA1, CA3, in the hilus, and along the hippocampal fissure. (b) Ipsilateral hippocampus of a saline-injected control mouse two days after injection. The distribution of reelin mRNA-positive neurons is similar to that in (a). (c–f) Ipsilateral hippocampus two days (c), seven days (d), two weeks (e) and six weeks (f) after kainate injection. (c) A dramatic reduction of reelin mRNA expression is evident all over the hippocampus two days after kainate injection. Except a few labeled cells, which are still present along the hippocampal fissure, no reelin mRNA-positive neurons can be detected in CA1 and in the hilus. (d) Seven days after kainate injection, a few reelin mRNA-positive neurons can be observed along the hippocampal fissure (arrows). (e, f) Numbers of reelin mRNA expressing cells remain low along the hippocampal fissure after two (e) and six (f) weeks following kainate injection, in parallel with a progressive development of GCD. GCL, granule cell layer. Scale bars: a–f, 100 μm. Adapted from Heinrich *et al.*, 2006; copyright by the Society for Neuroscience.

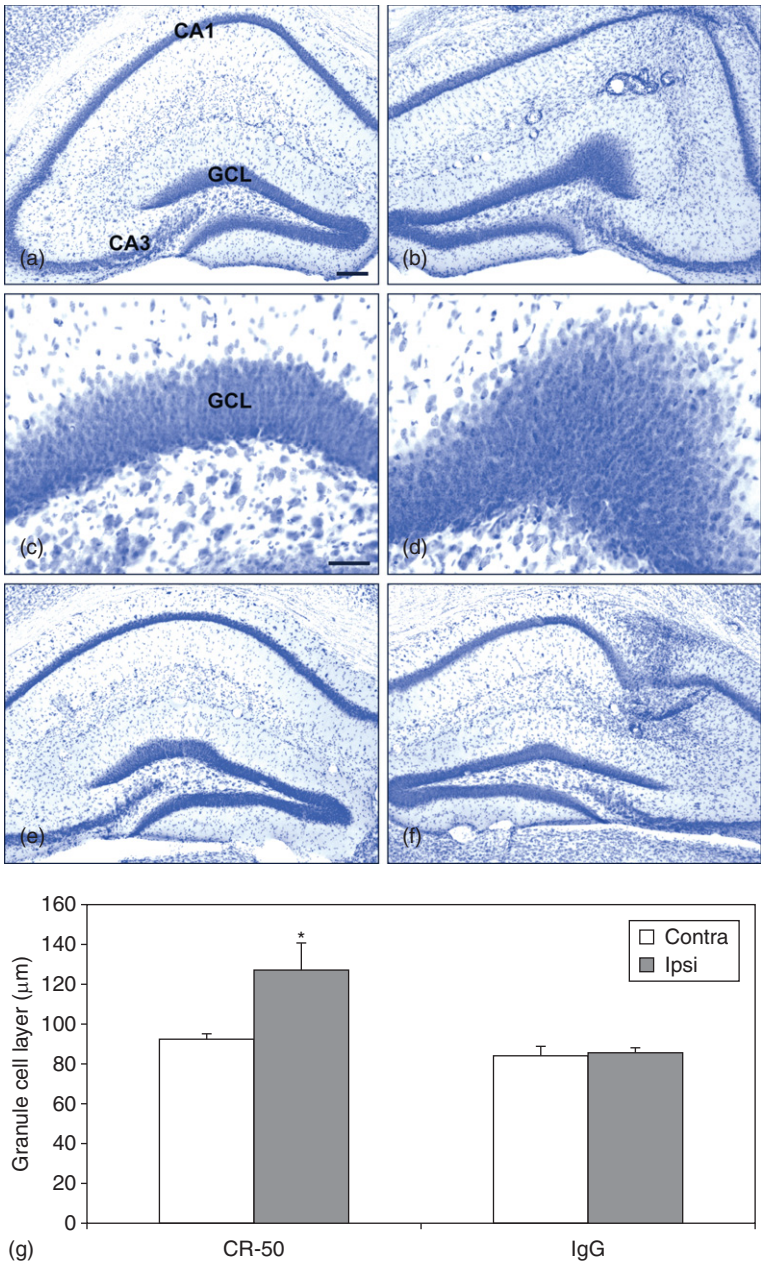


Figure 4 Effect of chronic CR-50 antibody infusion into the dentate gyrus of adult mice. Antibodies were chronically administered by osmotic pumps inserted in the right hippocampus over a period of seven days followed by another seven days of survival. Morphological changes were visualized by cresyl violet staining. Infusion of CR-50 resulted in a massive increase in the width of the granule cell layer (b, d) as compared to the contralateral side (a, c). The widening of the granule cell layer was confined to the site of injection, indicating that a local neutralization of reelin altered the lamination of adult granule cells. No change in lamination was observed after infusion of mouse control IgG (e, f). (e) Contralateral side. (f) Side of IgG infusion. Scale bars: a, b, e, f: 150 μm; c, d: 40 μm. (g) Quantitative evaluation of GCL width in CR-50 ($n = 6$) and IgG-infused hippocampi ($n = 5$). The width of the GCL was measured in the ipsi- and contralateral hippocampus in five sections/animal, and five measurements were taken per hippocampus. Mean values ± S.E.M. are shown. In the CR-50-injected animals, the width of the GCL was significantly increased as compared to the contralateral side ($p < 0.0277$) using the two sample Wilcoxon rank-sum test. Comparisons were of the unpaired two-sided type. Adapted from Heinrich *et al.*, 2006; copyright by the Society for Neuroscience.

CR-50 injection (Figure 4). These results clearly show that the expression of reelin is not only required for the normal development of dentate lamination but for the maintenance of a compact granule cell layer in the adult brain.

Summary and Conclusions

Correlating the results of studies on tissue samples from epileptic patients and in experimental animals and mouse mutants sheds light on the mechanisms leading to granule

cell dispersion in epilepsy. A similar loss in granule cell lamination in reeler mutants and in human GCD prompted our study of reelin expression in tissue samples from patients. The decreased reelin expression we observed in human GCD led us to study reelin expression in an animal model of epilepsy. We found both a decreased reelin expression and GCD only on the side of kainate injection (not on the contralateral control side). Interestingly, there was ongoing postnatal neurogenesis on the contralateral side, but a cessation of neurogenesis on the kainate-injected side. From these findings, we concluded that injection of the glutamate agonist kainate induced seizure activity associated with GCD, paralleled by a decreased reelin expression. A causal link between the development of GCD and decreased reelin expression was found in our experiments with antibodies neutralizing reelin. Infusion of CR-50 antibody, but not of unspecific IgG, was sufficient to induce GCD in adult, naïve mice.

In summary, we propose the following scenario for the development of GCD:

1. Seizure activity in epileptic patients, or in experimental animals induced by unilateral kainate injections, interferes with reelin expression. Since there does not seem to be a substantial death of reelin-synthesizing neurons (very few degenerating Cajal-Retzius cells were observed using Fluoro Jade staining, unpublished observations), we conclude that seizure activity decreases reelin expression.
2. The decreased reelin expression in adult animals or human patients allows mature granule cells to invade the molecular layer. This result is consistent with a 'stop signal function' of reelin. Reelin-expressing Cajal-Retzius cells in the marginal zone of the neocortex and in the molecular layer of the dentate gyrus prevent migrating neurons from invading these zones during development. In reeler mutants lacking reelin, the marginal zone of the neocortex is densely populated by neurons, and the characteristic dense packing of dentate granule cells is lost. Reelin appears to have a similar function in the adult organism: Decreased reelin expression, and thus a loss of the 'stop signal,' allows granule cells to invade the molecular layer. These aberrantly migrating cells were not, in our studies, newly generated neurons but differentiated granule cells.
3. A more general conclusion from these studies is that there are molecules, such as reelin, which are important for the maintenance of the structural organization of the brain. Their malexpression may result in secondary structural changes such as granule cell dispersion. It is tempting to speculate that not only seizure activity, but also a variety of other noxious stimuli, may interfere with reelin expression (or with the expression of other control genes) and lead to structural changes in the adult brain that are reminiscent of developmental defects and contribute to neurological or psychiatric diseases.

Future Directions

Our results have shown that reelin is important for the maintenance of the laminated organization of the dentate gyrus. In epileptic patients with decreased reelin expression, in kainate-injected mice, and in the reelin-deficient mutant reeler, dentate granule cells do not form a compact cell layer but are dispersed. Can the application of reelin rescue granule cell dispersion?

Using slice cultures of reeler mutants as a model, we were able to show that the scattered distribution of the granule cells can be rescued by wildtype co-cultures. On the basis of these findings, we have recently started to treat kainate-injected mice with infusions of recombinant reelin. As described, mice with unilateral injections of kainate develop epileptic seizures and show decreased reelin expression and granule cell dispersion on the injected side but not on the contralateral side. Our first results from experiments to treat these animals with recombinant reelin are very promising. However, there is much to be learned before we will know whether or not reelin treatment constitutes a new therapeutic avenue.

The results reported here have shown that reelin expression is sensitive to epileptic activity. There may be other noxious factors that interfere with the expression of reelin or other molecules important for the maintenance of normal network structure and function. Studies are in progress analyzing potential effects of virus infections on reelin expression. Along this line, it will be important to determine, by molecular approaches, how reelin expression is regulated. Finally, future studies should address the effects of reelin signaling on the cytoskeletal reorganization required for the actual migration process. In spite of increasing knowledge about the signaling cascade activated by reelin binding to its receptors, very little is known about the ways in which reelin acts on the cytoskeleton to control cell movement and arrest.

See also: **Development/Malformations**: Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis; **Genetics**: Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Pediatric Epilepsy**: Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation; **Plasticity**: Synaptic Reorganization in the Hippocampal Dentate Gyrus; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Focal Malformations of Cortical Development: Pathogenesis and Pathways

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Introduction

Focal malformations of cortical development (focal MCD), including focal cortical dysplasia with balloon cells, hemimegalencephaly, and gangliogliomа, are highly associated with epilepsy. Despite their prevalence in both pediatric and adult epilepsy populations, little is known about the molecular and developmental pathogenesis of these lesions or their causal relationship with intractable epilepsy. Of particular interest is the fact that focal brain malformations virtually always occur sporadically, with no defined family pedigrees or twin pairs identified to date. Monozygotic twins are typically discordant for these lesions. Only rarely does hemimegalencephaly occur in the setting of another genetic disease (such as Proteus syndrome or tuberous sclerosis), and early attempts at causative gene identification have been unsuccessful.

One attractive hypothesis is that focal brain malformations form as a consequence of a somatic gene mutation occurring during brain development within a cortical progenitor cell. Thus, new experimental strategies must be defined to identify gene mutations within brain tissue rather than in peripheral blood samples. Histopathological similarities between focal MCD and the autosomal dominant disorder tuberous sclerosis complex suggest that the pathogenesis of focal MCD may be related to the mammalian target of rapamycin (mTOR)-signaling cascade. Thus, comprehensive analysis of genes and proteins in this pathway may identify candidate molecules to explain how these lesions form during brain development.

Background

Focal MCD are a common cause of pharmacoresistant epilepsy. In a recent classification scheme, focal MCD were classified as putative disorders of cell proliferation. Tubers in TSC, focal cortical dysplasia with balloon cells (FCD), hemimegalencephaly (HMEG; **Figure 1**), and gangliogliomа (GG) are of particular interest because

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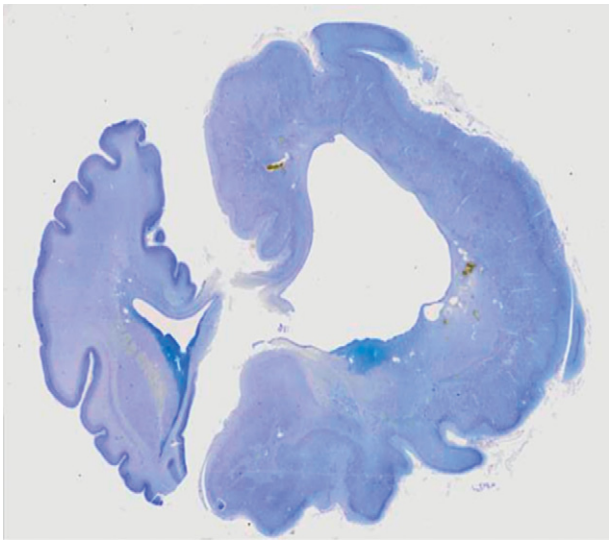


Figure 1 Hemimegalencephaly in a 1-month-old female. Note dramatic enlargement of one hemisphere (specimen courtesy of E. Aronica).

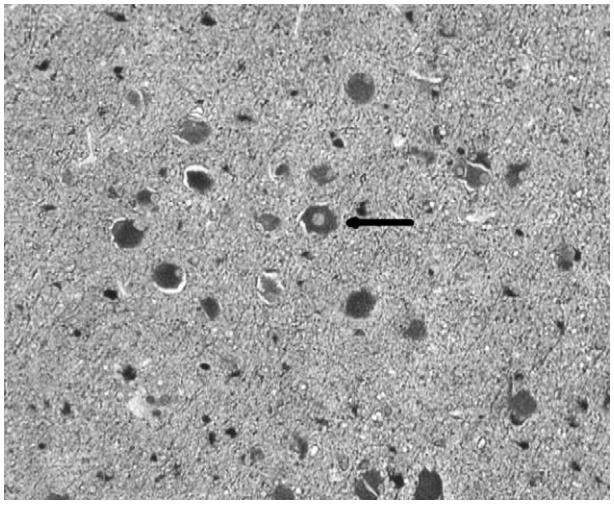


Figure 2 Balloon cells (arrow) in FCD labeled with NeuN antibodies. Note characteristic cytomegaly and laminar disorganization.

these MCD affect restricted regions of the cerebral cortex, share histopathological features, affect children and adults, and are highly associated with intractable epilepsy. TSC is an autosomal dominant disorder resulting from mutations in one or two genes (*TSC1* and *TSC2*), whereas FCD, GG, and HMEG are sporadic disorders that form by unknown mechanisms. It is estimated that 10–30% of resected neocortical epilepsy specimens are diagnosed as FCD. HMEG is highly associated with severe intractable neonatal seizures and infantile spasms, a devastating epilepsy syndrome in infants that often requires urgent neurosurgical intervention. GG represents the most common pediatric tumor associated with intractable seizures. The healthcare impact of these brain malformations (tubers, FCD, GG, and HMEG) is significant, and includes issues such as cost, morbidity, and even mortality.

Histopathology in Focal MCD: A Unifying Feature?

Tubers, FCD, GG, and HMEG are characterized histologically by disorganized or absent cortical lamination, loss of radial neuronal orientation in relation to the pial surface, and the presence of large, dysmorphic neurons ('cytomegalic' neurons). However, a unifying histopathological feature of these developmental lesions is the presence of unique cells that exhibit dramatic cytomegaly; these cells are known as balloon cells (BCs) in FCD (Figure 2) and HMEG, as atypical ganglion cells (AGCs) in GG, and as giant cells (GCs) in tubers.

Morphologically, BCs, AGCs, and GCs are not similar to any known cell types in the normal human cortex. These cell types are larger than dysmorphic, cytomegalic neurons or normal-appearing neurons in FCD, GG, and HMEG and lack the neuronal morphology characteristic of dysmorphic, cytomegalic neurons. The cell soma of BCs is quite large (often exceeding 120 μm) and exhibits a laterally displaced nucleus that may even be multinucleated. Unlike cytomegalic neurons, BCs do not extend clearly defined axons or dendrites and express phenotypic features suggestive of a mixed astrocytic and neural lineage. For example, the expression of neural protein markers such as neurofilament and α-internexin, as well as neurotransmitter receptor subunits and neurotransmitter uptake proteins, suggests a neural phenotype for BCs, AGCs, and GCs. At the same time, the expression of glial markers such as S-100, vimentin, and glial fibrillary acidic proteins in BCs, AGCs, and GCs suggests an astrocytic phenotype. In some cases, cytomegalic cells also express both neuronal and glial markers, suggesting a mixed glioneuronal lineage (or that these cell types can be further delineated into glial- or neural-derived lineage subtypes). The highly aberrant morphologies and phenotype of BCs, AGCs, and GCs suggest that genesis of these cells may be critical to all other histological findings in FCD, tubers, and HMEG.

Intersecting and Parallel Cell Cascades Modulate Cell Size

Recent insights into the roles that the *TSC* genes play in governing cell size and growth have shed light on cytomegaly as a feature of tubers, FCD, and HMEG. The *TSC1* gene encodes the protein TSC1 (also known as hamartin), and the *TSC2* gene encodes the protein TSC2 or tuberin. Hamartin and tuberin form a functional protein–protein complex that modulates several pivotal cellular pathways

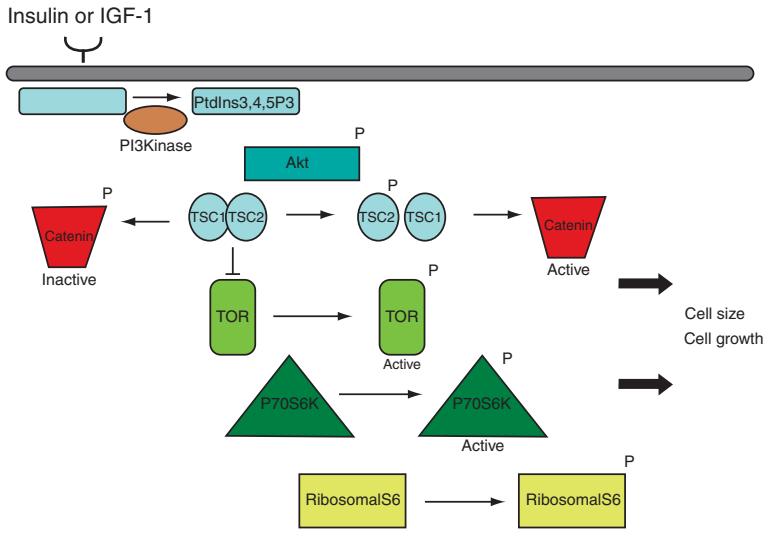


Figure 3 Overview of the TOR and Wnt-1/β-catenin paths. Note that hamartin–tuberin play pivotal negatively modulatory roles in both cascades.

that govern cell size, cell proliferation, and organ growth. For example, the hamartin–tuberin heteromer is a pivotal regulatory complex in the PI3K–Akt–TOR–p70S6-kinase–ribosomalS6 cascade (Figure 3).

Hamartin–tuberin functions downstream from insulin- and insulin-like growth factor receptors, PI3kinase, and Akt to constitutively inhibit the phosphorylation of proteins – including m TOR (mammalian target of rapamycin), p70S6 kinase, and ribosomal S6 – that contribute to ribosomal assembly and protein translation. This inhibition may occur as a direct stepwise cascade or may occur via yet-to-be-identified intermediary steps. Negative modulation of this cascade by the hamartin–tuberin complex results in growth suppression and restricted cell size. When tuberin is inactivated by Akt-mediated phosphorylation in vitro, hamartin and tuberin dissociate, resulting in Rheb (Ras homolog expressed in brain)-mediated phosphorylation of TOR, p70S6kinase, and ribosomal S6 proteins (Figure 3). A similar effect occurs in the setting of loss of function mutations, in either *TSC1* or *TSC2*, that lead to failed heteromer complex formation. For example, enhanced phosphorylation of TOR, p70S6 kinase, and ribosomal S6 is observed in neuroepithelial cells derived from *Tsc2* knockout mice, and increased phospho-ribosomal S6, in particular, has been identified in renal angiomyolipoma cells from TSC patients. Phosphorylation (activation) of TOR, p70–S6-kinase, and ribosomal S6 is observed following tuberin inactivation by Akt-mediated phosphorylation in vitro or in cells lacking tuberin or hamartin in vitro. Loss of function mutation in the Drosophila *dTSC2* mutant *gigas* leads to enlarged ommatidia, head, and wings.

TSC1 and TSC2 have been shown to act as a constitutive inhibitor of the Wnt/β-catenin pathway, which like the PI3K–Akt–TOR–p70S6kinase–ribosomalS6 cascade is pivotal in the regulation of cell growth. β-Catenin is a transcriptional signaling modulator of the Wnt pathway via effects on T-cell factor (TCF)-mediated gene transcription. Wnt binding to its receptor, frizzled, leads to transport of nonphosphorylated β-catenin to the nucleus where it functions as a transcriptional activator. Several downstream genes, including cyclin D1 and c-myc, have been identified that are transcriptionally activated by the Wnt/β-catenin cascade. β-Catenin is active when dephosphorylated, but is targeted for degradation in the cytoplasm after phosphorylation by a GSK-3/APC/axin1 complex at critical residues encoded in exon 3. Binding of Wnt to frizzled leads to phosphorylation of disheveled (dvl-1), which inhibits GSK activity. Total β-catenin protein levels were found to be elevated in TSC2-related renal tumors. The TSC1–TSC2 complex also inhibits Wnt-1 stimulated Tcf/LEF luciferase reporter activity. TSC1 and TSC2 coimmunoprecipitate with GSK3 and axin, suggesting a direct effect of TSC1–TSC2 on β-catenin. Enhanced expression of cyclin D1 mRNA has been reported in single microdissected GCs in tubers, supporting the role of tuberin in β-catenin modulation. Transgenic mice, in which the Wnt/β-catenin pathway was constitutively activated, were shown to exhibit megalencephaly and abnormal cortical gyral patterning. Elevated levels of cytoplasmic β-catenin have been demonstrated immunohistochemically in BCs in FCD. The *PTEN* gene negatively modulates both PI3Kinase-Akt and Wnt/β-catenin pathway activity, and targeted knockout of the PTEN gene in mice leads to megalencephaly and seizures. Finally, recent evidence suggests that the PI3K-mTOR cascade may intersect and function in parallel with three additional cell cascades related to cell

growth or proliferation – the mitogen activated kinase (MAPK)/p42/p44, JNK, and Jak–Stat pathways. These cell-signaling cascades may be activated by PI3K or Akt, bypassing Rheb or p70S6kinase, and lead to phosphorylation of ribosomal S6 protein. There is evidence to support MAPK activation in TSC. Thus, elucidation of select cell cascades – whose direct cellular function is to control cell size – offers a number of compelling candidate molecules that may be involved in the generation of enhanced cell size in FCD and HMEG.

Molecular Mechanism of FCD and HMEG Formation

Clearly, cytomegaly is a pivotal histopathological feature of FCD and HMEG, and likely arises by persistent or aberrant activation of a cell cascade such as the PI3K–Akt–TOR–p70S6kinase–ribosomalS6, Wnt/β-catenin, (MAPK)/p42/p44, JNK, or Jak–Stat pathways. However, FCD does not result from mutations in either *TSC1* or *TSC2*. Thus, it seems plausible that altered regulation, either up- or downstream of the PI3K–Akt–TOR–p70S6kinase–ribosomalS6 or Wnt/β-catenin pathways, may be implicated in the formation of FCD and HMEG, and specifically in the generation of BCs, during brain development.

How might FCD and HMEG form during embryogenesis? We propose that FCD and HMEG likely arise from an abnormality occurring during cell proliferative stages of cortical development. This hypothesis provides a conceptual framework that has driven several novel methodological approaches. Etiologic possibilities, including hypoxia-ischemia, intrauterine infection, nutritional deficit, or environmental toxins such as cocaine or ethanol, have been considered as inciting events responsible for FCD and HMEG. There have been recent studies to suggest that cytomegaly can reflect a reactive process in response to tissue injury. For example, 'mega-neurons' have been reported around the perimeter of porencephalic cysts. These cells are clearly neuronal in that they express neuronal marker proteins and are quite morphologically distinct from BCs. While reactive hypertrophy may reflect an injury process, the cell-specific activation – in TSC – of a cascade clearly linked to enhanced cell size provides highly plausible evidence that activation of these cascades (or a related parallel cascade) contributes to cytomegaly in FCD and HMEG.

Recent speculations have suggested the compelling hypothesis that FCD and HMEG result from a somatic gene mutation occurring during cortical development. No FCD or HMEG pedigrees have been identified, and monozygotic twins appear to be independently discordant for FCD and HMEG. Thus, FCD and HMEG may result from a mutational event occurring in a neural or astrocytic progenitor cell. On the basis of lineage analysis experiments in our lab, we postulate that a somatic gene mutation occurs in a single progenitor cell at a critical stage in cortical development, and that this cell continues to divide and yield progeny carrying a mutant gene. The effect of the gene mutation on these daughter cells would include cytoarchitectural abnormalities, cellular dysmorphism, and laminar disorganization observed in FCD and HMEG.

Somatic mosaicism provides a plausible explanation for the salient features of FCD and HMEG, including sporadic occurrence, histological heterogeneity, and restriction of the lesion to a given brain region or to one hemisphere. First, since a somatic mutation occurs long after meiotic recombination, these events are not germline mutations and thus are confined only to the progeny of cells derived from the original progenitor cell sustaining a mutation. From a practical perspective, this means that causative mutations in FCD and HMEG are not likely to be identified in leukocyte DNA. In addition, these disorders are not heritable. Second, the cellular heterogeneity found in FCD and HMEG suggests that distinct populations of cells may form by separate molecular mechanisms, and thus it is likely that FCD and HMEG contain cells derived from the initial progenitor that sustained a gene mutation that are directly adjacent to other cells born from distinct precursors cells that did not sustain a somatic gene mutation. Third, because the effects of a gene mutation are confined to a restricted population of cells, cytoarchitectural abnormalities remain within a focal brain region. The differences in the amount of cortex that is affected in FCD and HMEG can be accounted for *when* a somatic mutation occurs. For example, an earlier-occurring mutation would, in theory, render an effect on cell morphology and migration over a longer developmental time epoch, and thus lead to a more broad region of abnormality in the cortex (as observed in HMEG). In contrast, a later-occurring mutation might lead to a more focal or restricted region of structural abnormality (as in FCD).

Experimental Methodologies

Since FCD and HMEG are sporadic disorders, it is difficult to implement conventional gene screening approaches in which family pedigrees are collected for genetic analysis. Thus, analysis of DNA extracted from human FCD and HMEG brain tissue specimens provides the most direct avenue to study the molecular pathogenesis of these lesions. While there are numerous animal strains that exhibit cerebral cortical malformations, none contain cellular equivalents of BCs. One clear and highly likely benefit of human studies will be the potential identification of candidate genes that can be targeted for mouse model systems in the future.

Studies to define the pathogenesis of focal MCD include three primary experimental strategies: (1) localization and quantification of individual proteins in focal MCD, using immunohistochemistry and Western assay, (2) analysis of mRNA expression using cDNA and oligonucoleotide arrays, and (3) assaying gene sequence in select MCD specimens obtained during epilepsy surgery or at postmortem examination. Using these three approaches, the expression of specific candidate gene families – such as cytoskeletal elements, neurotrophins, neurotransmitter uptake sites, cell-signaling molecules, and neurotransmitter receptor subunits – that are directly relevant to cortical development and epilepsy has been assayed in FCD and HMEG.

Analysis of focal MCD specimens resected during epilepsy surgery is the most common approach to studying the neurobiology of these lesions. There are several important caveats to analysis of resected specimens. First, the expression of cell-selective protein markers in focal MCD may not demarcate specific cell types or lineages, but instead may reflect aberrant gene transcription or protein translation due to the abnormal phenotype of cytomegalic cells or to up- or downstream changes in the expression of other genes that are distinct from lineage identities. Alternatively, the profound abnormalities of cortical lamination in focal MCD may cause altered expression of protein markers distinct from the molecular events leading to lesion formation. It is conceivable that the presence of seizures alone, or antiepileptic drug exposure alone, can alter protein expression. Thus, comparison with a nonseizure control group is essential. It is also important to keep in mind that differential expression of individual proteins could represent regional expression variability in the normal cortex, so it is essential to compare homologous regions of cortex.

Recent Developments

Over the past 5 years, several key developments have added new insights into our understanding of focal cortical malformations. For example, immunohistochemical analysis of focal MCD specimens resected during epilepsy surgery has identified numerous differentially expressed proteins in focal MCD; some provide new insights into pathogenesis, while others shed light on the mechanisms of epileptogenesis. These results have included information regarding distinct cell-signaling cascades, cell lineage markers, and expression of neurochemical markers such as neurotransmitter receptor subunits and uptake sites.

Perhaps most revealing has been the finding, independently verified in three laboratories, that the mTOR cascade is activated in tubers, FCD, HMEG, and gangliolglioma in a cell-specific fashion. The detection of hyperphosphorylated S6 protein is largely confined to cytomegalic cells in each lesion type. In TSC, the TSC1 and TSC2 proteins are direct upstream negative modulators of mTOR. Thus, the detection of hyperactived mTOR signaling in all four lesion types, as evidenced by hyperphosphrylation of S6 protein (a downstream marker for mTOR activation) provides a potential pathogenic link between these phenotypically similar disorders. The cell specificity of mTOR activation suggests the challenging concept that focal MCD may be a mosaic of cells with differential activation of TOR.

Expression of Phospho-S6 in Focal Malformations: mTOR Cascade Activation

On the basis of known functions of TSC1 and TSC2 and their role in mTOR cascade activation, we have recently found that cells meeting morphological criteria for GCs express phosphorylated p70S6kinase (phospho-S6K) and ribosomal S6 protein (phospho-S6), whereas cytomegalic neurons and astrocytes do not. There is a virtual absence of phospho-S6K and phospho-S6 immunoreactivity in normal control cortex or in nontuber brain regions from postmortem TSC brain specimens, highlighting the specificity of phospho-S6K and phospho-S6 labeling in GCs. These results provide evidence that GCs represent a unique subpopulation in tubers that express phospho-S6K and phospho-S6, and demonstrate a clear association between phosphorylation of p70S6kinase and ribosomal S6 protein and loss of hamartin or tuberin expression.

Phospho-S6 is selectively expressed by BCs in FCD (**Figure 4**), GG, and HMEG – but not in adjacent cytomegalic neurons or astrocytes. The number of phospho-S6-labeled BCs was quite consistent across FCD samples.

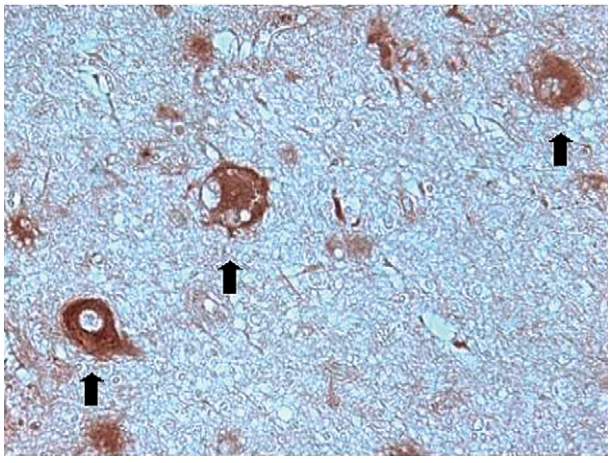


Figure 4 Phospho-S6 expression in BCs (arrows).

As in TSC, phospho-S6 is expressed in a highly cell-specific fashion, suggesting that the pathogenic process leading to lesion formation may not occur ubiquitously but rather in a select subgroup of cells. Thus, if we hypothesize that mTOR hyperactivation is a central mechanism of lesion formation in TSC as well as other focal MCD, then analysis of candidate gene expression and a search for candidate gene mutations must be confined to those cells that express phospho-S6.

Single-Cell Microdissection for mRNA Expression Analysis in Focal Malformations

A pivotal experimental strategy in our lab is the application of single-cell microdissection (Figure 5). Single-cell microdissection permits phenotypic classification of individual cells (by immunohistochemical labeling) so that alterations in gene expression can be determined in defined cell types. Thus, potential contamination of gene expression by the heterogeneous cell types in focal MCD in a homogenate is eliminated.

We have used this approach to define mRNA expression in fixed, paraffin-embedded brain specimens obtained intraoperatively during epilepsy surgery or at postmortem exam. For mRNA amplification studies, single cells are first microdissected using a stainless steel or borosilicate microscalpel driven by a joystick micromanipulator and then aspirated into a second pipette tip (the Eppendorf TransferMan apparatus). An oligo-dT24 primer coupled to a T7 RNA polymerase promoter is used to generate cDNA from poly (A) mRNA with a high-fidelity avian myeloblastosis reverse transcriptase. The cDNA then serves as a template for mRNA amplification incorporating ^{32}PCTP. Radiolabeled mRNA is used to probe large- or small-scale laboratory-generated or commercial cDNA arrays containing candidate genes of interest. We have demonstrated the success and reproducibility of the mRNA amplification protocol in surgical specimens and in postmortem brain tissue despite intervals to autopsy of as long as 18 h.

In a recent study, we used phospho-S6 protein as a marker for GCs and BCs and found that differential expression of 28 candidate genes distinguished BCs from GCs. Gene expression profiles were observed in four patterns, including changes in both GCs and BCs, in GCs alone, in BCs alone, or no change in expression. The expression profile of five mRNAs was identical between GCs and BCs whereas the expression of 12 mRNAs was selectively altered in BCs, and 12 distinct mRNAs were selectively altered in GCs. There was only modest variability among the data points in each cell group, and many of the individual mRNA abundances were similar within each cell type. One interesting finding that supports our hypothesis that activation of select pathways may lead to ribosomal S6 phosphorylation and cytomegaly was that both IGF-1 (insulin-like growth factor-1) and IGF-2 were significantly upregulated in BCs. These growth factors are upstream of PI3K–Akt, β-catenin, MAPK, JNK, and Jak–Stat cascades, and are regulated in a feedback loop by these pathways. A similar study was performed in which differential gene expression was defined in microdissected phospho-S6-labeled cells in FCD. Those investigators reported changes in numerous genes, including the gene for neurofilament, suggesting that the cell specificity of S6 protein phosphorylation is accompanied by cell-specific changes in gene expression.

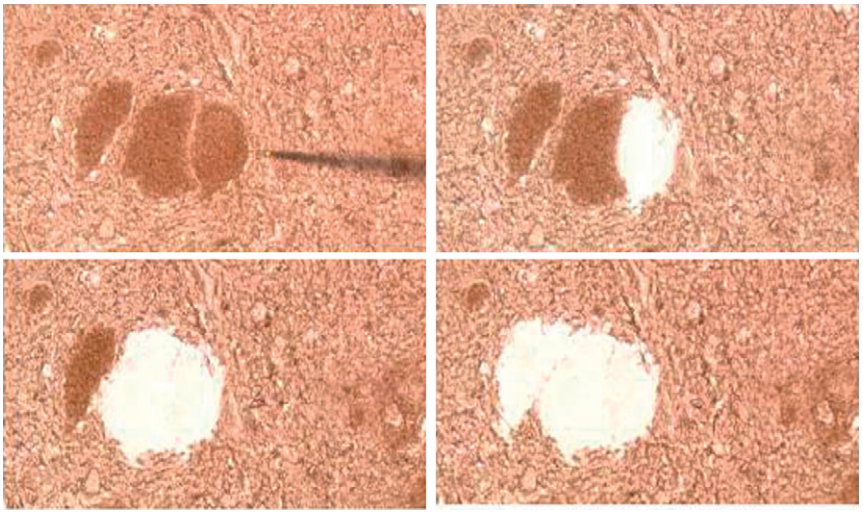


Figure 5 Microdissection of single NeuN immunolabeled cells from FCD sample. Note clean dissection with microscalpel (arrow) and aspiration of single cells in close proximity.

Recent data from our laboratory have used mRNA expression profile analysis to define differential expression of numerous candidate genes in HMEG. These studies were carried out in tissue homogenates, but nonetheless revealed changes in expression of several interesting classes of genes, including neurotransmitter receptor subunits and uptake sites, as well as transcription factors and cell-signaling molecules. We have implemented a functional cluster analysis to look at distinct classes of genes in TSC, FCD, and HMEG, and found that altered expression of genes can be clustered according to four categories (cell adhesion molecules, cell-signaling molecules, growth factors/growth factor receptors, and transcription factors) in these three lesion types.

Differential expression of 16 genes (including mRNAs for glutamate transporter (EAAC1) and receptor subunits (NMDA2C, mGluR5)), growth factor (hepatocyte growth factor), and growth factor receptors (platelet-derived growth factor receptor β, fibroblast growth factor receptor) was detected in GG neurons compared with control neurons. In astrocytes within GG, altered expression of p75NGF, mGluR3, TGFβ3, and Glt-1 mRNAs was detected. These findings demonstrate that altered gene transcription occurs in noncytomegalic cells in focal malformations.

Stem Cell Markers

One consistent finding in focal MCD has been the detection of protein markers in cytomegalic cells that are typically expressed by embryonic neurons or neuroglial precursor cells. For example, vimentin, phosphovimentin, nestin, and CD34 are normally expressed in progenitor cells in early stages of cortical development, but are then shut off in mature cerebral cortex. Over the past 10 years, expression of nestin, vimentin, phosphorylated vimentin, Mcm2, and CD133 has been identified in cytomegalic cells in focal malformations. These data suggest that cytomegalic cells in focal MCD may have failed to fully differentiate, may retain embryonic features, or may indeed represent a unique neural stem cell.

Challenges for the Future

One particular limitation for this research has been tissue specimen availability. It would seem logical for a repository of resected tissue to be generated so that individual samples can be stratified or classified according to tissue pathology or other clinical features. By more completely classifiying tissue samples, the chances of identifying a gene mutation or aberrant cell pathway are dramatically increased. To fully define the pathogenesis of focal MCD associated with epilepsy, additional high-throughput methodologies will be needed to screen for candidate gene mutations. Another limitation is that current technologies to screen for changes in mRNA expression or DNA sequence alterations are limited by paraffin fixation methods. Thus, development of new approaches to aid with in silico analysis of gene expression (or mutation in paraffin-embedded tissue specimens) is of paramount importance.

See also: **Development/Malformations**: Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue

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Introduction

The cerebral cortex, particularly in early development, is exquisitely sensitive to external insults; in response to such insults, it may become hyperexcitable and capable of generating paroxysmal activity, even though its constituent neurons have no obvious morphological abnormalities. It is thus not surprising that the search for 'epileptic' neurons in cortical or hippocampal tissue has been unsuccessful. Disruption of the balance between excitatory and inhibitory networks is now favored by most scientists as the mechanism by which epileptic activity is generated. However, in focal cortical dysplasia (CD), the presence of abnormal cell types has raised the suspicion that these cells could be epileptogenic.

Focal CD was first described in 1971 by Taylor and associates as a histopathological abnormality in surgical specimens from epileptic patients, characterized by cortical dyslamination, pyramidal cell disorientation, and the presence of abnormal cells – in particular, cytomegalic neurons and balloon cells. Although the potential role of these cells in epileptogenesis had been recognized, technical limitations prevented their electrophysiological characterization. With the introduction, in the 1990s, of infrared videomicroscopy in combination with differential interference contrast optics (IR-DIC), it became possible to visualize individual cells in living brain slices. IR-DIC and whole-cell patch clamp recordings constitute a powerful approach for the characterization of different cellular types in specific brain regions. In CD, this methodology allowed for the first time a systematic electrophysiological characterization of abnormal cells. The in vitro dissociated cell preparation has also been used to characterize, in more detail, abnormalities in ionic channels and receptors in cells from CD tissue. This article will summarize studies, describing some of the properties of these abnormal cells and providing a hypothesis about how CD tissue may be able to generate epileptic discharges.

Background

Electrophysiological recordings from human tissue resected for the treatment of pharmacoresistant epilepsy have been performed for many years. However, after a considerable number of such studies, the evidence for alterations in intrinsic membrane properties of cortical or hippocampal neurons in epileptic tissue was tenuous at best. Attempts to find abnormal cells in CD tissue, even from severe cases, were also unsuccessful, partly because of technical limitations as well as the inclusion of a heterogeneous population of epilepsy cases. Pioneer work by Avoli and associates, mostly in adult surgical patients, showed that CD tissue is particularly susceptible to epileptogenic agents (as compared to tissue resected from temporal lobe epilepsy cases), but tissue susceptibility could not be correlated with the presence of abnormal cells as these studies used blind recording techniques and neuronal staining was not attempted. Other studies also failed to find abnormal cells or synaptic transmission in tissue from children with catastrophic epilepsy, even when cells were intracellularly labeled with biocytin or Lucifer yellow. However, the patient population investigated in these studies was heterogeneous and included multiple pathologies.

Methodological Aspects

The Pathological Substrate in CD

CD is not a homogeneous pathological entity. In fact, several types have been described. To provide a first approximation, the pathology group at UCLA divided CD into mild and severe. More recently, CD has been classified in different subtypes, including CDI, where only architectural abnormalities are found, and more severe cases (where, in addition to cortical dyslamination, the sample contains abnormal cells) – CDIIA (with cytomegalic neurons) and CDIIB (with balloon cells). Even within the same patient, areas removed at surgery have different degrees of abnormality. Tissue typically has been separated into the most and the least abnormal areas, as based on electrocorticographic (ECoG), MRI, and PET studies. Areas with the highest degree of ECoG abnormalities correlate with severely abnormal MRI and pathological findings. Further, successful surgical outcome correlates well with removal of the most abnormal tissue. It is in these highly abnormal samples where the likelihood of finding abnormal cells is very high. Tuberous sclerosis complex (TSC), an autosomal dominant

disease caused by mutations in *TSC* genes (which encode hamartin and tuberin), shares histopathological similarities with severe CD; abnormal cells are abundant, particularly in the vicinity of the cortical tuber.

IR-DIC Optics and Electrophysiological Recordings

Correlative morphological and electrophysiological studies of pediatric CD tissue were first undertaken in our laboratory 10 years ago, with the goal of finding abnormal cellular types in severe CD and other pathological substrates. Individual cells in live tissue slices are visualized with IR-DIC optics. One limitation of this technique is that the age of the tissue plays an important factor. Younger tissue, with less myelination, is more accessible to adequate visualization compared to older tissue. Typically, the tissue samples most accessible to IR-DIC visualization are from patients younger than 5 years of age. Also, the health of the cells and the possibility of maintaining the slices for long periods of time in vitro are best in the young tissue samples. In our cohort, many cases are less than 2 years old (the youngest cases are only 2 months old), which permits electrophysiological recordings under optimal conditions.

Samples of CD tissue are prepared for in vitro analysis using the acute slice preparation approach. It is very important to slice the tissue sample as soon as possible after resection; slices (350 μm) should be cut within 10–15 min after obtaining the sample. The slices are allowed to recover for at least 1 h before recordings are obtained. For current clamp recording, a K^+-gluconate-based electrode solution is used; for voltage clamp recordings, a Cs^+-methanesulfonate-based electrode solution is used. After the recovery period, a slice is placed in a custom-made chamber attached to the fixed stage of an upright microscope. Potentially abnormal cells are visualized (40x lens) and selected for recording. Cytomegalic pyramidal neurons are easy to recognize by their large size, whereas balloon cells are harder to distinguish; they are usually large and round and appear pale.

Recent Results

One of the hallmarks of CD, in nearly all cases, is pyramidal cell disorientation, as manifested by a rotation of the cell body and deviation of the apical dendrite from the normal perpendicular orientation with respect to the pial surface. In some cells, disorientation is very subtle (~15–30° rotation), but in other cases it is more noticeable and complete inversion of the soma is not uncommon. A number of electrophysiological properties of disoriented pyramidal neurons were examined. The basic membrane properties of these cells are very similar to those of normal pyramidal neurons, and the cells do not appear to exhibit signs of hyperexcitability. Although it is possible that disoriented pyramidal neurons display subtle differences in synaptic inputs compared to correctly oriented pyramidal neurons, current evidence indicates that disorientation per se may contribute to – but is probably not a causal factor underlying – epileptic activity. This interpretation is consistent with the fact that a mouse model of cortical migration disorder, the Reeler mouse, does not display spontaneous seizures.

Another type of morphological finding in CD tissue is the presence of pyramidal neurons with tortuous or sinuous neurites. These neurons have normal somata but the apical or basilar dendrites, and sometimes also the axon, display segments that zigzag for variable distances, as though they were unable to find a specific target or pathway during neuritic growth. This feature could be another marker of migration disorder. The role of these neurons in the generation of paroxysmal discharges is still unknown, but electrophysiological recordings did not reveal clear signs of hyperexcitability.

Balloon cells are some of the most bizarre cells in CD tissue. They are found in ~50% of pediatric CD tissue from surgical cases and are very common in TSC and in severe CD (CDIIB). Morphologically, they are reminiscent of fibrillary astrocytes, but some features are more neuronal. They lack dendritic spines and an axon is not visible. They usually display very abundant, tortuous processes that extend for several hundreds of microns and become progressively thinner. Immunocytochemical studies revealed that balloon cells display neuronal and glial markers alone or in combination, suggesting that balloon cells did not 'commit' to a neuronal or glial phenotype and remained undifferentiated. For some time, investigators speculated that balloon cells could be the culprits – i.e., the basis of epileptogenicity – in CD. However, electrophysiological recordings demonstrated that these cells are unable to generate inward currents, do not respond to excitatory amino acids, and do not display spontaneous synaptic activity. In more recent studies, balloon cells were found not to be completely inert; they produced membrane oscillations in the presence of the K^+ channel blocker, 4-aminopyridine, similar to those generated by glial cells. The role of balloon cells in the generation of epileptic activity in severe CD is still an open question. However, based on the fact that these cells are unable to generate action potentials, their role in epileptogenesis is probably only minor.

The presence of cytomegalic pyramidal neurons is another hallmark of CD and occurs in ~60% of pediatric CD cases. These cells are 2–3 times larger than normal pyramidal neurons, and also display very thick processes, including the axon hillock. Spines are present and can be abundant in some dendritic segments, but are scarce in

other dendritic segments. Cytomegalic pyramidal neurons have abnormal membrane properties. Due to their large size, the membrane capacitance and time constants are significantly larger than those of normal pyramidal neurons, and their input resistance is very low. One of the main features of cytomegalic pyramidal neurons is their capacity to generate repetitive calcium oscillations. Because of the practical impossibility to obtain reasonable space clamp in these neurons, the dissociated cell preparation has been used to examine these calcium currents in more detail. These studies suggest that the large inward calcium currents in cytomegalic cells are due to upregulation of calcium channels. We speculate that upregulation of calcium channels gives rise to repetitive calcium oscillations in these cells when they are depolarized. These calcium oscillations could serve as an amplification device for the synchronization of cortical networks and the propagation of epileptic activity. However, these cells are also relatively hyperpolarized and, because of their low input resistance, it is difficult to depolarize these neurons via excitatory inputs. Immunocytochemical studies have revealed peculiar GABAergic basket formations around these neurons, and it has been hypothesized that GABA could depolarize these neurons to generate paroxysmal activity. The *N*-methyl-D-aspartate (NMDA) receptors in cytomegalic pyramidal neurons display reduced Mg^{2+} sensitivity, so that these receptors open (and permit calcium influx) at more hyperpolarized membrane potentials than normal cells – which can also lead to hyperexcitability.

In severe CD, particularly in cases of hemimegalencephaly, we recently described another type of abnormal neuron. Cytomegalic interneurons were found in the areas of greatest abnormality, in close proximity to the cytomegalic pyramidal neurons. The incidence of these cells in surgical material is presently unknown. Cytomegalic interneurons are 2–3 times larger than normal interneurons and can be labeled with GABAergic markers such as GAD, calbindin, and parvalbumin. They are generally basket cells and display increased numbers of dendrites compared to normal basket cells. These cells display some signs of hyperexcitability, such as non-inactivating Na^+ spikes when a depolarizing ramp voltage command is applied. An important feature of some cytomegalic interneurons is that these are the only cells in which spontaneous paroxysmal depolarizing shifts occur in vitro under physiological conditions. It is thus likely that cytomegalic interneurons play an important role in the generation of epileptic activity, assuming that GABA is excitatory in some pyramidal neurons from CD tissue.

Immature-looking pyramidal neurons can be observed in mild and severe CD tissue from patients generally younger than 2 years of age. Although, strictly speaking, these cells are not 'abnormal,' their presence in postnatal tissue was unexpected. They tend to occur in clusters, similar to the clones occurring during early neuroblast migration. Their morphological and electrophysiological properties are typical of immature pyramidal neurons, including incipient dendrites and spines, very high membrane input resistance, low capacitance, fast time constants, and small inward currents. But the main and most remarkable characteristic of these immature neurons is the presence of abundant, rhythmic spontaneous $GABA_A$ receptor-mediated synaptic activity. GABAergic synaptic events are depolarizing and may induce action potentials that can be blocked with bicuculline, a $GABA_A$ receptor antagonist. These observations support the notion that in early development GABA is the main excitatory neurotransmitter and led us to propose that CD tissue displays dysmature properties. Recordings of spontaneous synaptic activity from normal-appearing pyramidal neurons indicated that in CD tissue there is an over-abundance of GABAergic activity (relative to glutamate receptor-mediated activity). Paradoxically, anatomical studies have consistently found that the number of GABAergic interneurons is reduced in CD tissue. How can this reduction in GABAergic cells be reconciled with the observation of relatively high spontaneous GABAergic synaptic activity? One possibility is that synaptic reorganization of the remaining GABA interneurons occurs. Another is that in severe CD cases, cytomegalic interneurons provide rich innervation onto cytomegalic and normal-appearing pyramidal neurons. In fact, the presence of GABAergic basket formations surrounding cytomegalic pyramidal neurons suggests abundant GABAergic innervation and synaptic reorganization in CD tissue. The origin of this innervation is still unknown and could involve both intrinsic as well as extrinsic sources.

Hypotheses attempting to explain the intrinsic epileptogenicity of CD tissue range from those proposing that abnormal neurons can generate epileptic activity to those suggesting involvement of aberrant circuits. Needless to say, a combination of those factors is a more plausible explanation. The idea that cytomegalic pyramidal neurons and/or balloon cells are the generators of paroxysmal discharges has been entertained by several authors. Immunocytochemical studies seemed to support an important role of cytomegalic pyramidal neurons as they demonstrated abnormal expression and assembly of NMDA receptor subunits. That balloon cells could also generate paroxysmal activity was suggested based on morphological studies. However, as shown above, the balloon cells are unable to generate action potentials, and lack synaptic connections and axonal processes. Thus, their role in epileptogenesis can only be indirect. Immature cells are also candidates of hyperexcitable elements in younger, severe forms of CD. High membrane input resistances can amplify incoming signals. Immature properties also include depolarizing GABA

actions, and immunocytochemistry has shown altered expression of chloride transporters NKCC1 and reduced KCC2 in CD tissue. Recently described cytomegalic interneurons, in virtue of their hyperexcitability, have the potential to produce spontaneous depolarizing shifts, making them good candidates for generators of synchronous activity. If GABAergic basket formations surrounding cytomegalic pyramidal neurons are indeed depolarizing, GABA released from cytomegalic interneurons has the potential to activate upregulated calcium channels present in cytomegalic pyramidal neurons, which in turn could generate synchronous oscillations. A tentative scheme of an epileptogenic locus, constituted by a set of abnormal neurons and circuits in a brain with severe CD, is illustrated in Figure 1. In cases of mild CD, which do not contain abnormal cells, the generation of epileptic discharges has to rely on other abnormal functional properties. For example, aberrant excitatory and/or inhibitory circuits as well as abnormal receptor sensitivity in normal-appearing neurons could play important roles.

Directions for Future Research

Many unanswered questions still remain. For us, one of the most important questions is the origin of the abnormal cells in severe CD. When were they generated and, if this occurred during embryonic development, why do they remain in postnatal brains? Our studies suggest that CD is the consequence of abnormalities occurring late in neurogenesis and involve excessive generation of neurons and retention of preplate cells in the molecular layer and subplate regions. This retention could be the product of arrested development and/or decreased apoptosis. The fact that abnormal cells observed in severe CD resemble cells commonly found in the subplate supports our

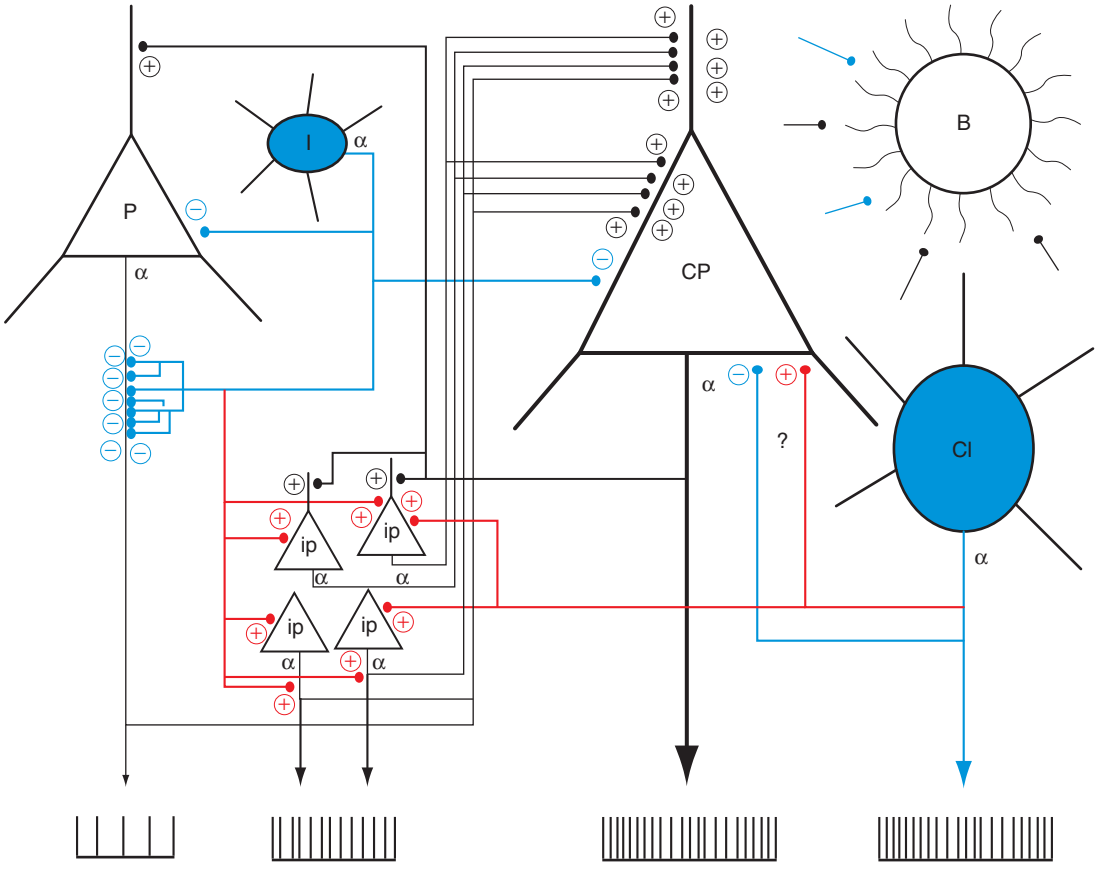


Figure 1 Diagram illustrates network hyperexcitability in an area of severe CD. It is hypothesized that cytomegalic (Cl) or normal GABAergic interneurons (I) generate pacemaker activity able to excite cytomegalic (CP) and immature (IP) pyramidal neurons. These clusters of immature pyramidal neurons, being excited by both glutamatergic (black) and GABAergic (red) inputs generate further excitation onto other pyramidal (P) neurons, thus creating a positive feed-back loop. Once cytomegalic pyramidal neurons are sufficiently depolarized they are able to amplify synchronous inputs and propagate abnormal activity to adjacent cortical areas. Balloon cells (B) do not participate directly as they do not receive synaptic inputs and do not appear connected to other cells. The question mark indicates that the effect of GABAergic inputs onto cytomegalic pyramidal neurons is not yet known. α indicates the axon.

hypothesis. Indeed, it is unlikely that cytomegalic neurons became hypertrophic as a consequence of epileptic activity; cases of mild CD (or other pathologies) also sustain epileptic activity, yet cytomegalic neurons are not observed. A number of studies support the hypothesis that balloon cells and dysplastic neurons are derived from radial glial progenitor cells.

The presence of cytomegalic interneurons in severe CD cases is particularly intriguing. These cells are unique in that they can generate spontaneous depolarizing shifts, making them suitable as generators of synchronous activity. It is interesting that in cell cultures from embryonic rat cerebral cortex, a distinct subpopulation of large GABAergic neurons, which constitute only a minority (<4% of all cultured neurons), is a key element in the generation of synchronous oscillatory network activity. In addition, these basket-like neurons are derived from the primordial plexiform layer and reside in the subplate at the time of birth, placing them in an ideal position to distribute activity in the local networks.

In conclusion, the presence of undifferentiated cells, retained embryonic neurons, immature pyramidal neurons, predominance of GABAergic synaptic activity, etc., all point to the idea that the abnormality in CD tissue is one of cortical dysmaturity. If true, then therapeutic approaches to CD-related epilepsies must take into consideration this important factor. For example, increasing GABA function in CD cases may not only be inadequate but even counterproductive.

Acknowledgements

We would like to thank the young patients and their parents for allowing use of resected tissue samples for research purposes. We also thank Raymond S. Hurst, Jorge Flores-Hernández, Elizabeth Hernández-Echeagaray, Nanping Wu, Irene Yamazaki, Marea K. Boylan, and Robin S. Fisher, for their assistance in electrophysiological data collection and morphological analyses. The expertise and dedication of the UCLA Hospital Pediatric Neurology Staff are also greatly appreciated. This work was supported by NIH grant NS 38992 from the NINDS.

See also: **Development/Malformations**: Cortical Malformations as a Cause for Epileptiform Activity: The Freeze Lesion Model; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis

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Introduction

A recurrent theme in epilepsy research is the contribution of neural progenitor defects to epilepsy-associated malformations of cortical development (MCDs). MCDs are associated with pharmacoresistant, intractable epilepsy, predominantly associated with seizure generation in the temporal lobe. MCDs were referred to as local neuroglial lesions in the 1990s. They include epilepsy-associated tumors and nontumoral malformations that have been recently classified based on the developmental stage (e.g., proliferation, migration, or differentiation) when a defect occurs, and subdivided based on their genetic mutations and/or histopathology.

Although MCDs encompass a broad spectrum of disorders, a subset of MCDs with abnormal glioneuronal proliferation and differentiation share similar molecular, histopathological, and clinical features, suggesting a common mechanism through which they contribute to epileptogenesis. These MCDs have been termed epilepsy-associated glioneuronal malformations, and include gangliomas (GGs), severe focal cortical dysplasia (FCD) with cytomegalic cells (called balloons cells), and tuberous sclerosis complex (TSC). These disorders are thought to result from combined defects in neural progenitor proliferation and differentiation, and neuronal precursor migration. Such defects can result from germline or de novo mutations, embryonic or neonatal insults, or a combination of both. It was initially thought that these defects occurred during embryonic life. However, it is becoming increasingly evident that both gliogenesis and neurogenesis within the subventricular zone (SVZ) in neocortex persist during neonatal life. This period is critical during brain development, since this is the time that infants are first directly exposed to the external environment. However, the contribution of neonatal progenitor defects to epilepsy-associated lesions has not been explored.

We propose to test whether defects in neural progenitors during neonatal life contribute to epilepsy-associated MCDs, and more specifically the MCDs (produced by glioneuronal proliferation and differentiation abnormalities) with known genetic mutations (e.g., of *TSC1* or *TSC2*). The results can then be compared to data obtained in studying defects in progenitor cells during embryonic life. Results from such studies should help us better understand the ontogeny of the epilepsy-associated MCDs and epileptogenic nature of these lesions.

Background

Abnormalities in SVZ-Derived Neural Progenitors Contribute to the Formation of Epilepsy-Prone Lesions during the Neonatal Period

Two relatively recent findings have radically changed our view on embryonic cell development and adult cell plasticity. During embryonic development, radial glia, long thought to serve as mere scaffold for migrating neuroblasts, are now recognized as neural progenitors. They can generate both cerebral cortical neurons and astrocytes. As a result, genetic mutations or insults to radial glia can have dramatic consequences on cortical development. For example, they can affect cell number or morphology, resulting in heterotopia (i.e., misplacement) of cortical neurons. In neonates and adults, it is now well-recognized that neurogenesis persists in two regions: the SVZ along the lateral ventricle (LV) and the dentate gyrus subgranular zone of the hippocampus. This continuing generation of new neurons confers an incredible level of plasticity, at the molecular, cellular, and network levels, to a brain long thought to have a fixed neuronal-cell population. Recent findings suggest a contribution of hippocampal neurogenesis to epileptogenesis, which is the subject of other articles in this volume. The contribution of SVZ neurogenesis and gliogenesis to epileptogenesis or seizure activity remains unclear.

The SVZ contains the largest pool of dividing neural progenitors in the adult mammalian brain, including humans. Neural progenitors generate highly proliferative intermediate progenitors (also called transit amplifying cells), which in turn divide to give rise to neuroblasts. These latter cells migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into interneurons. SVZ cells expressing glial fibrillary acidic protein (GFAP), a marker of astrocytes that is also expressed in human radial glia, act as neural progenitors. In this article, these GFAP-expressing cells in the SVZ will be called SVZ astrocytes for simplicity. It was shown that radial glia transform into SVZ astrocytes during the neonatal period, a process well-known for the transformation of perinatal radial glia into parenchymal astrocytes. Neonatal neurogenesis may provide a relatively 'simple' developmental process, the understanding of which may provide insight into the ontogeny of developmental disorders.

It was reported that seizure activity in pilocarpine-injected adult rats increases neurogenesis to the olfactory bulb and also triggers the production of glioblasts in the SVZ. These glioblasts then migrate into the hippocampus where they differentiate into astrocytes and oligodendrocytes. However, it remains unknown whether SVZ cells contribute to epilepsy-prone lesions or exacerbate seizure activity occurring in the cortex. Gliogenesis within the SVZ is clearly ongoing during the neonatal period. In addition, there is increasing evidence that SVZ neural progenitors produce cerebral cortical interneurons during neonatal life as well as neurons in the piriform cortex in adults.

We propose to examine whether SVZ neural progenitors contribute to the formation of epilepsy-associated MCDs during neonatal life. This is a critical period when genetic mutations or insults could alter the behavior of SVZ progenitors, resulting in aberrant cell production and misplacement. As indicated earlier, our studies use a model of TSC to examine neonatal progenitor contribution to epilepsy-associated MCDs with abnormal glioneuronal proliferation and differentiation (see another article in this volume. for a discussion of another TSC model in which mutations in astrocytes result in seizure activity).

TSC as a 'Model' of Epilepsy-Associated MCDs with Abnormal Glioneuronal Proliferation and Differentiation

MCDs with abnormal glioneuronal proliferation and differentiation share similar clinical, histological, cellular, and molecular features. Clinically, patients with these types of MCDs usually have partial seizures during childhood that are thought to originate from within or surrounding the cortical lesions. In addition, these patients display low rates of remission and high rates of medical intractability. In TSC patients, epilepsy is the most common and devastating complication of the disorder, observed in more than 75% of the patients. Seizures usually begin in infants (during the first year of life, corresponding to $\sim$2 weeks in mice) as infantile spasms that are pharmacoresistant. At the histological level, these malformations display glioneuronal hamartias and benign tumor-like lesions called hamartomas. Hamartias correspond to abnormal cellular arrangements – also called dysplasia, nodules, or tubers when located in the cortex. FCD is characterized by the presence of 'balloon cells,' whereas TSC is associated with 'giant cells' in the cortical tubers (FCD with balloon cells is often indistinguishable from TSC at the histological level). The simultaneous appearance of tumors, the detection of neurodevelopmental antigens in cytomegalic cells, and the predominant occurrence of these defects in the temporal lobe and along the LV (i.e., where neurogenesis persists) suggest a defect of neural progenitor development. Although the presence of tubers in the 20-week-old human fetus suggests a defect during embryonic life, de novo mutations may occur at any time during early brain development and the lesions may progress during neonatal life. There are three types of lesions in TSC: cortical tubers, subcortical heterotopic nodules, and subependymal nodules. The first two are thought to be static (hamartia), although subtle anatomical but functionally important changes are not likely to be detected. The last (hamartoma) one is progressive and may transform into subependymal giant-cell astrocytoma (SEGA) in 15% of the patients. At the molecular level, TSC is an autosomal dominant neurocutaneous disorder with germline or predominantly de novo somatic mutations in one of two genes, *TSC1* or *TSC2*, the protein products of which are hamartin and tuberin, respectively. The tubers display positive staining for these gene products, suggesting a relatively subtle mutation that leads to abnormal function. However, the mechanisms of lesion and giant-cell formation remain unclear. The Knudson 'two-hit' model of tumorigenesis (i.e., second-hit mutation resulting in loss of heterozygosity) is thought to explain SEGA formation (SEGAs display loss of heterozygosity). Interestingly, the other MCDs with abnormal glioneuronal malformation (i.e., FCD and ganglioglioma) also display *TSC1* or *TSC2* polymorphisms. Importantly, these MCDs exhibit dysregulation of signaling pathways downstream of tuberin and hamartin, consistent with the idea of a similar (or common) molecular mechanism of pathogenesis.

Methods

In our laboratory, we are using a combination of technical approaches to achieve two goals: (1) to develop an approach to selectively knock out *TSC1* or *TSC2* in neural progenitors and their progeny during the neonatal period, and (2) to examine whether these specific mutations give rise to abnormal progenitor growth, leading to hamartia (cortical tubers) with giant cells and hamartoma. Results will then be compared with those observed with mutations generated at different embryonic time-points.

To address our first goal, we will take advantage of transgenic mice where the *Tsc1* or *Tsc2* gene is floxed. By in vivo electroporating a plasmid or injecting a virus containing a Cre sequence and a fluorescent protein (e.g., red fluorescent protein, RFP), we can selectively knock out the gene of interest in electroporated or infected cells. To address the second goal, we will perform immunohistochemistry, on RFP-positive cells, to identify different neuronal, glial, and progenitor markers. We will investigate the rate of proliferation of *Tsc1/2*-null neural progenitors in the SVZ by using the mitotic marker, bromodeoxyuridine (BrdU), and determining the number of proliferative cells. The identity and properties of RFP-positive cells will then be determined using the patch clamp technique in acute brain slices containing the SVZ and cortex. These studies are at an

early stage; the in vivo electroporation technique in neonates is presented in the research results below, together with recent data suggesting the contribution of SVZ cells to a freeze-induced cortical lesion in neonates.

Recent Results

Several years ago, we thought of a possible contribution of neonatal progenitors to the formation of cortical lesions based on data using the cortical freeze-lesion model. In this model, a freezing probe was placed on the skull of a postnatal day (P) 1 mouse pup for 5 s, resulting in the formation of a focal lesion in the cortex. Three weeks later, this lesion displayed enhanced hyperexcitability but the mice did not exhibit clinical seizures, consistent with previous studies on freeze-lesioned rats. As shown in **Figure 1(a)**, the lesion is strongly immunoreactive for GFAP. When acute slices from lesioned animals were bathed for 2 h in BrdU, a mitotic marker taken up by cells in S-phase, a stream of cells in the SVZ, along the LV and below the corpus callosum (CC), were marked (**Figure 1(b)**). This result suggested that some proliferative cells were migrating out of the SVZ, possibly toward the lesion.

BrdU could be taken up by dying cells in the slices because of their leaky membranes. However, the predominant labeling in the SVZ and below the CC cannot be explained simply by cell injury because SVZ cells remain healthy in acute slices. These data indicated to us that the SVZ may contribute to 'feeding' cells to the cortical lesion. Although this model is attractive for its simplicity and the formation of a hyperexcitable lesion that resembles a microgyrus, it does not involve genetic defects in the formation of epilepsy-associated MCDs. In addition, it does not offer an easy way to alter gene expression in SVZ cells. We thus decided to develop a model of TSC in which the TSC genes can be selectively knock out in neural progenitors.

We first examined whether neonatal SVZ cells express the *Tsc1* and *Tsc2* gene products, hamartin and tuberin, respectively, using immunostaining. We found that tuberin expression was low or not present in SVZ cells in a P1 section (green labeling in **Figure 2(a)** and **2(b)**). Nevertheless, some glutamate–aspartate transporter (GLAST)-immunopositive cells (red labeling in **Figure 2**) along the ventricle (i.e., either radial glia or newly-formed ependymal cells) expressed tuberin. Neurons in the striatum and the lateral septal nucleus expressed high levels of tuberin, suggesting that tuberin levels become higher as cells differentiate into neurons. SVZ cells, in particular cells along the ventricle (presumably radial glia), expressed hamartin, as did striatal neurons (green labeling in **Figure 2(c)**).

To knock out *Tsc1* or *Tsc2*, we will take advantage of transgenic mice where *Tsc1* or *Tsc2* have been floxed (the floxed *Tsc1* mice ($Tsc1^{C/C}$ mice) are commercially available from Jackson Laboratory). Then, one of three approaches can be used to selectively introduce Cre recombinase into SVZ cells: (1) injection of a viral vector (containing the Cre recombinase cDNA) into the LV that will infect SVZ cells, (2) crossing $Tsc1^{C/C}$ mice with GFAP-Cre^{ER2} or nestin-Cre^{ER2}, where intraperitoneal injections of tamixofen will induce Cre expression and deletion of TSC1 in GFAP or nestin-expressing cells, respectively (GFAP is expressed in SVZ neural progenitors but also in parenchymal astrocytes; nestin is predominantly expressed in SVZ cells in neonate and adult mice), and (3) In vivo electroporation of a Cre recombinase-containing plasmid into neonatal SVZ cells. We decided to use the third method because of its simplicity and efficiency, as already shown by in utero electroporation. As a proof of principle, we electroporated a CAG-GFP plasmid at $4\,\mu g\,\mu l^{-1}$ into P2 SVZ cells. Following anesthesia of pups by hypothermia, a glass pipette (30–50-µm beveled tip) was inserted into the LV. Plasmid ejection and electroporation were performed by applying a positive pressure at the tip of

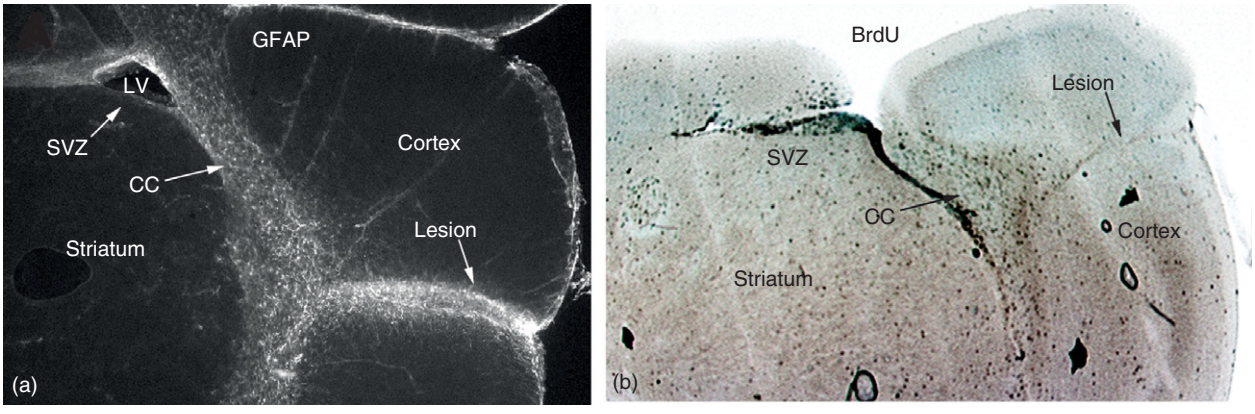


Figure 1 (a) GFAP immunostaining in a mouse coronal section at 21-day post lesion, which was performed at postnatal 1. (b) BrdU immunostaining in a mouse coronal section that was incubated for 2 h in BrdU, fixed, and stained. The section was prepared 18 days post lesion performed at postnatal day 1. CC, corpus callosum; LV, lateral ventricle.

the glass pipette and five 50 ms pulses (150 V) via electrodes placed on top and bottom of the head. Two days post electroporation, SVZ cells (as well as cells on top of the ventricle) displayed strong GFP fluorescence (**Figure 3(a)**). Radial glia were among the electroporated cells, as observed by the presence of GFP-fluorescent and GFAP-immunopositive radial processes crossing the parenchyma and reaching the cortical surface (**Figure 3(b)** and **3(c)**). Thus, electroporation provides a simple procedure by which we should be able to knock out TSC genes in SVZ cells, including radial glia, which are neural progenitors in neonatal mice.

Future Goals

Our immediate goal is to determine whether knocking out *Tsc1* in neonate SVZ neural progenitor cells results in lesion formation in the cortex (i.e., cortical tubers), and whether this lesion is associated with neuronal hyperexcitability, and perhaps clinical seizures or lowered seizure threshold. We also hope to identify cytomegalic cells that display features of the giant cells in TSC patients, as well as the formation of hamartoma along the SVZ (that may transform into SEGA either spontaneously or upon tumorigen exposure). However, we may not find defects in cortical lamination because neonatal neurogenesis is likely restricted to interneuron formation at a time when the cortical layers are already established. In addition, to better mimic the human disorder, it may be important to apply a two-hit injury model. We would then use one of the two following procedures. First, cross *Tsc1* heterozygote mice (noted $Tsc1^{-/+}$) with $Tsc1^{C/C}$ and then perform in vivo electroporation in neonatal $Tsc1^{-/C}$ mice. Interestingly, the $Tsc1^{-/+}$ mice do not develop the neural abnormalities seen in human TSC patients but these mice contain 50% more astrocytes than do littermate control mice. Second, electroporate Cre recombinase-containing plasmids

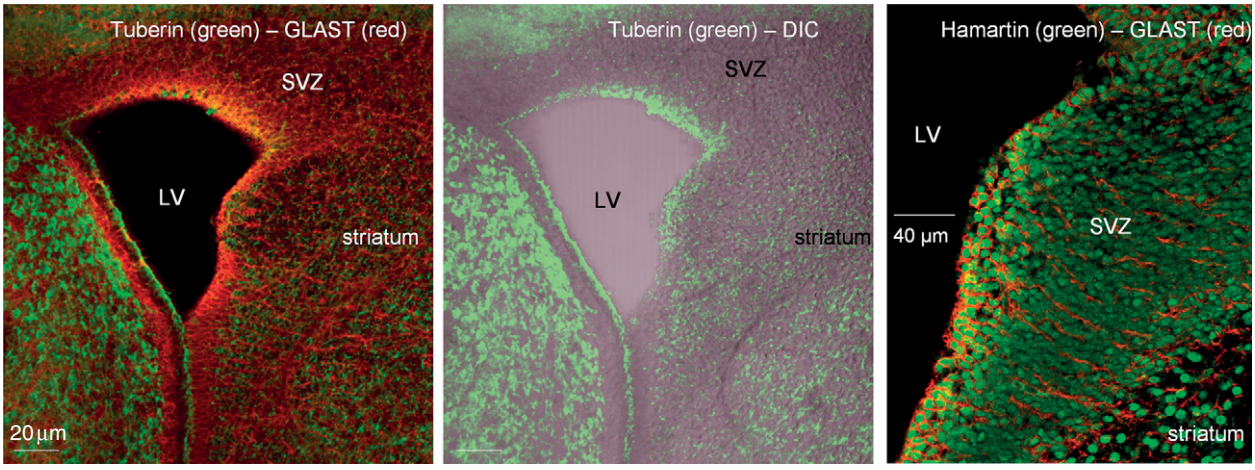


Figure 2 Tuberin (a and b) and hamartin (c) immunostaining in coronal section from a P1 mouse. Co-immunostaining was performed for GLAST (red) and overlaid with the tuberin or hamartin staining ((a) and (c), respectively).

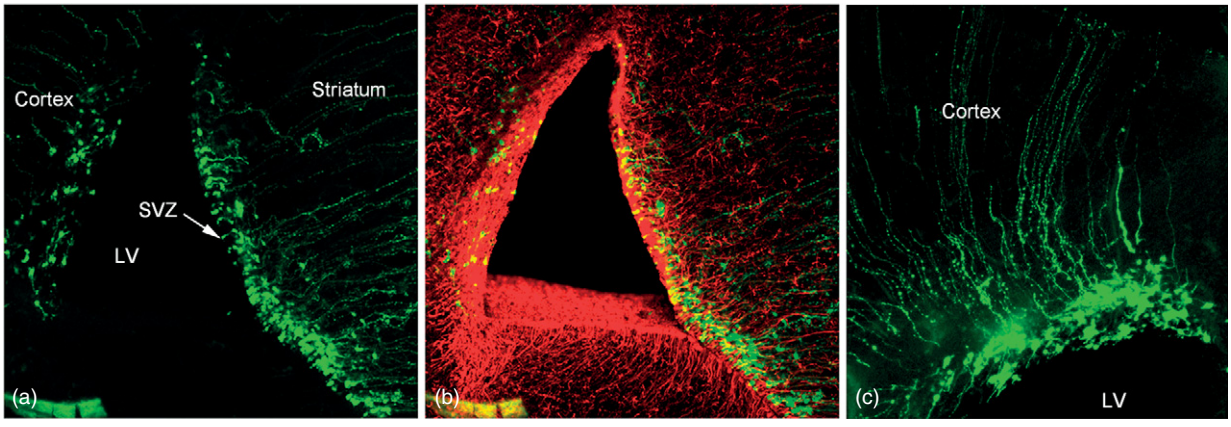


Figure 3 Images of GFP fluorescence (a-c) overlaid with GFAP staining in (b). Photographs were taken 2 day post-electroporation of a CAG-GFP plasmid in P2 mice.

in utero. As not all the cells will receive the plasmid, it could be interesting to apply a second electroporation during neonate life.

Once a lesion has been obtained, a series of experiments could then be performed to histologically characterize them and determine the state of neuronal excitability within and surrounding the lesions. Using immunohistochemistry, the presence of giant cells and proliferation in the lesions will be assessed. An increase in astrocyte proliferation is a common finding in MCDs with abnormal neuroglial proliferation and differentiation. Neuronal excitability can be assessed using electrophysiology or imaging in acute brain slices. In particular, voltage-sensitive dyes have shown tremendous improvement in their sensitivity and could give a more global view of neuronal excitability. Finding cytomegalic cells could help us understand the function of such cells in cortical circuits, and perhaps in hyperexcitability or seizure initiation. Finally, when possible it is important to compare the murine model to the human disease. Immunohistochemistry is the easiest technique to study human tissue, but it is also possible to use imaging in acute slices to better understand the mechanism of seizure initiation and propagation in human tissue (in parallel with the mouse study). Collectively, we hope that establishment of the described model of MCDs will provide a valuable tool to better study the ontogeny of these disorders and the epileptogenesis associated with the lesion. These approaches are critical for designing better diagnostic methods and therapeutic approaches to control seizure initiation or spread in affected children.

Acknowledgements

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See also: **Development/Malformations**: Epilepsy-Associated Reelin Dysfunction Induces Granule Cell Dispersion in the Dentate Gyrus; Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; Seizures and Adult Neurogenesis; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy.

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The Tish Rat: An Animal Model of Cortical Malformation in the Study of Epilepsy

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Introduction

Cortical dysplasia is defined as 'a malformative disorganization of the cytoarchitecture of the cortex relative to neurons'. It reflects disturbances in normal cortical development, which can be the result of aberrations in processes such as migration, proliferation, pruning of arborization, differentiation, and programmed cell death of neurons. There exists a wide range of histopathological and anatomical variations of human cortical malformations, reflecting the differential influence of the type, timing, and severity of the external insult or genetic mutation. One

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Cortical dysplasia is defined as 'a malformative disorganization of the cytoarchitecture of the cortex relative to neurons'. It reflects disturbances in normal cortical development, which can be the result of aberrations in processes such as migration, proliferation, pruning of arborization, differentiation, and programmed cell death of neurons. There exists a wide range of histopathological and anatomical variations of human cortical malformations, reflecting the differential influence of the type, timing, and severity of the external insult or genetic mutation. One

such variant involves a mutation in the doublecortin gene, which results in the development of subcortical band heterotopia. This disorder is also known as 'double cortex', since there exists a second, abnormally laminated and disorganized layer of cortex (heterotopia) located inferior to the normal-appearing cortex that maintains proper orientation and lamination of its primary neurons.

Studies performed on human brain tissue suggest that dysplastic tissue is hyperexcitable, as compared to surrounding neocortical tissue. However, specific mechanisms responsible for epileptogenic activity associated with cortical malformations remain to be elucidated. It is possible that hyperexcitable dysplastic and heterotopic neurons may be unable to establish their normal synaptic connections with nearby neurons and thus create dysfunctional synaptic activity. It could be at this level of circuitry that the abnormal synchronization of neuronal populations occurs, which in turn may underlie the genesis of epileptiform activity in cortical malformation.

It is important to also consider potential molecular mechanisms that may underlie the epileptogenicity of brains with malformations. Limited studies have demonstrated alterations in the subunits of glutamate and GABA receptors found within human dysplastic cortex. In addition, reduced inhibitory input from interneurons has been demonstrated in dysplastic cortical tissue resected from patients with intractable epilepsy. These alterations and reductions may result in an imbalance between excitation and inhibition, thus leading to a net increase in excitatory drive with epileptogenic potential.

Unfortunately, the amount and types of experimental information collected from human brain samples regarding epileptogenesis in cortical malformations are restricted. The data have suggested some mechanisms; however, the intrinsic limitations encountered when using human samples have impeded rigorous studies to define specific mechanisms. To help answer these questions, experimental, and genetic animal models of cortical malformation have been developed. Although these models do not utilize human tissue, they do provide a greater degree of control, standardized sample collection, the possibility for age-matched experiments, and large sample sizes that allow for statistical analysis of experimental results. In this article, we focus on the genetic model of cortical malformation, known as the 'tish' (telencephalic internal structural heterotopia) rat.

Background

The Tish Rat: Morphology, Genetics, and Development

The brain of the tish rat displays bilateral subcortical heterotopia reminiscent of those found in double cortex. The heterotopia consists of misplaced cortical neurons and glia that retain several basic features of normal neocortex, including topographic connectivity with subcortical targets. However, the heterotopic neurons fail to orient and laminate as in typical neocortex. Overlying the heterotopia is an area of neocortex termed 'normotopic cortex' that is thinner than normal neocortex, but maintains proper orientation and lamination of its primary neurons.

The initial characterization of the genetic disorder in the tish rat indicated that a spontaneous autosomal recessive mutation is responsible for the cortical heterotopia. More recent findings are consistent with an autosomal mutation with partial penetrance. This genetic basis distinguishes the tish rat from human double cortex, which is usually the result of an X-linked mutation.

Investigation into the developmental basis for the subcortical heterotopia in the tish rat has revealed that several important developmental milestones are achieved normally. Similar to control neocortex, only one preplate is formed, and this structure subsequently splits during expansion of the normotopic cortical plate into a single subplate and a marginal zone containing reelin-secreting Cajal–Retzius cells. The heterotopic cortical plate, therefore, forms without a dedicated preplate. In contrast to normal cortical development, the defining feature of tish neocortex is the presence of an additional proliferative zone located below the cortical plate and corresponding to the position of the intermediate zone in normal animals. This second proliferative zone has been shown to incorporate BrdU and to contain mitotic cells; therefore, it is hypothesized to give rise to many of the projection neurons and/or glia within the malformed neocortex. Local interneurons are unlikely to be generated in the heterotopic proliferative zone because they derive from the ganglionic eminences, which do not exhibit a misplaced proliferative zone.

Seizure Activity in the Tish Rat Brain

Spontaneous partial seizures with variable secondary generalization have been documented in the tish rat, which makes it an appropriate subject of study for epilepsy associated with cortical malformations. The distribution and site of seizure initiation zone were examined by using depth electrode recordings and postmortem analysis of c-*fos* mRNA expression to locate regions of aberrant discharge activity during seizures. The results of these studies demonstrated that the overlying normotopic neocortex and heterotopic cortex begin seizure activity almost simultaneously. Electrophysiological experiments using tish brain slices suggest that although epileptiform discharges occur in both brain regions, it is the normotopic cortex that is more prone to exhibit epileptiform activity and is responsible for recruiting the heterotopic area into aberrant discharge activity.

Considering these interesting results, experiments using anterograde and retrograde tracers were performed in order to examine the connectivity between the different regions (heterotopic and normotopic) of the tish cortex and both cortical and subcortical targets. The results show that both the normotopic and heterotopic cortices make functional topographic connections to subcortical targets, including thalamus and spinal cord. These experiments also demonstrated connectivity between the normotopic and heterotopic cortices, thus providing a potential avenue for the spread of epileptogenic discharges between hyperexcitable normotopic cortex and the heterotopia as well as subcortical targets.

Methods

Spontaneous seizure activity has only been noted in tish rats above the age of 30 days. This finding led our laboratory to examine a developmental stage prior to seizure development, specifically animals at 15 days of age (P15), in search of physiological abnormalities that might eventually lead to the development of spontaneous seizures in adult animals.

We used whole-cell patch clamp recordings of pyramidal neurons from live brain slices to characterize GABAergic inhibitory postsynaptic currents (IPSCs) at P15 in the tish brain. Layer V pyramidal neurons were selected from normotopic cortex because they are a major source of efferent projections from the neocortex, and have been implicated in epileptogenesis. In addition, this cell type could be targeted in both the normotopic and heterotopic areas for recordings; the cells in the normotopic area were identified on the basis of size, shape, and laminar position while the cells in the heterotopic area were defined on the basis of size, shape, and position within the outer one-third of the heterotopia. The recordings from these cells were compared with those collected from the same cell type found in wild-type animals of the same background strain, referred to hereafter as control.

By combining patch clamp recordings with pharmacological manipulations, we can isolate events in postsynaptic neurons that result from the action potential-dependent release of multiple GABA-containing vesicles from a presynaptic interneuron, otherwise known as spontaneous IPSCs (sIPSCs). Other pharmacological agents allow us to examine events that result from the action potential-independent release of individual vesicles of GABA at a single synapse from a presynaptic interneuron. These events are known as miniature IPSCs (mIPSCs). By measuring parameters (such as amplitude and frequency) that characterize sIPSCs and mIPSCs, we can gain knowledge about presynaptic activity from interneurons as well as synaptic function at the single synapse level.

Once recordings were completed, the brain slice was fixed and stained for a label that was incorporated into the solution filling the recording electrode. This procedure allowed a post hoc characterization of the morphologies of the cells from which recordings were made. Detailed morphologies of the reconstructed cells could then be established to ensure their consistency with morphology of layer V pyramidal cells.

Immunohistochemical experiments were also performed in order to investigate potential structural foundations of hyperexcitability in tish rat brains. Glutamic acid decarboxylase isoform 65 (GAD-65) is the rate-limiting enzyme in GABA production and is concentrated in the terminals and occasional cell bodies of GABAergic interneurons. Visualization of GAD-65 immunoreactivity allows the investigator to gain insight into the quantity, quality, and location of inhibitory interneuron terminals on principal cells. These types of immunohistochemical experiments also allow us to visualize a wide variety of cellular components that are distributed throughout the different regions of the tish brain. For instance, there are markers for multiple subtypes of inhibitory interneurons, and visualization of these markers provides insight into differential representation of the subtypes of interneurons found in the hetertopic and normotopic neocortices.

Recent Results

Spontaneous IPSCs recorded from normotopic neurons in tish brain slices were smaller in amplitude and less frequent as compared to those recorded from layer V neurons in control slices. The smaller amplitudes could result from a decrease in the occurrence of multiquantal, multiterminal release events in the tish brain compared to controls; however, smaller quantal size cannot be ruled out. In order to determine the quantal size in control and tish normotopic neurons, we measured the amplitude of action potential-independent mIPSCs. The amplitudes of mIPSCs recorded from normotopic neurons and control neurons were similar, indicating that quantal size was comparable in the two populations of neurons. The smaller amplitudes of sIPSCs recorded from normotopic neurons were therefore not due to smaller quantal size but rather due to a reduced number of GABA-containing vesicles being released from interneuronal terminals.

The reduced frequency of sIPSCs suggests that GABAergic terminals from normotopic cortex are not releasing vesicles at the same rate as in control cortex. It is possible that, establishing conditions that increase the probability of vesicle release (i.e., increasing calcium and lowering magnesium concentrations) might overcome this attenuated release from GABAergic terminals onto normotopic neurons. The frequency of sIPSCs recorded from control cells increased in response to these conditions, which reflects a robust increase in the release of presynaptic vesicles from control interneurons. In contrast, sIPSC

frequency was only modestly and nonsignificantly increased in normotopic cells in tish cortex under the same conditions. Thus, despite an enhanced probability of release, the frequency of sIPSCs in normotopic cells did not achieve a level comparable to that recorded from control neurons in standard recording conditions. We then investigated possible mechanisms that might be responsible for abnormal vesicular release.

Neurotransmitter release from presynaptic terminals is mediated by entry of Ca^{2+} into the terminal via voltage gated Ca^{2+} channels. Consequently, we tested whether the expression and/or function of Ca^{2+} channels on GABAergic presynaptic terminals in the normotopic cortex was reduced compared to control cortex. In interneurons, high voltage-activated N-type calcium channels are located specifically on synaptic terminals, are tightly coupled to exocytosis, and play a predominant role in action potential-mediated GABA release. This role for N-type channels contrasts with the situation in other central neurons in which P/Q type channels play a dominant role in synaptic release. One possible explanation for disturbed GABA release from interneuron terminals in tish normotopic cortex is that the expression of N-type channels is altered. Pharmacological experiments confirmed the presence and function of N-type channels in tish normotopic interneuron terminals and indicated that P/Q type channels do not participate substantially in regulating GABA release from terminals innervating layer V pyramidal cells. Thus, a shift of calcium channel subtypes did not occur in the interneurons innervating tish normotopic neurons.

Another plausible explanation for the decrease in presynaptic GABAergic neurotransmission is that fewer GABAergic terminals were making synapses onto normotopic neurons. To test this concept, immunohistochemical techniques were used to visualize GAD-65-positive terminals in control and tish neocortices. These experiments demonstrated that the intensity of GAD-65 immunoreactivity in layer V of the tish normotopic cortex was reduced relative to that observed in control tissue. GAD-65 IR terminals surrounding layer V pyramidal neurons in tish normotopic cortex displayed dispersed, punctate immunoreactivity that was less intense than that seen surrounding control layer V cells. The reduced perisomatic labeling in the tish cortex is consistent with a reduction in the number of GABAergic terminals innervating layer V pyramidal neurons.

This reduction in GAD-65-positive terminals in the tish normotopic cortex could reflect an overall reduction in GABAergic interneurons, a selective reduction in one or more interneuron subtype, and/or an attenuation of immunogenicity for GAD-65 in one or more types of interneuron. To examine these issues, we utilized a previously described classification system that identifies three discrete interneuron populations based on their selective expression of the following markers: parvalbumin, somatostatin, and cholecystokinin. The results demonstrate a selective decrease in parvalbumin-expressing interneurons (see Figure 1), with no significant change in somatostatin- or cholecystokinin-expressing interneurons.

The heterotopia was also examined to assess their electrophysiological and neuromorphological characteristics. The results indicated that: (1) inhibitory synaptic transmission onto large heterotopic pyramidal neurons was somewhat weaker than that observed in large pyramidal neurons in layer V of control tissue; (2) calcium channel expression was similar to that found in control and normotopic cortex; (3) the intensity of the GAD-65 immunoreactivity appeared more diffuse around the heterotopic pyramidal neurons as compared to control, but not as weak as that observed surrounding the tish

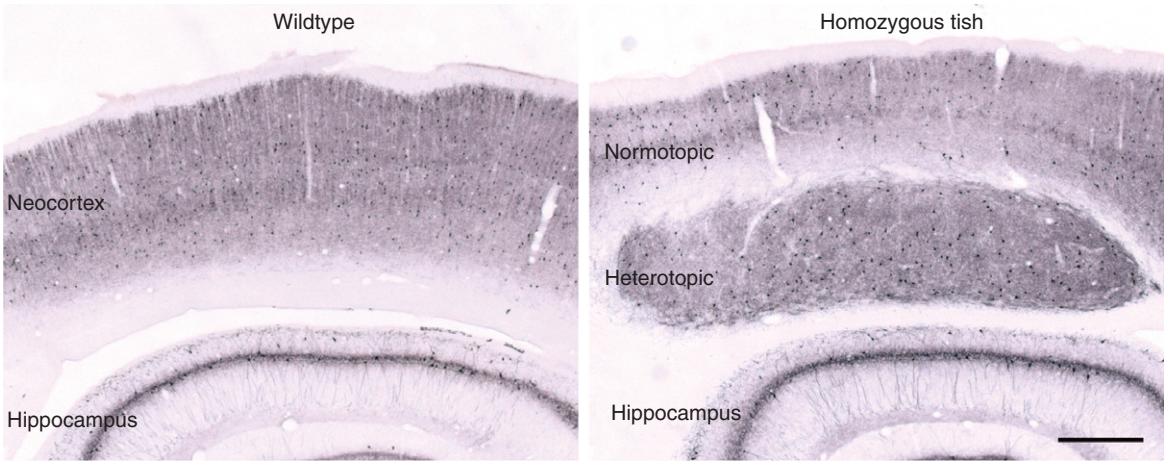


Figure 1 Coronal sections are shown from wild-type and homozygous tish brains stained for parvalbumin immunoreactivity. The tish phenotype is characterized by a thinned normotopic neocortex and an underlying heterotopic neocortex. Fewer parvalbumin-positive interneurons are observed in the tish normotopic neocortex in comparison with the wild-type neocortex.

normotopic layer V neurons; (4) all three subtypes of interneurons were present in the heterotopia of the tish brain, but their distribution appeared more random than in the laminated areas of the neocortex; and (5) a profound reduction of parvalbumin-positive cells was not seen in the heterotopia. These results suggest an overall reduction in GABAergic inhibitory drive on heterotopic neurons, which was less profound than the prominent reduction observed in normotopic neurons.

In summary, these recent results demonstrate that GABAergic synaptic inhibition is reduced in a seizure-prone animal model of cortical malformation. Studies performed in humans and other animal models have implicated disruptions in the GABAergic system, such as altered inhibitory drive, alterations in GABA interneuron populations, changes in $GABA_A$ receptor subunits, and changes in GABA reuptake transporters. A key finding in this unique model is that inhibitory failure of GABAergic innervation onto primary neurons in normotopic and heterotopic cortices actually precedes the onset of seizures. This type of disturbance thus provides not only a mechanism for ongoing seizure activity, but also represents a rational substrate for epileptogenesis in a dysplastic brain.

Future Goals

A question that still remains unanswered is what events precipitate the onset of seizures in tish animals. Alterations in inhibitory drive have been described in immature animals, but it remains to be elucidated whether these changes persist into adulthood, or whether there are further changes that may contribute to the onset of seizures. A rigorous study of adult tish brain inhibitory physiology, similar to that described for P15 animals, will help in further understanding these issues.

Our studies have focused on the presynaptic aspect of the GABAergic synapse; however, the postsynaptic side of inhibitory synaptic function (e.g., $GABA_A$ receptors) remains unexplored. These receptors and related molecular components of the postsynaptic complex represent an important area of study because during normal development of the forebrain, modifications occur in $GABA_A$ receptor subunit mRNA and protein expression that, in turn, influence synaptic transmission. It is plausible that there could be alterations in the $GABA_A$ receptor subunit composition that contribute to altered inhibitory neurotransmission in the tish brain.

The disturbances in interneuron distribution and their innervation of primary neurons are critical features of the seizure-prone tish neocortex. In light of the lengthy tangential migration of interneurons from the ganglionic eminences during embryonic development, it is intriguing to hypothesize that the errors in tish interneuron distribution in the P15 brain could have their origins in disrupted tangential migration. A number of molecules are known to stimulate or otherwise coordinate the process of interneuron migration, including various neurotrophic factors, hepatocyte growth factor/scatter factor, class 3 semaphorins, and the chemokine CXCL12 (also known as SDF-1). In particular, CXCL12 is important for maintaining migrating interneurons in the marginal and subventricular/intermediate zones, allowing them to invade the laminar cortex only after they have reached their appropriate mediolateral position within the cortex. Alterations in CXCL12 gradients via genetic knockout result in dispersal of migrating interneurons, their premature entry into the cortical plate, and their disturbed laminar positioning in the adult animal. These findings highlight the importance of this particular guidance molecule in determining interneuron distribution. Considering that CXCL12 is produced, in part, by neurogenic intermediate progenitor cells in the subventricular zone, and that a malpositioned population of proliferative cells exists in the tish intermediate zone and normotopic cortical plate, the changes in interneuron distribution in tish neocortex may result from errors in CXCL12 gradients caused by heterotopically located intermediate progenitor cells. In an effort to better address this possibility, ongoing and future studies in our laboratory will address the distribution of specific progenitor populations and their contributions to the construction of the neocortex. In addition, since CXCL12 is one of several cortical molecular signals that might regulate the migration and positioning of interneurons, an important goal of future studies will be to define the critical cellular and molecular regulators that are modified in the developing tish cortex.

Finally, the studies so far undertaken on tish brain physiology have focused on GABAergic inhibitory synapses. However, human and animal studies of cortical malformation have also implicated alterations in excitatory neurotransmission in the hyperexcitability associated with epilepsy. It would be worthwhile to investigate alterations in excitatory glutamatergic physiology in the tish brain, as well as structural entities that may underlie abnormalities associated with a disturbance between excitatory and inhibitory influences.

See also: **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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E

EPILEPTOGENESIS

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Epileptogenesis and Plasticity

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Introduction

Epileptogenesis and plasticity of neuronal networks are two related phenomena because some epileptogenic lesions show clear reorganization of neuronal connections and growth of neuronal elements. This is conspicuous in epileptogenic foci induced by kindling, but even more so in 'post-status epilepticus' (SE) experimental models of chronic epilepsy. In this article we consider, *first*, the relations between kindling epileptogenesis and a classic form of neuronal plasticity, long-term potentiation (LTP). *Second*, we examine the evidence regarding how restructuring of synaptic connections may underlie epileptogenesis. *Third,* we analyze the relations between developmental plasticity and epileptogenesis. *Fourth,* the question of mechanisms of neurogenesis and the role of growth factors is discussed. *Finally* we summarize some conclusions and formulate some open questions that merit being further investigated.

Background

Electrophysiological Evidence for a Relation between LTP and Kindling Epileptogenesis

Experimental evidence support that repeated tetanization (i.e., high-frequency electrical stimulation) of several brain structures, particularly the limbic cortex, at regular intervals, leads finally to the development of an epileptogenic focus. This epileptogenic process is accompanied by a progressive change in evoked synaptic responses that has a striking resemblance to what is seen during the establishment of LTP in vivo. This progressive change has been shown by recording local evoked potentials (EPs) for many hours and even for days after tetanization of the Schaffer collaterals. In particular, potentiation was shown for the synaptic potentials elicited by the stimulation of the Schaffer collaterals, using the paired-pulse paradigm and recording field EPs in the CA1 area. The tetanus consists of a burst of high-frequency pulses, similar to the stimulus used to induce LTP in vivo in the rat, but at somewhat higher stimulus intensity. Using a kindling procedure involving once daily tetanization, investigators (not surprisingly) found that the first tetanic stimulus induced LTP (i.e., an increase in the peak amplitude of the field EP). In one series of experiments, the degree of LTP increased gradually up to the 4th daily stimulation session, but then became variable or nonsignificant. This transient character of LTP, induced in the kindling paradigm, was interpreted by some researchers as indicating that LTP cannot be the 'cause' of kindling epileptogenesis. Nevertheless, LTP may play a role in the initiation of the

plastic processes that eventually lead to kindling epileptogenesis. We find it interesting that the occurrence of epileptiform after-discharges and of the first behavioral signs of seizures takes place at about the same time that the LTP effect appears to saturate and becomes rather variable. At this time, additional changes in the EPs are noted. In particular, the late components of the EP are modified in a manner that has been shown to correspond to a decrease in a population inhibitory postsynaptic potential (IPSP) in the CA1 area. In contrast, in the dentate gyrus, paired-pulse stimulation of the angular bundle shows an increased paired-pulse depression that can be interpreted as a strengthening of inhibition (especially at the septal pole of the hippocampus). Several factors may be involved in this increased inhibition, including sprouting of interneurons, sprouting of granule cell axons that may form synapses preferentially with inihibitory interneurons, or increased number of $GABA_A$ receptors in this region. These findings lead to two main conclusions: First, kindling epileptogenesis and LTP may share some common plastic neuronal changes. And second, kindling epileptogenesis involves additional processes concerning LTP. Thus, kindling cannot be considered just a simple amplification of the same processes underlying LTP, but rather involves *additional plastic changes*, among which a restructuring of synaptic connections plays an important role. There is evidence that ultrastructural changes of synapses may occur in LTP, particularly enlargements of the spine head and shortening of the dendritic spine neck, both changes can be associated with changes in the strength and time course of synaptic currents and intracellular calcium dynamics. In addition, time-lapse imaging has provided the evidence of increases in the density of spines. How these ultrastructural changes are related to the induction or maintenance of LTP is still an open question. In contrast to LTP, however, kindling epileptogenesis is associated with much more prominent and extended morphological changes. In kindled rats, changes in the form of synaptic contacts have been described at the ultrastructural level in the dentate gyrus of the hippocampus; in particular, there is an increase in the ratio of perforated axo-spinous synapses (i.e., synapses with discontinuous postsynaptic densities) to nonperforated synapses. The consequence of this structural change would be, in theory, an increase in the gain of excitatory synapses.

Methods

In most of the experiments described later, the SE model for temporal lobe epilepsy in the rat has been used. In this model, SE is induced by electrical stimulation of the angular bundle or by kainate or pilocarpine injection. The SE is followed by a latent period of about 1–2 weeks (in the case of the electrical stimulation), after which spontaneous seizures start to develop. This seizure progression can be monitored by hippocampal EEG and field potential recordings. Using in situ hybridization and immunocytochemistry at different time points after SE, we can gain some insights into the factors that can contribute to epileptogenesis and seizure progression.

Recent Results

In the 'post status epilepticus' models the occurrence of *sprouting of mossy fibers* and the formation of aberrant mossy fiber synapses onto apical dentate granule cell dendrites in the inner molecular layer of the dentate gyrus (a zone where mossy fiber terminals synapses are not found in normal animals) is an even more conspicuous structural change. These mossy fibers can be viewed by Timm staining for heavy metals (zinc is abundant in these terminals), or by dynorphin-A immunocytochemistry. The sprouting of mossy fibers may contribute to the formation of a recurrent excitatory circuit, which could facilitate the spontaneous recurrence of focal seizures and propagation of the epileptic state. Nevertheless, sprouted mossy fibers may also innervate inhibitory interneurons which, after an initial loss of inhibition following SE, could contribute to recovery of inhibition in this subarea of the hippocampus during epileptogenesis. An interesting new finding is that stimulation of granule cells can cause inhibitory synaptic potentials in postsynaptic pyramidal cells after epileptic seizures (but not before), indicating a change in the neurotransmitter signalling of these cells from purely glutamatergic to a combination of glutametergic and GABAergic effects (refer 'See also' section). This change in phenotypic expression may compensate for the increased excitability associated with mossy fiber sprouting, since the GABAergic transmission of these 'post-seizure' granule cells may hyperpolarize target cells, shunt incoming excitatory signals, and enhance tonic inhibition at both pre- and postsynaptic sites.

In addition to sprouting of mossy fibers, the granule cell layer of the dentate gyrus displays another conspicuous structural change, namely the *dispersion* of the granule cell layer, and the formation of granule cell basal dendrites (which normally appear only in early stages of development). The processes that lead to these structural changes are still unknown. However, recent studies suggested that granule cell dispersion does not result from increased neurogenesis but rather reflects a displacement of mature granule cells, most likely caused by a local deficiency in the protein reelin that is associated with neuronal migration and the development of normal dendritic trees.

Moreover, kindled seizures are also associated with axonal and synaptic remodeling in other subareas of the hippocampal formation, including the hilus of the dentate gyrus, the CA3 and CA1 regions, and the entorhinal

cortex (EC). What induces this abnormal plasticity, characterized by sprouting of axonal collaterals that invade a foreign territory, is still an unsettled issue. Mossy fiber sprouting appears as a reaction to the loss of the normal post-synaptic targets of the mossy fibers, namely the hilar cells. Indeed, in the hilus of the SE rats, there is a severe loss of mossy cells as well as elimination of parvalbumin, claretinin, and somatostatin-immunoreactive interneurons – all cell types that normally receive mossy fiber input.

The occurrence of sprouting in this hippocampal area, may be induced by a transient decrease of the chemorepellant Semaphorin (Sema3A). Sema 3A is normally secreted by axons from the EC and serves to prevent axon collaterals from entering the molecular layer of the dentate gyrus. It is interesting that Sem3A expression is downregulated in a model of temporal lobe epilepsy caused by electrical stimulation that induces 'status epilepticus.' Indeed, shortly after induction of 'status epilepticus,' Sema3A messenger RNA almost disappears in the stellate cells of the EC, although this loss was transient. The downregulation of Sema3A appears concomitantly with an upregulation of GAP-43 in the granule cells. GAP-43 is known to stimulate mossy fiber sprouting and is enhanced in many epilepsy models. Thus, in this temporal lobe epilepsy model, the chemorepellent Sema3A disappears from the molecular layer at the same time as granule cell axons are prompted to form sprouts. We were surprised to know that if the electrical stimulation of the angular bundle, used to induce seizures, does not result in a complete status epilepticus, the downregulation of Sema3A does not occur but the upregulation of GAP-43 does – but there is no sprouting. This result suggests that the downregulation of Sema3A in the molecular layer of the dentate gyrus is a molecular event needed for recurrent mossy fiber sprouting. Also in the SE model induced by kainic-acid injections, the chemorepellant Sema3C content of CA1 has been shown to be downregulated within the first week of epileptogenesis. These findings indicate that an inactivation of the Semaphorin signalling may play a role in the formation of new aberrant synaptic contacts.

Seizure Progression and Selective Neuronal Loss in Parahippocampal Regions

Although mossy fiber sprouting has been proposed to be a prerequisite for chronic seizure activity in experimental temporal lobe epilepsy, and may occur regardless of the kind of initial epileptogenic insult (electrical stimulation, stroke, or percussion injury), the extent of hilar cell death also appears to be an important factor that could contribute to seizure progression, as illustrated in **Figure 1**.

Although most studies have focused on cell death in hippocampus, extensive cell death has also been observed in other limbic regions, including thalamic nuclei, amygdala, and piriform- and entorhinal cortices. Inhibitory GABAergic cells have been reported to be reduced in epileptic foci in both experimental animals and epileptic patients. A subset of GABAergic cells, the parvalbumin (PV) immunoreactive neurons, exert strong perisomatic inhibition and are involved in both feedback and feedforward inhibition. Extensive PV cell loss has been reported in rats that have developed a progressive form of epilepsy and exhibit frequent daily seizures in the chronic epileptic phase. The density of PV-IR neurons is, however, not significantly decreased in the medial EC layer III (**Figure 2(e)**), even though most of the surrounding layer III principal neurons are lost (particularly in rats that develop a progressive form of epilepsy) (**Figure 2(b)**). The disappearance of these principal layer III neurons may leave the PV interneurons without their main excitatory innervation, leading them to become 'dormant,' similar to what has been suggested for basket cells in the dentate gyrus. The decrease of PV-IR neurons in EC layer II (and the less PV 'rich' layers V/VI), together with dormant cells in EC layer III, might have a profound effect on net inhibition in the EC. Under normal circumstances, layer III is thought to act as a powerful 'gate,' limiting the responsiveness of the superficial EC layers. In the absence of this gate, superficial layers may become hyperexcitable to deep layer entorhinal input. Support for this hypothesis has been provided by several electrophysiological studies.

The selective cell loss is less extensive in post-SE rats that did not develop a progressive form of epilepsy (although an occasional seizure can be observed in these rats). Neuronal death is induced mainly by the initial SE (not by later spontaneous seizures), and the loss of PV containing neurons does not progressively increase along with the number of seizures. Therefore, the significant correlation between decreased PV immunostaining and lifetime seizure load appears to be related to the fact that rats with longer SE duration show more extensive cell loss and are more likely to develop a progressive form of epilepsy.

Developmental Plasticity and Epileptogenesis

An interesting possibility to account for epileptogenesis is that the reaction of brain tissue to an epileptogenic insult may cause the neuronal system to recapitulate some mechanisms that were active during its most intense period of plasticity – i.e., during early development. This recapitulation may lead to changes in neuronal systems that are inappropriate for the adult brain, and that become manifest by the occurrence of epileptic seizures, as well as other possible functional impairments. In this respect, a number of interesting changes have been found in epileptic tissue, including the reappearance of embryonic forms of sodium channels, and the appearance GABAergic depolarizing signalling characteristic of early

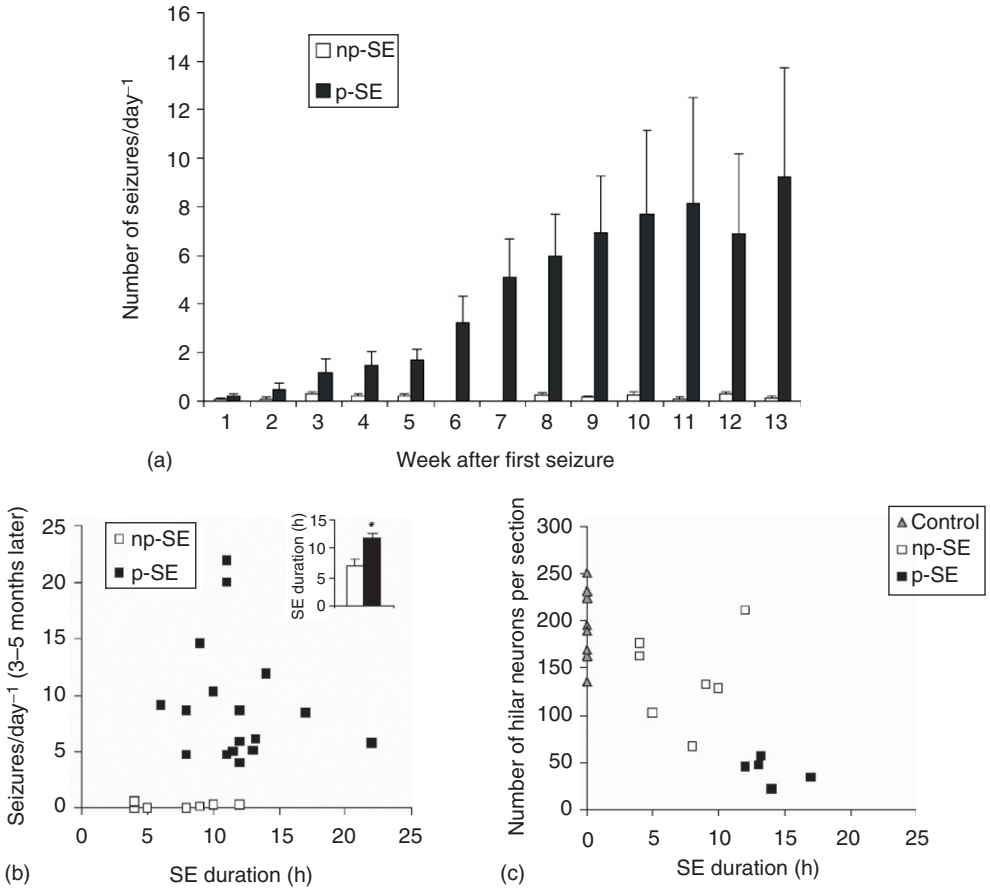


Figure 1 (a) Data from SE rats, induced by repeated electrical stimulation of the angular bundle, that after a latency period develop spontaneous seizures; in some rats seizure development is progressive, with an increase of seizures' frequency and duration (p-SE), but in some other seizures occur only occasionally and do not show a progressive trend (np-SE). (b) Relation between the duration of the SE and the later individual seizure activity in progressive epileptic rats (black squares) and nonprogressive epileptic rats (white squares) during the last week before perfusion. The average group data (inset 1(a)) indicate that rats that displayed a progressive form of epilepsy had experienced a significant longer SE than rats that had a nonprogressive form of epilepsy. (c) Relation between the duration of the SE and the number of hilar Nissl cells at dorsal hippocampus after SE. There is a significant negative correlation ($r = -0.66$, $p < 0.01$) between the duration of the SE and the hilar cell number of the three groups (Adapted from Gorter *et al*. 2003 and van Vliet *et al*. 2004).

development. It has been previously shown that inactivation properties of the fast Na+ ionic current shift to a more depolarized potential after kindling epileptogenesis, and that this feature has some resemblance to sodium current properties in neonatal rat neurons. This finding, in turn, led to an investigation of the cell-specific expression of mRNAs encoding 'neonatal' II and III alpha-isoforms, and a comparison of these isoforms with 'adult' sodium channels (NaCh I, II, III). For these studies, we used in situ hybridization, at different time points after electrically induced seizure activity, in the SE rat brain. Both types II and III sodium channel genes encode two isoforms, as a result of a mutually exclusive splicing of a single exon. This alternative splicing is developmentally regulated. An increased expression of neonatal sodium channels II and III alpha-isoform mRNAs after the induction of status epilepticus was found in neurons of CA1, CA3 and in the dentate granule cell layer. These changes persist, although less intensely, in the chronic phase when rats display spontaneous seizures. In addition, this enhanced expression of neonatal sodium channels II and III was observed also in microglial cells. The increased expression of neonatal isoforms of sodium channels in both neurons and microglia represents a plastic regression to early stages of development. This plasticity may reflect a critical mechanism for enhancing neuronal excitability in the course of epileptogenesis, and may be relevant for the pharmaco-resistance that is frequently found in epileptic patients with mesial temporal lobe epilepsy. Furthermore, a period of status epilepticus can produce other plastic changes in ionic currents. For example, during the latent period of epileptogenesis, most CA1 pyramidal cells that normally fire in a regular pattern acquire low-threshold bursting behaviour, generating high-frequency clusters of 3–5 spikes as their minimal response to depolarizing stimuli. This burst firing pattern is initially

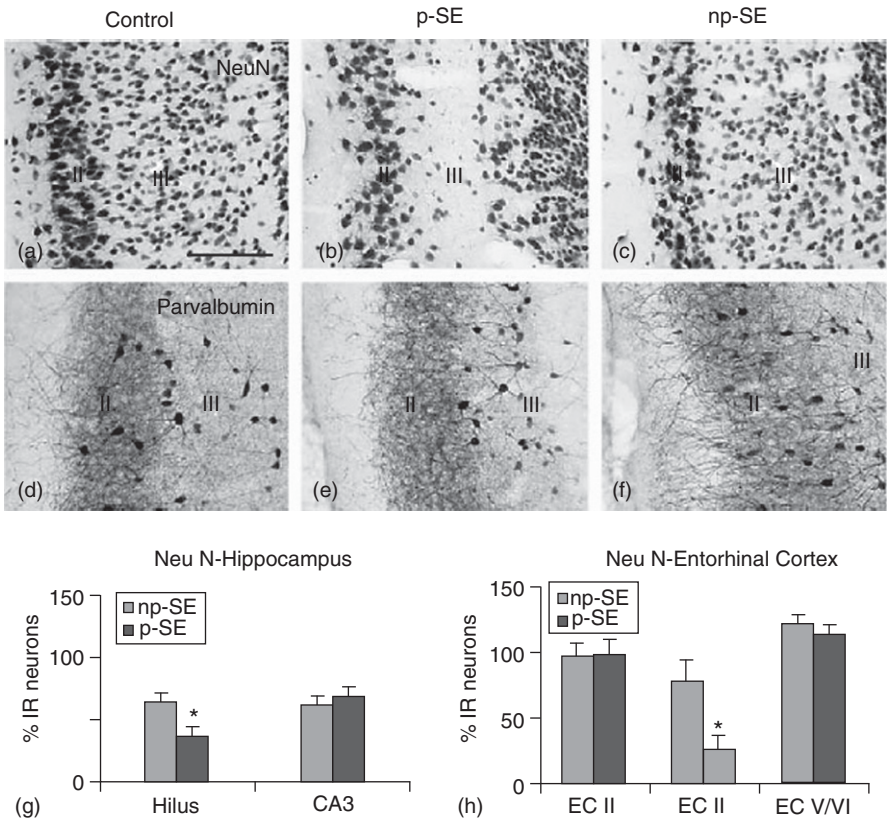


Figure 2 Examples of NeuN ((a)–(c)) and PV ((d)–(f)) immunostainings in the superficial entorhinal cortex (EC) layers (II and III) of a control rat ((a), (d)), progressive post-SE rat (p-SE; (b), (e)), and nonprogressive post-SE rat (np-SE, (c), (f)). Note the preservation of PV-positive staining in layer III in both p-SE and np-SE rats, while NeuN staining is extensively decreased only in p-SE rats. Scale bar in panel a = 250 μm. ((g) and (h)) NeuN densities in various hippocampal and entorhinal areas in rats with progressive and nonprogressive epilepsy (control densities are 100%). A significant decrease was observed in hilus (g) and EC layer III (h) in p-SE rats compared with controls. Np-SE rats also showed reduced NeuN staining in the hilus but not in the EC layer III. (Adapted From van Vliet *et al*. 2004).

mediated by a T-type Ca^{2+} current, but gradually becomes dependent on a persistent Na+ current. This plastic transition may contribute to the development of temporal lobe epilepsy.

Another example of a reiteration of a developmental program is the overexpression of a sodium-potassium chloride transporter (NKCC1) – as evident in subicular pyramidal cells removed from MTLE patients. NKCC1 is known to facilitate the accumulation of Cl^- in neurons, producing a GABA receptor-mediated depolarizing response which is characteristic of the immature brain. The subicular cells of adult epileptogenic tissue appear to reproduce the behavior of the embryonic brain with respect to NKCC1 expression. NKCC1 overexpression has also been found in tissue of epilepsy patients with developmental lesions such as focal cortical dysplasia (type IIB), gangliolioma and hemimegalencephaly. This protein may contribute to the occurrence of seizures. In the electrically induced SE rat model, a gradual upregulation of NKCC1 gene expression was found during the chronic epileptic phase in both CA3 and EC. The switch from depolarizing to hyperpolarizing GABAergic synaptic activity depends not only on the expression of the K-Cl co-transporter, NKCC1 but also of the KCC2 transporter. The latter becomes expressed only a few days after birth in the rat; and it causes the reversal potential of chloride to shift in the hyperpolarizing direction. In this context, it is interesting to note that de-afferentation (e.g., resulting from the neuronal loss) can cause a downregulation of the neuronal KCC2. An increase in expression of brain-derived neurotrophic factor (BDNF) (see later text) may also contribute to the shift of the membrane equilibrium potential for $[Cl^-]$ in hippocampal cells associated with seizures.

Neurogenesis and the Role of Growth Factors

A plastic change associated with epileptogenesis is the enhancement of *neurogenesis* – a process that occurs throughout life in the dentate gyrus, but is highly accentuated after epileptic seizures. Some of the newborn cells mature as adult granule cells and appear to integrate into the local circuits; other cells do not integrate appropriately and appear to be 'displaced.' Newborn granule cells also show increased NKCC1 expression which leads to a

GABA-mediated depolarizing action. The molecular mechanisms responsible for new neurogenesis are still unclear, but neurotrophins are likely contributors. Neurotrophins play a significant role during neural development, and are associated with normal plasticities associated with, e.g., learning and memory. A particularly relevant neurotrophin is the BDNF that acts by binding to the high-affinity receptor tyrosine kinase, TrkB. The activity of BDNF is quite complex; besides the full-length receptor, TrkB, there exist spliced truncated forms (trkB.T1 and trkB.T2), both of which bind BDNF but lack tyrosine kinase activity. These spliced variant receptors inhibit the function of the full-length receptor. The expression of BDNF is enhanced after limbic seizures. BDNF, through Trk receptors, can downregulate KCC2 protein levels in hippocampal neurons. In addition, BDNF decreases the amplitude of IPSCs in hippocampal excitatory neurons, and induces hyperexcitability in the hippocampus. Transgenic (tg) mice overexpressing BDNF also exhibit increased severity of seizures. Furthermore, kindling in BDNF knockout mice is retarded. Finally, the inactivation of the BDNF efficacy, by intraventricular administration of antibodies to trkB receptor, during induction of kindling reduces kindling epileptogenesis. Experiments in mice in which status epilepticus was induced by kainate support the hypothesis that *decreased* BDNF signalling *reduces* epileptogenesis. Furthermore, the potential role of BDNF in mossy fiber sprouting has been addressed by studies in BDNF overexpressing transgenic mice; in these experiments, no difference was seen in induction of mossy fibre sprouting after kainate-induced SE in mice with truncated trkB receptor compared to wild type mice – showing that BDNF is not the primary inducer of sprouting after status epilepticus.

Plastic Changes that are Responsible for the Formation of a Mirror Focus

Many years ago, Morrell hypothesized that a mirror focus may be formed in the course of an epileptic process – i.e., a secondary epileptogenic focus may be formed on the side of the brain contralateral from a primary focal epileptogenic area. This phenomenon of *secondary epileptogenesis* is an example of how in an adult brain plastic changes may be induced in normal tissue by seizure activity in a remote, but connected, brain area. The phenomenon of secondary epileptogenesis is well described in experimental animals but its relevance to human epilepsy has been the subject of controversy. Nevertheless, it has been shown that secondary epileptogenesis is not uncommon in humans, although it does not occur in all cases. An experimental study using an ingenious in vitro preparation, has given new insights into the plastic mechanisms mediating the formation of a mirror focus. Using this preparation (composed of a three compartment chamber in which two intact hippocampi and the connecting fibers are placed), investigators showed that induction of a 'mirror focus' required activation of NMDA receptors as well as persistent changes in GABAergic synapses which became depolarizing due to a shift in the chloride reversal potential (as described above).

Plasticity Related Genes and Epileptogenesis

Microarray studies in animal models of temporal lobe epilepsy yield results that are in general agreement with respect to genes associated with the immune response, inflammation and proteolysis. The expression of these genes is enhanced especially during the first weeks after the 'initial insult.' Another conspicuous change is the decrease or change in expression of various GABA receptor subtypes. In another article, we discuss in more detail the gene expression changes that are related to synaptic plasticity and epileptogenesis (refer 'See also' section).

Future Directions

An initial epileptogenic insult, such as a brain inflammation, trauma, ischemic lesion, or tumor, may cause neuron loss, de-afferentiation of surviving cells, and proliferation of glial cells. These cellular alterations can cause changes in gene expression, promoting inflammatory processes and neurodegeneration, but also the upregulation of neurotrophic factors, such as BDNF, enhanced neurogenesis, and the sprouting of new synaptic connections. This replay of phenomena associated with growth in early stages of development is accompanied by the reappearance of several processes that are characteristic of embryonic phases, such as neonatal forms of sodium channels, of the sodium-potassium chloride co-transporter NKCC1 (with the consequence that GABAergic processes becoming depolarizing), and of GABA expression in normal glutamatergic cells (e.g., the granule cells). These processes are necessary during the early stage of development, where they contribute to the growth and formation of neuronal connections, but become disruptive when they re-appear in the adult brain, where they affect the dynamical equilibrium of the neuronal networks. Thus, conditions that may finally lead to the emergence of epileptic seizures may be created. Thus, we postulate that this kind of epileptogenic lesion results from *plastic developmental changes in reverse direction.* Other processes characteristic of this replay of early developmental stages will likely emerge as more such researches is pursued. A general issue that merits further investigation is how the chain of reactions that goes from the early inflammatory and immunological responses to an initial epileptogenic insult results in a series of plastic changes that revive early stages of development (with respect to ionic channels, neurotransmitter signaling, growth of neural cells and of synaptic connections).

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Synaptic Potentiation and Inhibition after Partial Seizures.

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Gene Expression in Immature and Mature Hippocampus After Status Epilepticus

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Introduction

Prolonged seizures or status epilepticus (SE), as well as repeated brief intermittent seizures associated with epilepsy, have been shown to cause neuronal damage. A single systemic administration of kainic acid (KA) in mature rodents causes seizures that can last for several hours, followed by a latency period of about 2 weeks between status and the onset of spontaneous seizures, and finally a chronic epileptic state characterized by spontaneous recurrent seizures. In young rats prior to postnatal day 18 (P18), however, KA injection does not result in spontaneous recurrent seizures or cell death. Rather, these young rats exhibit a limited pathology characterized by a lowered seizure threshold. Studying such age-dependent differences, therefore, offers a strategy not only for understanding epileptogenesis, but also for identifying factors that contribute to seizure

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Synaptic Potentiation and Inhibition after Partial Seizures.

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susceptibility in mature animals and neuron-protection in younger animals.

We focused on the contrasting responses of immature and mature brains to the same stimulus, KA-induced seizures, to identify target genes involved in epileptogenesis and cell death processes. We used high-throughput, high-density oligonucleotide gene arrays (microarrays), to study the molecular basis of the detrimental effects of seizures. The comparison between transcriptional changes in immature brain and those in the mature brain following SE may offer insight into mechanisms of neuroprotection against seizure-induced neuronal injury. Some of the highly regulated genes identified in our array analysis were confirmed by a real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Background

Gene Expression Profiling Using Microarray Technology

Advances in both array technology and knowledge of genomic sequences have opened a new way to study the nervous system. One of the benefits of these innovations is high-density oligonucleotide arrays that contain more than 400 000 sequences, typically 25-mers, in $20 \times 20\,\mu m^2$, in a total area smaller than half inch square. We used the Affymetrix Rat Genome U34A GeneChip®, which has 8799 genes and expressed sequence tags (EST) and 16 probe pairs targeted to the most unique regions of genes, a design that increases detection redundancy and specificity.

The array interrogates complex nucleic acid samples and effectively counts the number of different RNA molecules that are present in a sample. The process is highly parallel, as all sequences are counted simultaneously, and is quantitative over a limited range. The relative concentration of a given sequence fragment between two samples can be determined for thousands of genes at a time, based on hybridization reaction and a posthybridization fluorescence scan of the array. Instead of a 'one gene at a time' approach, microarrays allow us to look broadly and to gain a new and broadly-encompassing view of genome-wide changes in neuronal gene expression. This strategy is particularly suitable for a study of complex diseases such as epilepsy, as it is likely that a large number of genes are simultaneously involved in the disease process.

Gene microarray technology has been successfully applied in many areas of medical research, most notably in cancer research to aid in classification, diagnosis, and prognosis predictions. By facilitating the identification of potential therapeutic targets from thousands of genes in a single experiment, array technology provides a new and valuable approach in searching for candidate target genes for epileptogenesis.

Lack of Histopathologic Markers for Epileptogenesis

Beginning within the second week after KA treatment, during the time referred to as the 'latent period,' there is temporal progression of axon growth in the dentate gyrus, which proceeds for several months. This progression is believed to correlate with the onset and peak of spontaneous seizures. A currently influential hypothesis suggests that hippocampal neuronal loss and mossy fiber sprouting play a critical role in the pathogenesis of chronic epilepsy. Recent evidence regarding the relationship between chronic epileptogenesis and hippocampal histopathology, however, has challenged this hypothesis. Studies using rat models have revealed that neither cell death nor mossy fiber sprouting is necessary or sufficient for the generation of spontaneous seizures after SE. These findings suggest that while neuronal injury and mossy fiber sprouting may be common sequelae of SE, and may accompany the epileptogenic process, they do not cause chronic epilepsy. Instead, the epileptogenesis process is preceded by, and may occur as a result of, a cascade of changes in gene expression. Temporal sequences of gene expression may incite a subsequent cascade of cellular events, ultimately leading to the occurrence of cell death, mossy fiber sprouting, and spontaneous seizures. Although molecular, cellular, and physiologic mechanisms operate together to contribute to epileptogenesis, our focus here is on the earliest events detectable following evoked seizures.

Role of Inflammation in Epileptogenesis

The contribution of inflammation in mediating many aspects of disease has been relatively well established. However, we have only begun to explore the involvement of inflammation in the pathogenesis of epilepsy and seizure-induced brain damage. For example, hippocampal sclerosis has been viewed as an inert scar, but recent neuropathological studies of resected hippocampal tissue reveal many hallmarks of active inflammation, such as astrocytic proliferation, microglia activation, and expression of cytokines. These and other findings raise the possibility that seizure-induced glial activation and neuronal degeneration are active and ongoing processes.

Clinical observations also report acute inflammatory reactions after seizures. Active inflammation has been detected not only in prototypical inflammatory epilepsies such as Rasmussen's encephalitis or limbic encephalitis, but also in patients with pharmacoresistant epilepsy of diverse causes. Proinflammatory cytokines, NF-κB, and interleukin 1β (IL-1β) and its signaling receptor IL-1R1 are highly expressed by neurons and glia in temporal lobe epilepsy, focal cortical dysplasia, glioneuronal tumor, and in tuberous sclerosis complex. Increased levels of cytokines such as IL-6, IL-1β, and IL-1-receptor antagonist (IL-1Ra), a

naturally occurring antagonist of IL-1β, have also been detected in plasma and cerebrospinal fluid from patients with recent seizures without evidence of infections. Involvement of the 'cytokine network' has also been implicated in febrile seizures. Whether cytokines contribute directly to pathogenesis of seizures and chronic epilepsy, or the presence of high levels of cytokines merely reflects activation following seizures, cannot be determined from these clinical observations. However, consistent with these clinical findings are related results from experimental studies. In many animal models of epilepsy, acute seizures cause glial activation and increased expression of transcription factors and cytokines that coordinate inflammatory responses. Proinflammatory cytokines can affect neuronal excitability, either directly – by interacting with glutamatergic neurotransmission, or indirectly – by activating gene transcription.

Methods

Phosphate-buffered saline or KA were given to P15 ($3\,mg\,kg^{-1}$) and P30 ($10\,mg\,kg^{-1}$) Long-Evans male rats. These age-specific doses of KA have been determined previously to result in less than 25% mortality while inducing seizures in over 60% of animals. Only animals with nearly continuous seizures for more than 30 min were included in the study. P15 is considered roughly equivalent to human infancy or early childhood. P30 animals were chosen because their response to KA injection is adult-like, with neuronal injury and occurrence of spontaneous seizures, although P30 might be more accurately considered pubescence rather than adult.

We assessed hippocampal mRNA expression using microarrays after KA-induced SE in immature (P15) and mature (P30) rats at five time points (1, 6, 24, 72, and 240 h). For qRT-PCR, separate sets of rats were sacrificed at 1, 6, and 24 h post seizure. Total RNA was isolated from individual hippocampal tissues. For microarray analysis, equimolar quantities of RNAs were pooled from four animals (eight hippocampi) for each sample to average out individual variability present in the rat brain (i.e., single nucleotide polymorphism 'noise'). Each sample was hybridized onto an Affymetrix rat U34A gene chip. Three independent hybridizations were performed per condition per age per time point – a total of 60 chips ($3 \times 2 \times 2 \times 5 = 60$). In our data, each replicate had a correlation coefficient of greater than 0.95, indicating highly reproducible results. Preparation of cRNA, array hybridization, and scanning were performed by an array consortium (NINDS/NIMH Microarray Consortium). Individual RNA samples were not pooled for qRT-PCR.

Microarray data analysis consisted of many levels of quality control and analysis. At the chip level, the fluorescent image of each chip was scrutinized using programs such as dChip (HSPH) (that allowed direct visualization of the chips) and Probe Profiler (Corimbia, Inc., Berkeley, CA) (that detected and corrected saturation artifacts and outliers). MAS, Affymetrix software, generated the raw expression value of each probe set from the scanned image. This expression value, the signal value of intensity, is a quantitative metric calculated for each probe set, representing the relative level of expression of a transcript, upon which further analysis was based. Probe-level analysis relies on the interpretation of probe sets hybridization performance (pairs of 16 perfect match and mismatch 25-mer oligonucleotides per probe sets) as a measure of whether signal intensities are significantly above background and specific to the gene of interest. The detection algorithm uses probe pair intensities and assigns a 'present', 'marginal', or 'absent' as a flag, after comparison to a predefined threshold. For analysis of each gene, normalization was performed by standardizing experiments between real-time biological variations (controls) and alteration in gene expression following KA treatment. First, the averages of the three control replicates were calculated to form the baseline gene expression, by which the signal intensity of each KA-treated probe set was divided. This normalized value of KA replicates, representing fold change from the control, was used for further analysis (see Table 1). GeneSpring (Silicon Generics) was used for normalization, hierarchical cluster analysis, and graphics generation. For group comparison between control and KA-treated samples, statistical analysis (significance analysis of microarrays (SAMs), Stanford University) was used to select genes that were significantly regulated by KA. SAM generates a q-value (our cutoff 0.05) that reflects a false discovery rate.

Results

Selection Process and List of Genes Selected

Selection of genes significantly regulated by KA-induced seizures was carried out in two steps. First, flag selection was performed as described in Methods. By selecting 'present' in at least two replicates out of three, for every time course and for both control and KA-treated samples, we eliminated genes with low precision, those with expression levels too close to the background. Although this was a rather stringent criterion, we found that it allowed us to include most probe sets with reliable and consistent performance, and minimized background noise. Forty to fifty percent of genes were selected by this process of 'scrubbing'. Second, SAM was used for statistical selection with the median false discovery rate (q-value) of less than 5%. Only known genes with greater than twofold changes were included in our list of selected genes. Figure 1 illustrates the selection process.

Table 2 shows the number of genes selected. At 1, 6, and 24 h after KA-induced seizures, there were over twice

Table 1 An example of normalization: Egr1 (early growth response 1) in P15 and P30 rats at 1 h

	Normalized value	*Control*	*Signal intensity*	*t*-test *p*-value	*Flag*
P15 rats					
Control 1	0.829	381.5	316.3	0.527	Present
Control 2	1.286	381.5	490.4	0.356	Present
Control 3	0.885	381.5	337.7	0.678	Present
KA 1	2.986	381.5	1139	0.0599	Present
KA 2	3.06	381.5	1167	0.0585	Present
KA 3	4.023	381.5	1535	0.0282	Present
P30 rats					
Control 1	1.073	332.5	356.7	0.65	Present
Control 2	0.972	332.5	323.2	0.891	Present
Control 3	0.955	332.5	317.6	0.805	Present
KA 1	6.456	332.5	2147	0.0164	Present
KA 2	9.509	332.5	3162	0.0075	Present
KA 3	7.232	332.5	2405	0.0115	Present

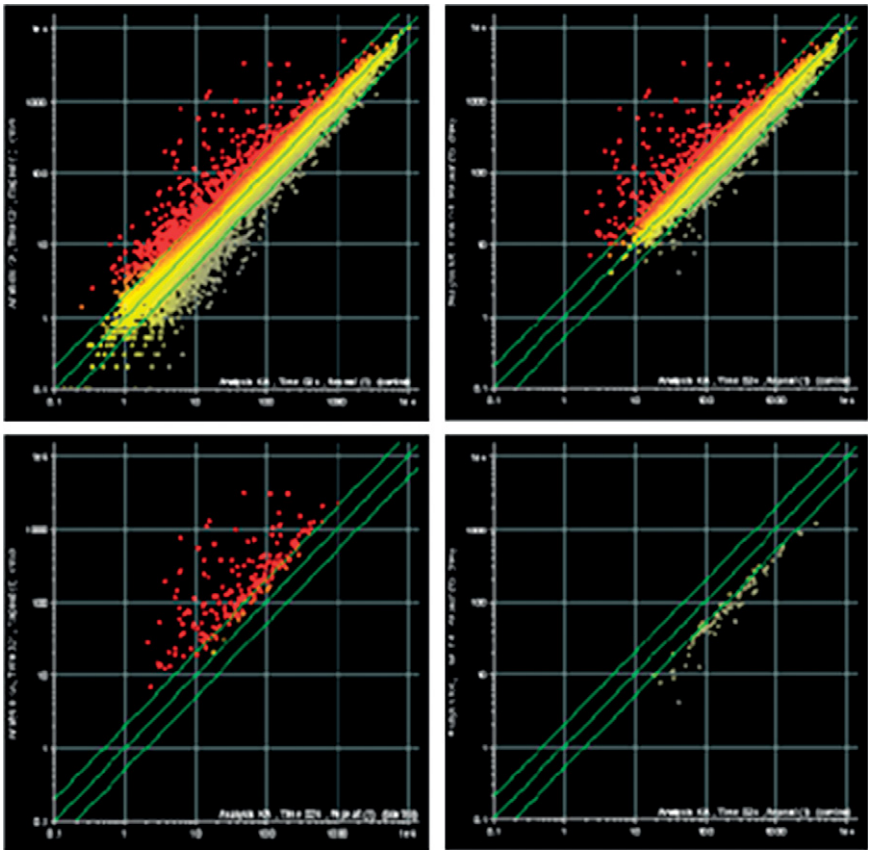


Figure 1 An example of selection (P30 24 h KA 1). Upper left: before selection (8799). Upper right: after flag selected (4525); lower left: after statistical selection up-regulated (207); upper right: down-regulated (78). Horizontal axis: control value; vertical axis: normalized KA value; green line: twofold.

as many genes up-regulated in P30 rats than in P15 rats. And in P15 samples, there was a virtual disappearance of genes significantly increased at 72 and 240 h, whereas a substantial number of genes remained up-regulated in P30 samples. The peak of gene expression for both P15 and P30 rats appears to be at 24 h.

Immediate-Early Genes

Gene induction appears to be the preferred process for acute seizure-regulated changes in neuronal function. There was an early wave of gene induction at 1 and 6 h, followed by a second wave of genes that were either induced or repressed at 24 h.

By far the most predominant class of induced genes, more than 80% in both P15 and P30 rats, was transcription factors or immediate-early genes (IEGs). They were turned on en masse at early hours after SE. They included Ania (activity and neurotransmitter-induced early gene)-2, Ania-3, Ania-6a, Ania-7, Arc (activity-regulated cytoskeletal-associated protein), Btg2 (PC3;Tis21), C/EBP (CCAAT/enhancer binding protein), c-fos, Crem (cAMP responsive element modulator, ICER), Egr1 (early growth response1, NGFI-A, zif268, Krox24), Egr2 (Krox20), Homer1a (vesl-1), v-jun, JunB, Narp (neuronal activity-regulated pentraxin), Nr4a1 (nuclear receptor subfamily 4, groupA, member 1, also known as NGFI-B), Nr4a2 (Nurr1), Nr4a3, Plat (tissue plasminogen activator), and Ptgs2 (Cox2). These genes include activity-regulated transcription factors and IEGs capable of modulating signaling cascade or pathways and protein networks. These genes are intimately associated with cellular and synaptic plasticity and can affect many biological processes. They initiate genomic response underlying long-term modification of neuronal physiology at later time points, thus translating a seizure activity into lasting changes in cellular function. In our data set, there were about twice as many IEGs induced at P30 than at P15, a clear indication that the KA-induced seizures exert more transcriptional changes in adult brain (see Table 3).

Table 2 Up-regulated known genes

	1 h	*6 h*	*24 h*	*72 h*	*240 h*
P15 (twofold, $q < 0.05$)	46	130	97	3	0
P30 (twofold, $q < 0.05$)	107	208	207	48	44
Common	45	105	52	1	0
P15 average fold change	4.68	5.83	5.26	2.36	0
P30 average fold change	8.22	9.35	7.89	3.30	7.09
Down-regulated known genes					
P15 (twofold, $q < 0.05$)	0	5	19	0	0
P30 (twofold, $q < 0.05$)	4	55	78	0	0
Common	0	3	3	0	0
P15 average fold change	0	0.42	0.45	0	0
P30 average fold change	0.46	0.42	0.41	0	0

Inflammation-Related Genes

The transcription factors and IEGs activated at early hours (1 and 6 h), while being crucial regulators of subsequent activity-dependent gene expression, are not unique to KA-induced seizures. Other stimuli such as kindling or brain injury are also known to induce similar transcription factors. Transcripts that are regulated at 24 h and beyond, however, appear to be more stimulus-specific. Most dramatically regulated at 24 h after KA-induced seizures, in both juvenile and adult rats, were inflammation-related genes. Notable were transcription factors known to be early mediators of immune and inflammation responses, as well as cytokines associated with microglial activation. GFAP and vimentin mRNAs were also significantly up-regulated, indicating astrocyte activation. Genes for tachykine, coagulation factor, cell-surface antigens (MHC class II, mast cell), and cell-surface glycoproteins (CD9, CD44, CD63) were also acutely increased after early-life seizures. After KA administration at P30, these inflammation-related genes were increased to a greater extent than at P15 and were often persistently elevated beyond 72 h (Figure 2). High and sustained levels of inflammatory gene expression after KA administration in adult rats contrasts with transient inflammatory response after KA administration in the juvenile counterpart.

Rapid up-regulation of cytokines and complement components implicates microglial activation after KA-induced seizure (Figure 3). Also, significantly increased were small inducible cytokines (Scya2) and tissue plasminogen

Table 3 Early growth response genes in P15 and P30 rats at 1 h

Common name	*Probe set name*	*Control 1*	*Control 2*	*Control 3*	*KA 1*	*KA 2*	*KA 3*	*Fold change*	*q*-value
P15 rats									
Egr1	M18416_at	316.3	490.4	337.7	1139	1167	1535	3.36	2.03
Egr1	rc_AI176662_s_at	180.2	218.6	202	539.7	628.6	543.2	2.85	2.03
Egr2	U78102_at	32.1	71.3	37.9	516.1	426.8	415.4	9.61	2.03
Egr4	M92433exon#1-2_s_at	145.4	205.4	155	1091	830.4	1051	5.88	2.03
P30 rats									
Egr1	M18416_at	356.7	323.2	317.6	2147	3162	2405	7.73	0.72
Egr1	rc_AI176662_s_at	124.9	141.4	106.4	1235	1315	1070	9.71	0.72
Egr1	U75398_s_at	122.4	105.4	100.7	331.2	376.1	470.4	3.59	0.72
Egr2	U78102_at	25.6	22.3	28.9	1047	969.5	820.6	36.94	0.72
Egr3	U12428_at	5.2	6.9	5.2	57.1	91.1	100.5	14.38	0.72
Egr4	Rc_AI145177_f_at	265.4	233.2	244.4	583.1	525.2	777.7	2.54	0.72

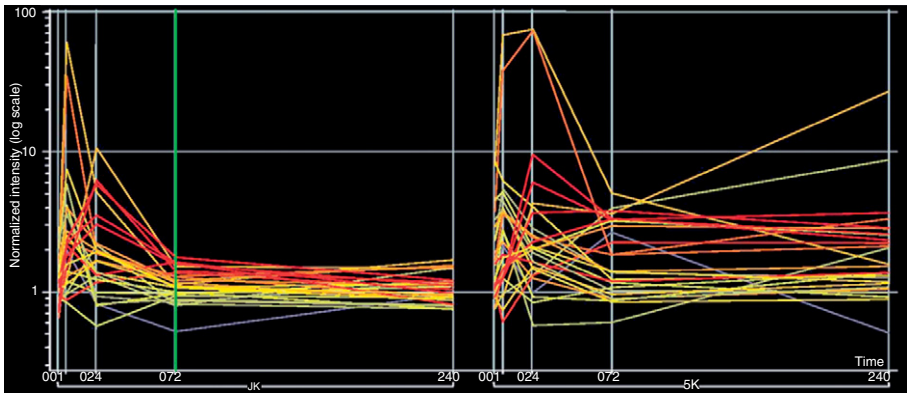


Figure 2 Summary graph of the time course of the fold changes in select inflammation-related genes significantly regulated in KA-treated samples compared with controls at 1, 6, 24, 72, and 240 h after KA administration at P15 (left panel) and P30 (right panel).

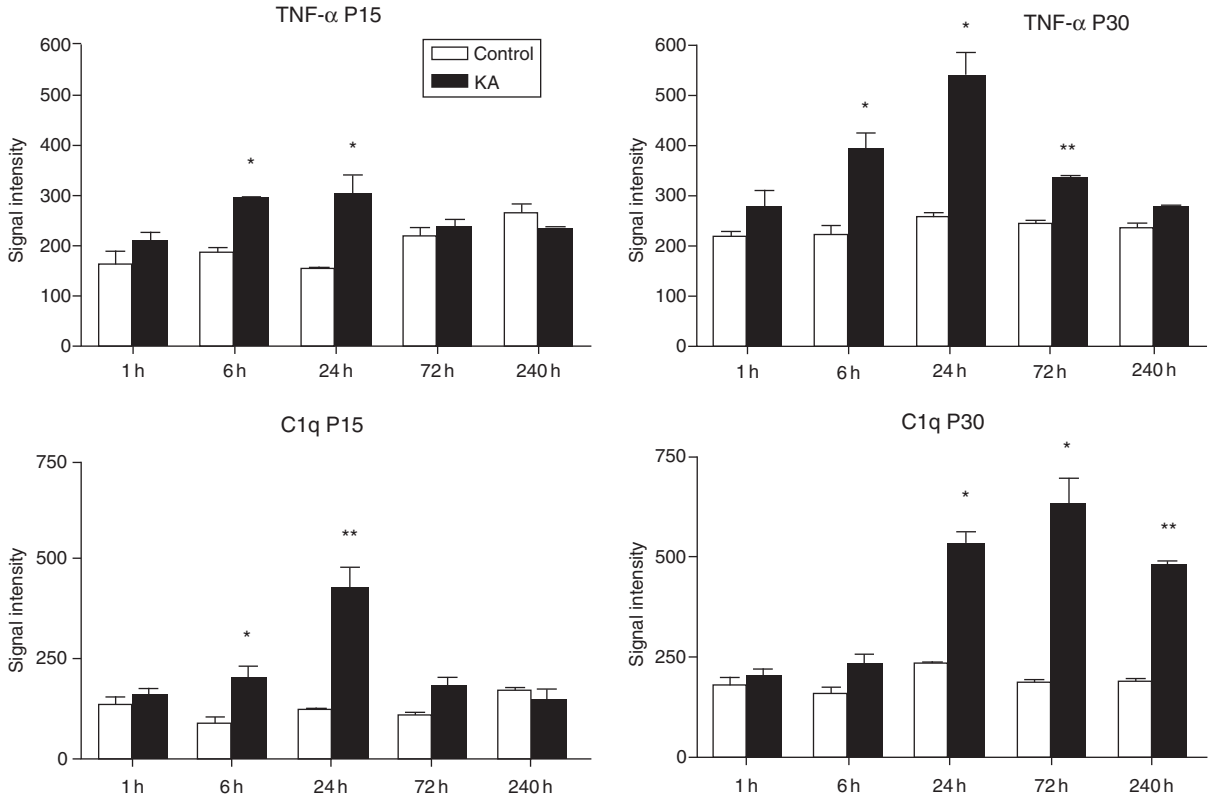


Figure 3 TNF-α and C1q.

activator (tPA or Plat), which showed acute transient increases at 6 and 24 h post KA administration in P15 animals and persistent increases (up to 72–240 h post-KA) in P30 animals. Astrocyte-specific transcripts, such as GFAP, S-100a4, S-100a10, and vimentin, were significantly increased after KA-induced seizures in immature and adult animals. While astrocytic activation was seen as early as 6 h in P15 rats, it was delayed until 24 h in P30 rats – and then remained markedly increased for 10 days, possibly reflecting glial activation following neuron degeneration at later time points in adult rats (**Figure 4**).

Transcripts for proinflammatory cytokines, IL-1β and IL-6, were uniquely increased after KA-induced seizures in adult animals. Delayed increases in MHC class II, C3, immunoglobulin Fc receptor gamma III (FcRgIII), and allograft inflammatory factor 1 (Aif1, marker for activated microglia) mRNA may indicate late transformation of microglia. In an experimental model of autoimmune disease, immune thrombocytopenia (ITP), disease induction requires the presence of low affinity FcRgIII, suggesting a pivotal role of this immunoglobulin receptor in the disease pathogenesis of autoimmunity. Whether FcRgIII

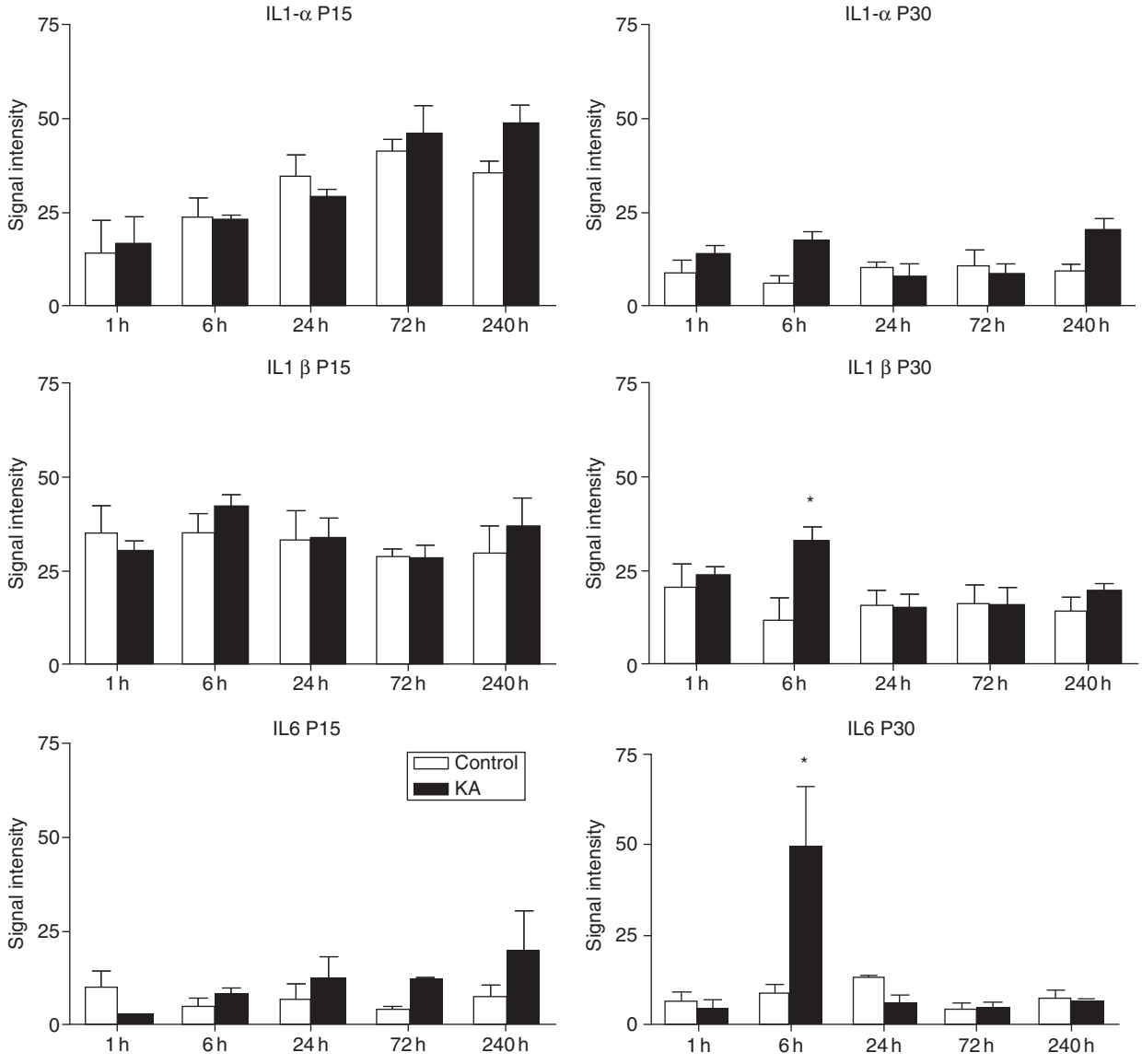


Figure 4 IL-1α, IL-1β, and IL-6.

activation may play a role in immunologic memory, and thus contribute to the long-term effects of early-life KA-induced seizures, remains to be determined.

Validation of Microarray Data

Just as any other technology, the microarray approach has its share of variances associated with various aspects of the process, including experimental design, reagents in hybridization, expression signal normalization, choice of base (house keeping) genes, the algorithm in probe set level analysis and fold change, and statistical methods in gene-level analysis. These variances require validation. There are many ways of validating microarray data, including northern blot, RT-PCR, real-time qPCR, and siRNA; in addition, one can use cross platform validation (i.e., assessing the results with a different microarray platform). Our choice for validation has been qRT-PCR. A relative quantification analytical method was used to analyze the real-time qRT-PCR data, by which the data are normalized to an endogenous reference gene. This normalized value is then used to compare control versus KA-treated samples. Microarray and qRT-PCR data are represented as fold change in KA-treated samples over controls. qRT-PCR results confirmed the expression level trends seen in microarray analysis for all 25 genes that we have examined. Fold changes were often higher in the qRT-PCR reaction than in microarray results, possibly reflecting a greater linear dynamic range afforded by qRT-PCR.

Summary and Future Goals

By using the microarray analysis of time-dependent sequential changes of genes, we found that transcription factors, cytokines, and complement that coordinate inflammatory responses are differentially regulated in juvenile and adult rat hippocampus after KA-induced seizures. Inflammation-related genes comprised the single most abundant functional group that is significantly regulated after KA-induced seizures in the hippocampus of both age groups, and also comprised the majority of genes that remained significantly elevated at 240 h in P30 rats. It is likely that neuronal death provoked further glial activation and a persistent inflammatory response 3–10 days after seizures. As a result, chronic, persistent, and active inflammation appears to occur in adult rats, as evidenced by the presence of mRNA coding for inflammatory cytokines and microglia-associated proteins. Because spontaneous recurrent seizures occur only in adult rats (not in juvenile rats) after KA-induced seizures, it is tempting to speculate that chronic active brain inflammation plays a critical role in future epileptogenesis. Although our results suggest a potential pivotal role of inflammation in spontaneous recurrent seizures and seizure-induced brain damage, they do not prove the causal role of these molecules for subsequent neuronal excitability and degeneration. We need to show the functional consequences of increased inflammatory cells and molecules in seizure-induced brain damage and in the epileptogenic process. That is, we need to demonstrate whether an acute seizure-induced inflammatory response leads to future seizure susceptibility and neuronal damage.

Microarray-based gene expression profiling provides a powerful tool to detect sequence of alterations that occur with time after seizures. Successful application of this method, however, requires the ability to sift through, manipulate, and analyze large volumes of data sets – and then to link them to the biological phenomena. Interpretation of microarray data can thus be a difficult and challenging task. In addition, the sensitivity of microarray technology for detecting genes of interest may be low. Many genes known to be expressed in the brain and important in epilepsy, such as channel genes, may not be reliably detected by this microarray approach. As tissue complexity (number of cell types) increases and transcript abundance (copies per cell) decreases, transcript may be diluted to levels below detection. Biological sensitivity of microarrays can be improved by further microdissection of cell type, such as dentate granule cells within the hippocampus.

As inflammatory responses involve a complex set of interactions, as well as integration among many soluble factors and cells, one must proceed with caution when considering antiinflammatory therapy. Chronic suppression of the inflammatory response may be just as harmful as unchecked chronic inflammation itself. Further, some inflammatory response can both heal and harm the CNS. When appropriately activated and deactivated, inflammation benefits the host defense response by removing damaged cells and promoting repair and recovery. However, uncontrolled or chronic glial responses can actually exacerbate the pathology, inducing further glial activation. When the balance between the beneficial and detrimental effects of activated glia is disrupted, the scales may tip toward neuropathological outcomes. Whether inflammation can be life-preserving or death-promoting appears to depend on the context, timing, and magnitude of the inflammatory reaction. For this reason, there is a concern that antiinflammatory strategies to reduce neuronal damage may interfere with neuronal regeneration and recovery, and thus render the intervention ineffective or even exacerbate the insult. It remains to be determined whether delayed intervention, which allows an early inflammatory response to occur, will maximize the benefit of modulating neuro-inflammation in preventing the seizure-induced epileptogenesis and neuronal injury. It is thus important to perform careful dose–response and time–course experiments to determine the efficacy of anti-inflammatory drugs and to optimize treatment protocol. Intervention in and modulation of the inflammatory response to acute seizures not only offer the potential to develop new neuroprotection strategies, but also allow investigators to examine the inflammatory processes involved in both injury and repair after a seizure.

See also: **Epidemiology**: Epidemiology: Status Epilepticus; **Epileptogenesis**: Multi-Hit Mechanisms Involved in Epileptogenesis: Role of Early-Life Seizures; Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Inflammation**: Inflammatory Responses and Cytokines; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Seizures**: Seizure-Induced Gene Expression; **Temporal Lobe Epilepsy**: Functional Genomic Analysis in Temporal Lobe Epilepsy.

Further Reading

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Multi-Hit Mechanisms Involved in Epileptogenesis: Role of Early-Life Seizures

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Introduction

The predominant and often sole abnormality found in surgically resected mesial temporal structures in patients with intractable temporal lobe epilepsy (TLE) is hippocampal sclerosis: severe hippocampal neuron loss and gliosis. Despite early recognition of this characteristic pathology more than 170 years ago, it remains unclear whether hippocampal sclerosis is the cause or consequence of repeated seizures. Perhaps the strongest argument for a causative role of mesial temporal sclerosis (MTS) in epilepsy is the remission of complex partial seizures after resection of sclerotic hippocampus. MTS, however, can result from a variety of insults, including hypoxia–ischemia, Pick's disease, Alzeimer's disease, general paresis, and pellagra – and most of these patients, in whom 'classic Ammon's horn sclerosis' was seen in postmortem material, never experienced seizures (Gloor, 1991).

The age at which an initial insult occurs may be a critical factor in epileptogenesis and hippocampal damage. Falconer was the first (1964) to suggest an etiological connection between genetics, prolonged febrile seizures (the most prevalent form of seizures during first 3 years of life), and subsequent development of MTS. Meencke, in his 1991 neuropathologic survey of age-specific epilepsy syndrome in 52 patients who died of other causes in adulthood, found hippocampal sclerosis in 46% of patients with infantile spasms (average age at onset, 3.5 months) and in 23% of patients with Lennox-Gastaut syndrome (average age at onset, 3.1 years) – but none in patients with childhood absence (average age at onset, 7.5 years) or in patients with juvenile myoclonic epilepsy (average age at onset, 15.5 years). Both clinical and laboratory studies, however, suggest that the immature brain is relatively resistant to seizure-induced brain damage. While early-life seizures may not be sufficient to cause epilepsy and hippocampal damage, molecular and cellular events that accompany early-life seizures may predispose the brain to future seizure-induced damage.

Background

Febrile Seizures and Risk of Later Epilepsy

Of 1706 children with a history of febrile seizures in the cohort of the US National Collaborative perinatal project, the risk of later epilepsy in a 7-year follow-up was 1–10%. In an extended 18-year follow-up study of 678 children with febrile convulsions, the risk of later epilepsy varied from 2.5% to 22%, and rose to 49% if a child had complex febrile seizures, an abnormal neurologic state, and family history of epilepsy. If the first febrile seizure occurred before the child was one-year old, later afebrile seizures and epilepsy were more likely. Preoperative neuropsychiatric evaluation also revealed that the early onset of seizures is correlated with significantly poorer cognitive function in patients undergoing temporal lobectomy for intractable epilepsy. Hippocampal sclerosis is found in only 1–5% of patients with adult-onset TLE compared with 50–80% of those with antecedent seizures, most often febrile, in infancy or early childhood. These studies show that children with febrile seizures are at increased risk for later epilepsy.

It is difficult, however, to distinguish between 'epilepsy triggered by fever' and the febrile seizure itself increasing the risk for later afebrile seizures. Moreover, a common genetic disposition may exist for both febrile seizure and epilepsy. In complex multifactorial disorders such as epilepsy, it is conceivable that both genetic susceptibility and injury (insult, life-event, or exposure) are necessary for

Koh S, Storey TW, Santos TC, *et al.* (1999) Early-life seizures in rats increase susceptibility to seizure-induced brain injury in adulthood. *Neurology* 53(5): 915–921.
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disease manifestation. For example, abnormal hippocampal shape and position can be found in healthy controls; however, a significantly greater proportion of such hippocampal abnormality is found in patients with partial epilepsy arising either from a cortical malformation or of a temporal lobe origin. The multiple hit hypothesis for disease expression predicts that the combination of diathesis, early-life brain insults, and later-life stress together can cause an individual to succumb to chronic epilepsy.

Epileptogenic Effects of Early-Life Seizures

Seizures that occur during a critical developmental period of enhanced neuronal excitability can increase long-term seizure susceptibility. Such a susceptibility caused by early-life seizures has been observed in different animal models. Hypoxia-, hyperthermia-, kainic acid- or flurothyl-induced seizures in the immature brain during the first 2 weeks of life cause permanent functional alterations and enhance long-term excitability within the hippocampal neuronal network. Further, intrauterine toxin-induced neuronal migration defects (simulating congenital brain malformations) can act as the first hit and predispose the brain to the damaging effect of seizures later in life.

Cellular and molecular mechanisms behind this heightened susceptibility to injury after early-life seizure remains unknown. Further, we do not understand the cellular and molecular mechanisms responsible for the subsequent occurrence of spontaneous seizures as a consequence of status epilepticus in adult animals. An influential current hypothesis posits that hippocampal neuronal loss and mossy fiber sprouting play a critical role in the pathogenesis of chronic epilepsy. Recent studies scrutinizing the relationship between chronic epileptogenesis and hippocampal histopathology, however, have challenged this hypothesis and shown that neither cell death nor mossy fiber sprouting is necessary or sufficient for the generation of spontaneous seizures after status epilepticus. While neuronal injury and mossy fiber sprouting may be common sequelae of status epilepticus, and accompany the epileptogenic process, they themselves do not appear to cause chronic epilepsy.

Role of Inflammation in Epileptogenesis and Seizure-Induced Neurodegeneration

The contribution of inflammation in mediating many aspects of the disease has been relatively well established for both acute CNS injury (such as cerebral ischemia and traumatic brain injury) and chronic CNS conditions (such as multiple sclerosis, HIV encephalopathy, and cerebral malaria). The active involvement of inflammation in the pathogenesis of epilepsy and seizure-induced brain damage has only recently been explored. Available evidence suggests that proinflammatory and antiinflammatory molecules are synthesized in glia during seizures, in the CNS regions where seizures initiate and spread. Such inflammatory molecules produced in the CNS may have a role in the pathogenesis of epilepsy. Recent studies also indicate (as has been observed in cerebral ischemia or traumatic brain injury) that cytokines may contribute to exacerbation of brain damage after seizures. Activation of resident microglia and subsequent increases in mRNA for cytokines and complements appear to be the key initiating events for seizure-induced inflammatory responses. We hypothesize that the inflammatory reaction provoked by early-life seizures primes the developing brain so that microglia are modified, leading to rapid reactivation by a second seizure in adulthood.

Methods

In our laboratory, we developed a 'two-hit' rodent seizure model in which systemic kainate (KA) or hypoxia was used to induce seizures in early life (postnatal day (P), P10–P21), and a second KA injection was given again in adulthood (P35–P45). The effect of early-life seizures on later seizure susceptibility was measured by examining seizure onset latency and grade of seizure severity in the adult animals. Seizure-induced neuronal injury was also examined using in situ detection of DNA fragmentation. This assay offers cellular resolution and yields dense nuclear staining of fragmented DNA, a robust and quantifiable marker of cell death. To determine whether seizure-induced inflammation plays a direct role in increasing seizure susceptibility after the experience of early-life seizures, we treated rats with minocycline, an inhibitor of microglia activation, for 7 days after the initial seizure. Microglia were subsequently visualized by Aif-1 immunocytochemistry.

Systemic administration of KA, which stimulates a subset of excitatory glutamate receptors, induces prolonged seizures in rodents. KA seizures originate in the hippocampus and cause characteristic patterns of neuronal death that resemble human hippocampal sclerosis. Hippocampal lesions are not due to direct toxicity of KA, but rather due to epileptic activity induced by the intraperitoneal injection of KA, as blocking the epileptic events with diazepam abolishes the neuronal loss. Seizure-induced cellular damage appears to be mediated by excessive excitatory amino acid release, which activates NMDA receptors and voltage-gated Ca^{2+} channels, both of which allow Ca^{2+} influx. The calcium influx then results in a cascade of biochemical changes that subsequently leads to cell death. Seizures induced by KA also cause axonal reorganization referred to as 'mossy fiber sprouting.' Most important, KA-induced status epilepticus is followed by a latent period between injury and the onset of spontaneous seizures, and finally a chronic

epileptic state. In immature rats younger than P18, however, KA injection (or hypoxia-induced seizures) causes neither cell death nor mossy fiber sprouting, although it induces status epilepticus at a dose one third the adult dose per weight. Furthermore, spontaneous recurrent seizures do not develop after status epilepticus until animals reach P20.

Results

Seizures in immature animals may cause subtle functional or molecular changes without causing cell death, and thus alter the response to seizures later in life. In our two-hit rodent seizure model, despite the lack of cell death, animals subjected to seizures during the second week of life become more susceptible to seizures in later life and sustain more extensive hippocampal neuronal injury after the second KA-induced seizure in adulthood (**Figure 1**). Furthermore, we have shown that the priming effect of early-life seizures could be reversed if acute seizures were blocked by an alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/KA-type glutamate receptors antagonist (**Figure 2**).

Recently, we began to examine the role of brain inflammation in mediating the epileptogenic effect of early-life seizures. Our focus was on microglia, resident immune cells in the CNS and the primary source and target of

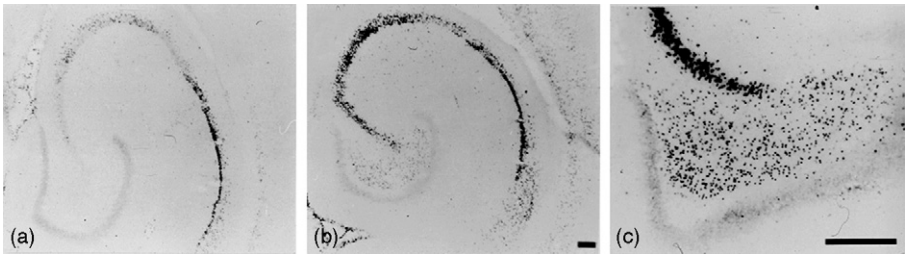


Figure 1 DNA fragmentation in the hippocampus 10 days after KA seizures on P45. (a) Animal treated with saline on P15 and KA on P45. (b) Animal treated with KA at P15 and P45 (KK). (c) Higher magnification view of KK hippocampus showing extensive hilar and CA4 neuronal injury. Scale bar = 200 μm. Reproduced from Koh S, Storey TW, Santos TC, *et al*. (1999) Early-life seizures in rats increase susceptibility to seizure-induced brain injury in adulthood. *Neurology* 53(5): 915–921, with permission from Lippincott, Williams & Wilkins.

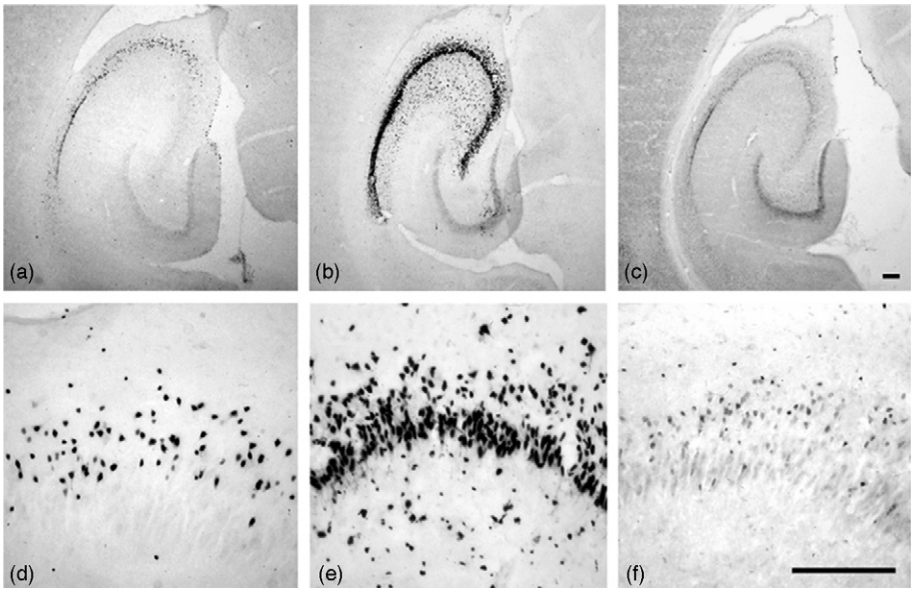


Figure 2 Blockade of perinatal seizures prevents the damaging effect of hypoxia on later-life seizure-induced neuronal injury. (a, d) KA alone at P30 without prior exposure to hypoxia at P10. (b, e) An animal exposed to hypoxia at P10 and KA at P30. Notice widespread seizure-induced DNA fragmentation throughout all subfields of the hippocampus of animal previously exposed to hypoxia. (c, f) An animal treated with topiramate, an anticonvulsant shown to attenuate AMPA/KA current, prior to hypoxia at P10 and KA at P30. Scale bars = 100 μm. Reproduced from Koh S and Jensen FE (2001) Topiramate blocks perinatal hypoxia-induced seizures in rat pups. *Annals of Neurology* 50(3): 366—372, with permission from Wiley.

many cytokines and chemokines. Within 24 h after KA-induced seizures, we observed retraction of processes and cellular hypertrophy of microglia, suggestive of an activated state. Further, the group that experienced early-life seizures (K/K) had a marked increase in the number of activated microglia compared with a group of control littermates who received single exposure to KA in adulthood (saline/kainate, S/K). These microglia were much larger and formed a complex, web-like network localized within the hippocampus especially over the CA1 region (**Figure 3**). The early experience of KA-induced seizures primed the developing brain such that there was a lowered seizure threshold to the second 'hit' (the KA injection in adulthood), as well as rapid microglial activation associated with that second KA-induced seizure (**Figure 4**). Increased susceptibility to later-life seizures is associated with rapid hyperplastic reactivation of microglia after the second KA. To show a causal link between initial microglial activation and seizure susceptibility, we treated the animals with an inhibitor of microglia activation, minocycline, after the initial (early-life) seizure, and determined whether minocycline reversed this priming effect of early-life seizures. Both increased seizure susceptibility and microglial hyperactivation caused by early-life seizures were abolished by minocycline treatment. Our data thus suggest that microglial activation and brain inflammation occur following KA-induced seizures, and that modulation of the inflammatory responses to seizures could be a target for specific types of pharmacological intervention to block the epileptogenic effect of early-life seizures.

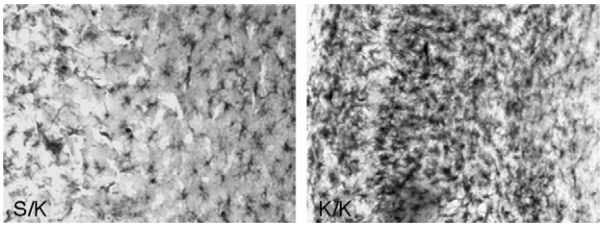


Figure 3 Reactivating effect of an early-life seizure on microglia. The rats were injected either with saline (S/K) or KA (K/K) at P21 and challenged with KA at P35. Microglia activation is increased in K/K.

Work in Progress and Future Goals

We found that the experience of early-life seizures primes the microglia so that a much more robust activation is elicited in response to later-life seizures. Microglial reactivation may be causally related to the epileptogenic effect of early-life seizures. Neuroinflammation provoked by seizures appears to be mediated via activation of resident CNS immune cells, microglia. Sequential propagation and widespread microglia activation have been described in rodent models of acute seizures. As rapid responders, imbalanced or chronic activation of microglia to full-blown phagocytic and potentially cytotoxic cells may *cause* neurodegeneration rather than microglia being activated as a *consequence* of neurodegeneration. In our model, we hypothesize that the experience of early-seizure facilitates this

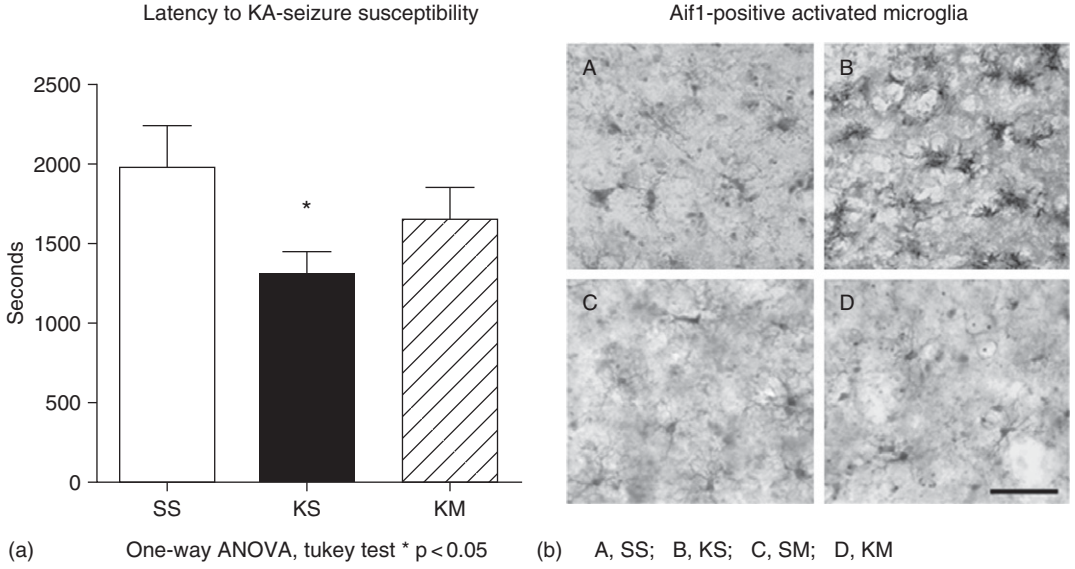


Figure 4 (a) Seizure susceptibility to KA on P35 was increased after KA on P21 (KS), compared with saline treated littermate (SS). This epileptogenic effect of early-life seizure was reversed by treating the animals with daily injection of minocycline for one week (10 mg kg^{-1}, i.p.) (KM). (b) Microglia activation 48 h after KA seizures at P35. High magnification view of hilar microglia of rat hippocampus. (A) SS: Saline at P21, KA at P35, (B) KS: KA at P21and P35, (C) SM: Saline at P21, minocycline × 7 days, KA at P35; (D) KM: KA at P21, minocycline × 7 days, KA at P35. Scale bar = 100 μm.

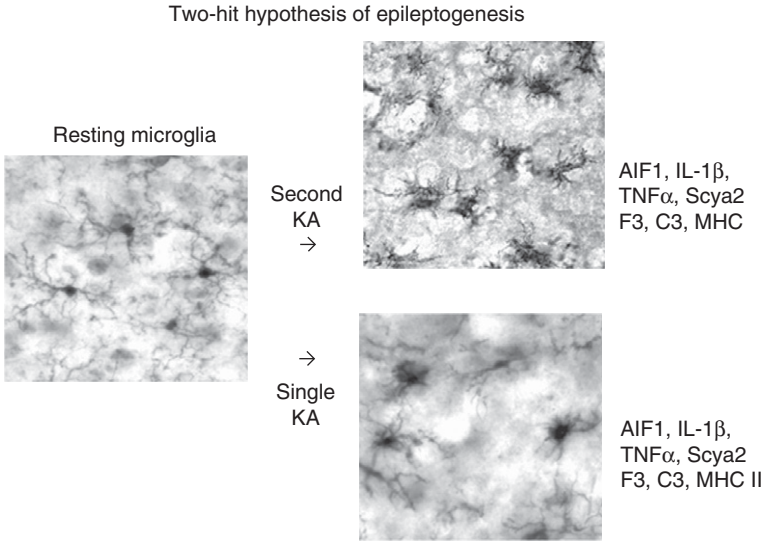


Figure 5 The 'two-hit' model of epileptogenesis.

reactive microgliosis, leading to excessive activation of microglia (Figure 5) and to subsequent increases in susceptibility to seizures. This proposed central role of microglia in seizure-induced neuronal excitability and degeneration suggests that an inhibition of microglia activation may be a potential target for therapeutic intervention. Although anti-inflammatory therapy may be antiepileptogenic, potential risks associated with immune modulation need to be weighed against its benefit.

The role of activated glia as a sensor and a cause of brain injury, as well as a mediator of neuronal excitability and epileptogenesis, will need to be directly assessed both in vitro and in vivo. Pure microglia cultures can be established with relative ease from early postnatal rodent brain. We can take advantage of a transgenic mice, $Cx3cr1^{GFP/+}$, in which all microglia are fluorescently labeled, and establish a neuron–microglia cocultures to demonstrate the contribution of activated microglia in neuronal excitability. Neuronal excitability can be measured by calcium influx (Fura-2-AM, a membrane-permeable calcium-sensitive dye) evoked by an acute application of potassium in the culture bath. Whether activated microglia mediate neuronal hyperexcitability can be tested in vivo with the use of cultured pure microglia. Induction of spontaneous seizures or a lowering of seizure threshold by stereotactic injection of hyperactive microglia into the hippocampus may provide direct evidence that activated microgila can cause seizures.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Epileptogenesis**: Gene Expression in Immature and Mature Hippocampus After Status Epilepticus; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; Developmental Aspects of Seizures; Seizure-Induced Injury and the Two-Hit Hypothesis.

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Patterns of Gene Expression in Epileptogenesis: Micro-Array Studies in Rats

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Introduction

To study changes in gene expression of multiple genes, the DNA microarray approach is now the most frequently used technique. A DNA microarray, or gene *chip*, is a collection of microscopic DNA spots – each of which represents a single gene – arrayed on a solid surface. Measurements with DNA microarrays use nucleic acid hybridization under high-stringency conditions. DNA arrays are commonly used for monitoring expression levels of thousands of genes simultaneously. Many epilepsy-related gene-expression studies compare gene expression between normal and epileptic hippocampal tissue. Although this tissue consists of an mRNA mixture of different cell types, expression profiles from selected brain regions can be used to describe changes in local networks. In this article, we focus on the large-scale microarray studies on (para)hippocampal (sub) regions in a rat model for temporal lobe epilepsy. In this model, epilepsy develops a few weeks after electrically induced status epilepticus (SE). To obtain insight into how epilepsy develops after an initial insult, we characterized the temporal profile of changes in gene expression during three crucial stages of epileptogenesis: the acute, latent, and chronic epileptic phases.

Background

Before the existence of microarrays, studies on gene expression related to temporal lobe epilepsy – in animal models – were mainly focused on individual genes that were induced by acute seizures or repeated electrical stimulations that led to an epileptic condition (e.g., kindling). In the late 1980s, many studies appeared (e.g., using in situ hybridization) that described seizure-related changes in gene expression in the rat brain. For instance, shortly after seizure occurrence, a marked upregulation was found in the expression of immediate early genes (IEGs; transcription factors). These IEGs stimulate the expression of genes such as growth factors, and also activate genes associated with apoptosis and stress. For a comprehensive review of the studies that report changes after kainate-induced SE, see Zagulska-Szymczak et al. in Further Reading.

The microarray technique has provided significant insights into the expression of a large number of genes in specific brain regions following a seizure. Tang and colleagues were the first to show the acute coordinate gene-expression changes in the parietal cortex 1 day after kainate-induced SE. They showed that most genes induced by kainate-induced seizures, were also induced by ischemia, intracerebral hemorrhage and hypoglycemia – a finding that suggests that excitotoxicity-mediated injury is common to all of these acute conditions. The rapidly activated genes included genes encoding for heat shock proteins, DNA damage repair proteins, proteases and protease inhibitors, metal ion and free-radical scavengers, and calcium-binding proteins. The induction of Hmox1, ceruloplasmin, GFAP, vimentin, matrix glia protein, and S-100 genes pointed to a generalized response of glial cells in all of these injury conditions.

More recently, special attention has been paid to changes in gene expression at increasingly longer time points after an initial insult, in order to better understand the evolution in time of the process of epileptogenesis. Attention to temporal changes in gene expression is essential in order to understand the dynamics of how the brain becomes epileptic, and can provide insight into the therapeutic potential of a specific target. Using long-term video and EEG observations, investigators have shown that most animals develop epilepsy within a few weeks after an initial SE evoked by injections of kainate or pilocarpine or via electrical stimulations of a specific brain region (often hippocampus or amygdala). In these so-called post-SE models, frequently used to study the development of temporal lobe epilepsy after an initial insult, we can roughly separate three crucial stages of epileptogenesis: (1) the *acute phase*, in which the animals exhibit seizures in the immediate aftermath of the status; (2) the *latent period*, which is characterized by the absence of spontaneous seizures and that ends with the first spontaneous seizure; and (3) the *chronic phase*, in which the animals exhibit spontaneous seizures. A few studies have described gene expression at these different phases of epileptogenesis. One group used the Research Genetics Rat array on whole hippocampal tissue obtained from the amygdala stimulation model of TLE. They studied the gene-expression profile at 1, 4, and 14 days after induction of SE, and showed that several of the differentially expressed genes were associated with neuronal plasticity, gliosis, cellular metabolism, and inflammation. Another group studied differential gene-expression patterns in dentate gyrus (DG) and CA1 hippocampal areas after

pilocarpine-induced SE, using the Affymetrics 34A array. In that study, at 3 days after SE, the majority of upregulated genes were related to mechanisms of cellular stress and injury; a greater degree of gene upregulation was seen in CA1 than in DG. At 14 days after SE, numerous transcription factors and genes involved in cytoskeletal and synaptic reorganization were differentially transcribed. During the chronic stage (>20 days post-SE), expression changes were observed in genes associated with different neurotransmission pathways. Although a direct comparison between these studies is not possible, there was general consensus that inflammation-related genes were also activated after the acute phase following SE.

Methods

Using the Affymetrics Rat RAE 230A, we studied gene expression at three specific stages after electrically induced SE, looking specifically in hippocampal CA3, entorhinal cortex (EC), and cerebellum (CB). Total RNA was obtained from the selected brain regions, quantified, amplified, and labeled according to the Affymetrix Small Sample labelling protocol. Samples were hybridized to the GeneChip. After 16 h of hybridization, the GeneChips were washed and stained on a fluidics station and scanned in a confocal scanner. The expression levels and present/absent calls were calculated for all 15 866 probe sets, with the affy package from Bioconductor in the R program for statistical computing, using the MAS5 algorithm. Comparisons were made between control and experimental samples taken at the same time points; the analysis required at least $p < 0.05$ for a significant difference. Because array analysis deals with large numbers of multiple comparisons, it is necessary to take into consideration the false discovery rate for multiple comparisons. The dataset consisting of the significantly altered genes was entered into GenMAPP (Gene Map Annotator and Pathway Profiler), a computer program that is designed for viewing and analyzing genome-scale data.

Results – Patterns of Gene Expression in the Electrical Post-SE Model

In the post-SE model, CA3 and EC are assumed to belong to the epileptogenic region while CB does not. The time points of our study included 1 day after SE (acute phase), 1 week after SE (latent period), and 3–5 months after SE (when rats exhibit frequent daily seizures). The RAE230A GeneChip contains many more probes (~16.000) than the previous arrays (e.g., the arrays used in the studies mentioned above use <8.000 probes). With that chip, we could demonstrate coordinate changes of groups of genes that belong to specific biological processes or pathways. Using specific web-accessible software for large-scale data analysis, we obtained an overview of the expression of genes that belong to a specific Gene Ontology (GO) term (biological process, molecular function, or structural component) at a specific time point. In this way, dynamic changes at each crucial stage of epileptogenesis could be described in this electrical post-SE model. **Figure 1(a)** shows a global overview of the distribution of up- and downregulated genes in CA3 at the three different phases. The number of genes that were differentially expressed is very high at the acute stage, and progressively decreases over time. Moreover, within a specific process (or function or component), there are genes that are activated but also genes that are downregulated, perhaps reflecting a homeostatic biological response to the molecular SE. The relative expression changes for each process, function, and cellular component (**Figure 1(b)**, pie charts) remain surprisingly stable over time. A similar distribution can be constructed for expression in EC.

To find significant trends during epileptogenesis, the percentage of genes that changed at a specific time point can be calculated for each GO term, and a statistical evaluation can be made. For instance, we ranked the GO terms by a *Z*-score, using the MappFinder program. This analysis reveals that the immune and inflammatory responses, among others, play a prominent role not only during the early phase and latent period but also during the chronic phase.

The Acute Phase (1 Day after SE)

As mentioned earlier, the general consensus is that the inflammatory response plays a prominent role (at least in those brain regions examined to date – mainly the hippocampal and parahippocampal subregions) shortly after SE. In the CA3 and EC regions of the post-SE model, we confirmed maximum activation of processes, such as transcription and translation, cell differentiation, progression through cell cycle, neuropeptide signaling, and immune responses, at this time point. Many genes related to cell adhesion, synaptic transmission, and ion transport have the strongest suppression at this acute phase, in both CA3 and EC. Such changes are not observed in the cerebellum. On the basis of the injury that is caused by SE, it is not unexpected that many acute changes include activation of inflammatory- and proteolysis-related genes. These changes are associated with increased gene expression of GFAP and vimentin, both genes that code for intermediate filament proteins present in astrocytes – which are, together with microglial cells, the main cellular entities in the brain responsible for release of inflammatory and proteolytic proteins. Vimentin expression is almost 10-fold increased at 1 day after SE and remains

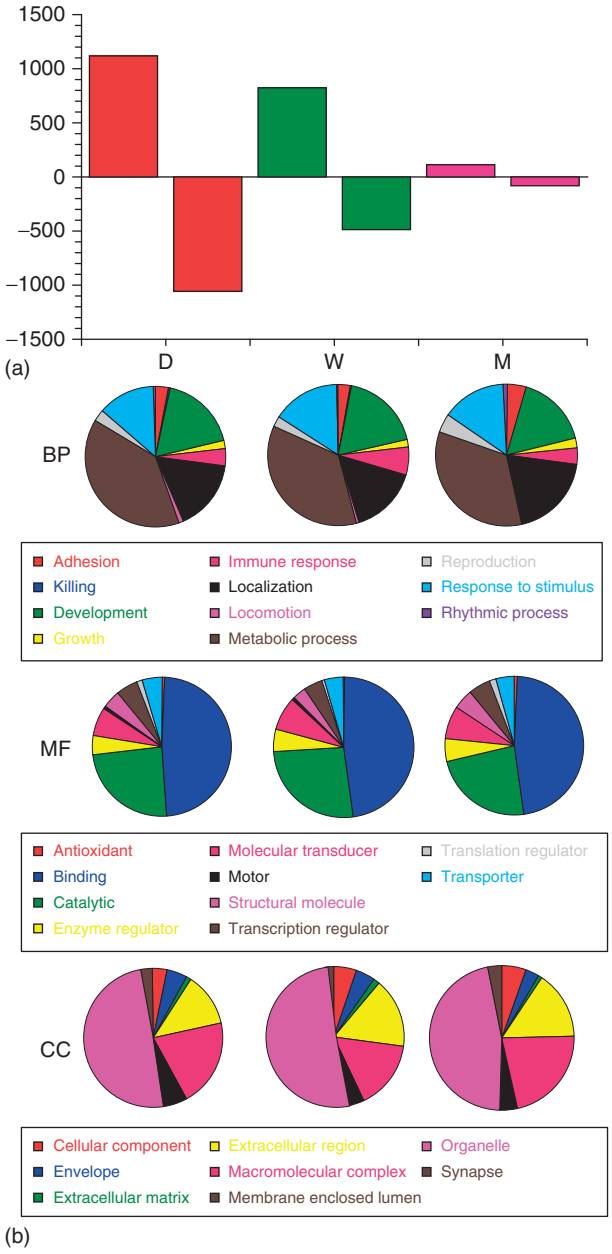


Figure 1 Global overview of the distribution of up- and downregulated GO terms in CA3 at the three different phases at 1 day (D), 1 week (W), and 3–5 months after SE (M: in the chronic phase). (a) Shows that the number of genes that were differentially expressed is high at the acute stage and progressively decreases over time. (b) The relative expression changes in each biological process (BP), molecular function (MF), and cellular component (CC) remain rather stable over time.

significantly increased during all three phases of epileptogenesis. Many genes that are upregulated during the acute phase show a slower time course, with maximum activation at a later time point (e.g., at 1 week; explained later). It should be noted, too, that increased expression of more rapidly activated genes with transient expression might have been missed in our study. Expression of many (but not all) proteases peaked at 1 day; these proteases include cathepsins (*Cts*), caspases (*Casp*), calpains (*Calp*), matrix metalloproteases (e.g., *Mmp9*), tissue-plasminogen activator (*Plat*; which can activate *Mmp9* transcription) and urokinase-plasminogen activator (Plau). *Stat3*, *CD14*, and *Spp1* (Osteopontin – which binds to *CD44* and is responsible for macrophage migration) also peaked at 1 day after SE. Noteworthy, several protease inhibitors were also upregulated, such as cystatins (*Cstb*, *Cstc*), tissue inhibitors of metalloproteases (*Timp1* and *Timp3*), and serine inhibitors (Serpins). The chemokine *CCl2*, which is also involved in macrophage migration, was the most markedly activated gene, while *IL1b* and *IL6* were among the most activated interleukin genes at this acute time point. The process of synaptic transmission is most significantly downregulated in the acute phase. This downregulation could reflect a homeostatic protective response, rather than an epileptogenic response. For example, the downregulation of glutamate receptor activity can help to limit further excitotoxic damage. Downregulation of the expression of these genes does not simply reflect neuronal loss, but consists of a transient repression of gene expression, since ion channel and receptor gene expression largely recover in later phases. The seizure-related increase of several neuropeptides can also be seen as a compensatory response, since several neuropeptides have antiepileptic properties.

The Latent Phase (1 Week after SE)

During the latent phase, which in this electrical post-SE model is relatively short (an average of <8 days), many cytokines, proteases, and their inhibitors were still significantly upregulated – and several of these genes showed maximum expression at this time point (e.g., *Tgfbr2*, *Casp1*, *Casp11*, *Ctsd*, *Ctsl*, *Mmp2*, *Aif1*, *CstC*). The immune response was the most significantly activated process at this time, both in CA3 and EC, which was not surprising considering the maximum microglial activation that can be detected. *IL1a*, *IL18*, *Casp1* expression, already significantly activated at 1 day after SE, peaked during the latent phase. Again, the cerebellum did not exhibit activation of these genes. Increased macrophage activity peaks at this time point as illustrated with Mpeg1 expression. Interestingly many genes belonging to the classical and alternative complement cascade displayed maximum expression at 1 week after SE. Overactivation of this system, which is a key component of the *innate immune* system, can mediate further inflammation and tissue damage. However, most of the significantly repressed genes at this time point are related to neurotransmitter receptor and ion channels, including multiple GABA-receptor subunits.

The Chronic Phase (3–5 Months after SE)

During the chronic phase, when post-SE rats exhibit daily seizures, many genes are still differentially regulated. Although in the acute and latent phases, hierarchical clustering analysis nicely grouped the gene profiles of CA3 and EC together, they were separated in the chronic phase. This 'dissociation' suggests that in the initial phases, the dominant changes are common to CA3 and EC, but in chronic phase, the two areas display distinct area-specific changes. Heat shock protein binding was the only GO term that was significantly upregulated in both CA3 and EC. Upregulation of aquaporin 4 (*Aqp4*), which is associated with a breakdown of the blood brain barrier (BBB), was increasingly evident during epileptogenesis. A few proteolytic and inflammation-related genes were also still activated during this phase (**Figure 2(a)**). Moreover, various 'activity-dependent' genes showed an increased expression after a transient 'silencing' during the latent period. These genes include *Sgk*, *Homer1*, *CryaB*, *Tnfaip6*, *Dnajb9* (*Hsp40*), *Akap8*, *Arf4*, *Slc2a1*, *Ddah1* (involved in nitric oxide biosynthesis), several neuropeptides (e.g., *Sst*, *Npy*, *Trh*, *Crh*, *Penk*, *Gal*), *pafah1b1*, growth factors, including *Bdnf*, *Cntf*, *Vgf*, *Ptgs2* (or *Cox-2*), *Agpt2*, *Egr1*, *Tieg*, and several genes involved in protein transport and folding (**Table 1**). Although some of the 'activity-dependent' genes are

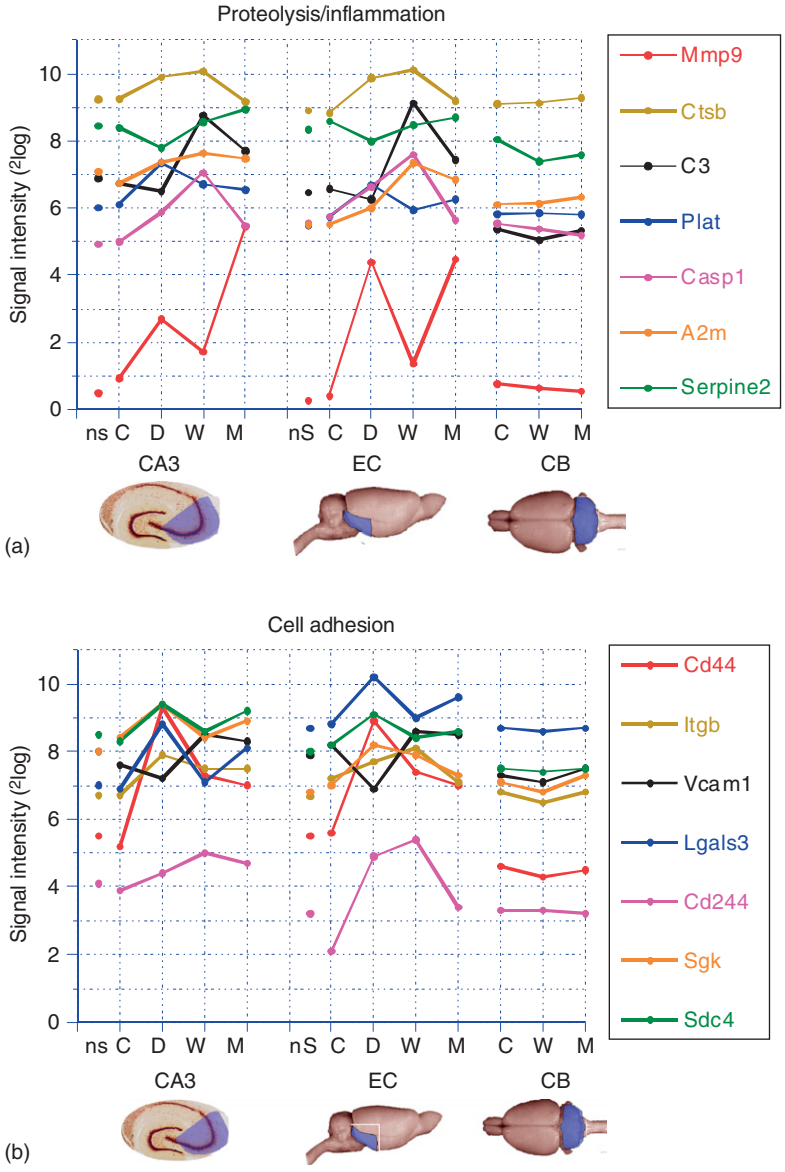


Figure 2 Examples of genes that are significantly activated in CA3 during the chronic phase and that are related to (a) proteolysis, inflammation and (b) cell adhesion. Note that many of the same genes are also activated in EC but not in cerebellum (CB) and that gene expression in stimulated rats that did not exhibit SE after stimulation (nS, 3–5 months after stimulation) were almost similar to controls.

Table 1 Gene symbols and gene names mentioned in this article, with corresponding biological process (BP) and corresponding fold changes at 1 day (D), 1 week (W) and 3–5 months (M) compared to control expression (C) in CA3 region

Gene symbol	*Gene name*	*BP*	*Fold-D/C*	*Fold-W/C*	*Fold-M/C*	*pCD–05*	*pCW–05*	*pCM–05*
A2m	Alpha-2-macroglobulin	Intracellular protein transport; inflammatory response	1.6	1.9	1.7	1	1	1
Agpt2	Angiopoietin 2	Angiogenesis	11.3	2.0	4.2	1	0	1
Aif1	Allograft inflammatory factor 1	Inflammatory response	1.3	6.3	0.9	0	1	0
Akap12	A kinase (PRKA) anchor protein (gravin) 12	Protein targeting	3.5	1.0	1.4	1	0	1
Aqp4	Aquaporin 4	Water transport	1.0	2.3	1.6	0	1	1
Arc	Activity regulated cytoskeletal-associated protein	Regulation of neuronal synaptic plasticity	3.1	0.88	1.1	1	0	0
Arf4	ADP-ribosylation factor 4	Intracellular protein transport	1.4	1.1	1.1	1	0	1
Arl4	ADP-ribosylation factor-like 4	Intracellular protein transport	1.9	1.2	1.5	1	0	1
Atrn	Attractin	Inflammatory response; heterophilic cell adhesion	0.6	1.1	1.4	−1	0	1
Bdnf	Brain-derived neurotrophic factor	Regulation of synaptic plasticity	4.2	0.9	2.5	1	0	1
C1s	Complement component 1, s subcomponent	Glial cell differentiation	3.5	3.5	2.7	1	1	1
C3	Complement component 3	Inflammatory response; complement activation	0.9	4.1	2.0	0	1	1
Camk2a	Calcium/calmodulin-dependent protein kinase II alpha subunit	Plasticity	0.8	0.8	0.5	0	0	−1
Camk2d	Calcium/calmodulin-dependent protein kinase II, delta	Plasticity	2.1	1.6	1.7	1	1	1
Capn2	Calpain 2	Proteolysis and peptidolysis	1.7	1.3	1.4	1	1	1
Casp1	Caspase 1	Regulation of I-kB kinase/NF-kB cascade	2.0	4.2	1.4	1	1	1
Casp11	Caspase 11	Proteolysis and peptidolysis	3.2	2.9	1.7	1	1	1
Casp2	Caspase 2	Proteolysis and peptidolysis	1.0	1.2	1.4	0	0	1
Ccl2	Chemokine (C-C motif) ligand 2	Inflammatory response; cell adhesion	640.1	23.3	3.2	1	0	0
Cd164	CD164 antigen	Immune response; cell adhesion	1.4	1.6	1.5	1	1	1
Cd24	CD24 antigen	Immune response; cell adhesion	1.1	1.5	1.6	0	1	1
Cd244	CD244 natural killer cell receptor 2B4	Lymphocyte activation	1.4	2.1	1.7	0	1	1
Cd44	CD44 antigen	Cell adhesion	17.1	4.3	3.7	1	1	1
Cd47	CD47 antigen	Cell–matrix adhesion	2.2	1.6	1.6	1	0	1
Cd9	CD9 antigen	Cell adhesion	3.7	2.5	1.6	1	1	1
Cfh	Complement component factor H	Complement activation	1.6	6.0	1.8	0	1	1
Cntf	Ciliary neurotrophic factor	Synaptic transmission; neurogenesis	1.3	1.5	1.4	0	1	1
Cryab	Crystallin, alpha B	Protein folding	2.2	1.0	1.5	1	0	1
Cst3	Cystatin C	NA	0.9	1.9	0.9	0	1	0
Cstb	Cystatin B	Regulation of apoptosis	1.7	1.1	0.9	1	0	0
Ctsd	Cathepsin D	Proteolysis and peptidolysis	1.1	2.2	1.1	0	1	0
Ctsl	Cathepsin L	Proteolysis and peptidolysis	2.7	2.7	1.2	1	1	1
Ctss	Cathepsin S	Proteolysis and peptidolysis	1.6	2.8	1.9	1	1	1

Dcamkl1	Double cortin and calcium/calmodulin-dependent protein kinase-like 1	Protein amino acid phosphorylation; neurogenesis	1.9	0.9	1.4	1	0	1
Ddah1	Dimethylarginine dimethylamin-ohydrolase 1	Nitric oxide biosynthesis	2.3	1.3	1.7	1	0	1
Dnajb9	DnaJ (Hsp40) homolog, subfamily B, member 9	Protein folding; anti-apoptosis	1.6	1.2	1.3	1	0	1
Egr1	Early growth response 1	Regulation of transcription	2.7	0.8	1.4	1	0	0
Egr2	Early growth response 2	Regulation of transcription	4.6	1.7	1.5	1	1	0
Eif2s2	Eukaryotic translation initiation factor 2, subunit 2 (beta)	Translational initiation	1.5	1.1	1.4	1	0	1
Emb	Embigin	Cell adhesion	1.0	1.0	1.4	0	0	1
Fn1	Fibronectin 1	Acute-phase response	1.8	1.2	1.8	1	0	1
Fosl1	Fos-like antigen 1	Regulation of transcription	4.7	1.2	1.1	1	0	0
Foxr1_pred	Forkhead box R1 (predicted)	Regulation of transcription	1.8	1.3	1.3	1	0	1
Gal	Galanin	Neuropeptide signaling pathway;	3.4	1.7	1.6	1	0	0
Gabra5	γ-Aminobutyric acid A receptor, alpha 5	γ-Aminobutyric acid signaling pathway; synaptic transmission	0.5	0.8	0.7	−1	−1	−1
Gabrd	γ-Aminobutyric acid A receptor, delta	γ-Aminobutyric acid signaling pathway; synaptic transmission	0.7	0.8	0.8	0	0	0
Gfra1	Glial cell line derived neurotrophic factor family receptor alpha 1	Cell surface receptor linked signal transduction;neurogenesis	1.6	1.2	1.5	1	1	1
Gpd1	Glycerol-3-phosphate dehydrogenase 1	Carbohydrate metabolism	5.1	1.2	2.0	1	0	1
Gpnmb	Glycoprotein (transmembrane) nmb	Osteoblast differentiation; cell adhesion	6.8	6.4	1.6	1	1	1
Gria1	Glutamate receptor, ionotropic, AMPA1	Regulation of synaptic plasticity	0.7	0.7	0.6	−1	0	−1
Hif1a	Hypoxia inducible factor 1, alpha subunit	Angiogenesis; regulation of apoptosis	2.3	1.3	1.2	1	1	0
Homer1	Homer homolog 1 (Drosophila)	Metabotropic glutamate receptor signaling pathway	4.6	1.2	2.9	1	0	1
Hspb8	Heat shock 22-kDa protein 8	Protein folding	4.2	1.1	1.3	1	0	0
Il13ra1	Interleukin 13 receptor, alpha 1	Cell surface receptor linked signal transduction	2.7	2.4	1.3	1	1	0
Il16	Interleukin 16	NA	0.3	0.8	0.9	−1	0	0
Il18	Interleukin 18	Angiogenesis; immune response	1.9	3.3	1.1	1	1	0
Il1a	Interleukin 1 alpha	Inflammatory response	1.7	1.8	1.0	1	1	1
Il1b	Interleukin 1 beta	Angiogenesis; inflammatory response	1.5	1.4	1.4	1	1	0
Il6	Interleukin 6	Immune response	7.9	1.1	0.9	1	0	0
Il6r	Interleukin 6 receptor	Immune response	1.0	2.2	1.0	0	1	1
Il6st	Interleukin 6 signal transducer	Immune response	1.2	1.6	1.3	0	1	1
Il9r	Interleukin 9 receptor	Signal transduction	1.1	1.2	1.3	0	0	1
Ilf3	Interleukin enhancer binding factor 3	Protein amino acid methylation	1.0	0.9	0.8	0	0	1
Insig1	Insulin-induced gene 1	Response to sterol depletion	1.4	0.5	1.7	1	−1	1

Continued

Table 1 Continued

Gene symbol	Gene name	BP	Fold-D/C	Fold-W/C	Fold-M/C	pCD–05	pCW–05	pCM–05
Itgb1	Integrin beta 1	Homophilic cell adhesion	2.2	1.7	1.7	1	0	1
Junb	Jun-B oncogene	Transcriptional activator activity	2.4	1.2	1.1	1	0	0
Kpnb3_pred	Karyopherin (importin) beta 3 (predicted)	Protein transport	2.7	1.2	1.3	1	0	1
Lgals3	Lectin, galactose binding, soluble 3	Heterophilic cell adhesion	42.9	15.4	5.8	1	1	1
Mapk6	Mitogen-activated protein kinase 6	Protein amino acid phosphorylation	1.6	1.1	1.2	1	0	1
Metap2	Methionine aminopeptidase 2	Proteolysis and peptidolysis	1.7	1.4	1.7	1	0	1
Mmp9	Matrix metallopeptidase 9	Proteolysis and peptidolysis	3.4	1.7	23.1	0	0	1
Mpeg1	Macrophage expressed gene 1	NA	1.5	4.3	2.1	1	1	1
Mustn1	Musculoskeletal, embryonic nuclear protein	Wound healing	2.3	1.1	1.5	1	0	1
Nars_predicted	Asparaginyl-tRNA synthetase (predicted)	Protein biosynthesis	1.8	1.0	1.2	1	0	1
Nid67	Putative small membrane protein NID67	Neurogenesis	2.4	1.1	2.0	1	0	1
Npy	Neuropeptide Y	Synaptic transmission; regulation of blood pressure	5.1	1.3	2.9	1	0	1
Nr4a1	Nuclear receptor subfamily 4, group A, member 1	Inhibition of caspase activation	1.9	1.1	1.4	1	0	1
Oazin	Ornithine decarboxylase antizyme inhibitor	Polyamine metabolism	2.8	1.2	1.5	1	0	1
Omd	Osteomodulin	Cell adhesion	1.0	1.7	1.6	0	1	1
Pafah1b1	Platelet-activating factor acetylhydrolase, isoform Ib, alpha subunit 45kDa	Neurogenesis	1.7	1.1	1.5	1	0	1
Pcdh3	Protocadherin 3	Homophilic cell adhesion	1.2	1.2	1.4	0	0	1
Pcdh8	Protocadherin 8	Homophilic cell adhesion	2.4	1.0	1.5	1	0	1
Pde4b	Phosphodiesterase 4B	Signal transduction	1.5	1.0	1.3	1	0	1
Pdlim3	PDZ and LIM domain 3	Actin filament organization	1.5	1.1	1.3	1	0	1
Penk-rs	Preproenkephalin, related sequence	Immune response; neuropeptide signaling pathway	3.8	1.2	2.2	1	0	1
Pgcp	Plasma glutamate carboxypeptidase	Proteolysis and peptidolysis	0.8	1.1	1.5	0	0	1
Plat	Plasminogen activator, tissue	Protein modification; proteolysis and peptidolysis	2.4	1.5	1.4	1	1	0
Plau	Plasminogen activator, urokinase	Proteolysis and peptidolysis	4.1	3.1	1.2	1	0	0
Plekhc1_predicted	Pleckstrin homology domain containing, family C (with FERM domain) member 1	Cell adhesion	1.5	1.1	1.3	1	0	1
Prkar2a	Protein kinase, cAMP-dependent, regulatory, type 2, alpha	Protein amino acid phosphorylation	1.5	1.1	1.4	1	0	1
Psmb8	Proteosome (prosome, macropain) subunit, beta type 8	Proteolysis and peptidolysis	2.8	3.4	1.4	1	1	1
Ptgs2	Prostaglandin-endoperoxide synthase 2	Regulation of inflammatory response	6.4	1.4	3.5	1	0	1

Ptprf	Protein tyrosine phosphatase, receptor type, F	Homophilic cell adhesion	1.7	1.2	1.6	1	0	1
Raph1_predicted	Ras association (RalGDS/AF-6) and pleckstrin homology domains 1	Cell–matrix adhesion	1.1	1.3	1.6	0	0	1
Rnp24	Coated vesicle membrane protein	Protein transport	1.5	1.2	1.3	1	0	1
Sdc4	Syndecan 4	Cell adhesion	2.1	1.3	1.9	1	0	1
Serpinb2	Serine (or cysteine) proteinase inhibitor, clade B, member 2	Wound healing	23.7	2.0	1.6	1	0	1
Serpine2	Serine (or cysteine) proteinase inhibitor, clade E, member 2	Neurogenesis; regulation of proteolysis and peptidolysis	0.7	1.1	1.5	−1	0	0
Sgk	Serum/glucocorticoid regulated kinase	Protein amino acid phosphorylation; sodium ion homeostasis	2.0	1.0	1.5	1	0	1
Slc12a2	Solute carrier family 12, member 2	Chloride transport; amino acid transport	1.1	1.4	1.5	0	1	1
Slc2a1	Solute carrier family 2 (facilitated glucose transporter), member 1	Response to osmotic stress; glucose transport	2.2	1.1	1.5	1	0	1
Slc38a2	Solute carrier family 38, member 2	amino acid transport	1.9	1.2	1.4	1	0	1
Slc3a2	Solute carrier family 3 (activators of dibasic and neutral amino acid transport)	Amino acid transport; cell growth	1.9	1.1	1.2	1	0	1
Sox11	SRY-box containing gene 11	Regulation of transcription; neurogenesis; gliogenesis	6.1	1.8	4.4	1	1	0
Spp1	Secreted phosphoprotein 1	Ossification; inflammatory response; cell adhesion	68.2	9.8	1.8	1	1	0
Sst	Somatostatin	Regulation of cell migration	1.8	0.7	1.4	1	0	1
Tacstd1	Tumor-associated calcium signal transducer 1	Defense response	3.6	1.2	1.9	1	0	1
Tagln	Transgelin	Cytoskeleton organization and biogenesis	10.5	2.2	2.4	1	0	1
Tcp1	t-Complex protein 1	Protein folding	1.4	1.0	1.3	1	0	1
Tf	Transferrin	Iron ion homeostasis	2.1	1.2	1.3	1	0	1
Tgfbr2	Transforming growth factor, beta receptor II	Transforming growth factor beta receptor signaling pathway	1.7	3.0	1.5	1	1	1
Tieg	TGFB inducible early growth response	Induction of apoptosis; regulation of cell proliferation	1.7	0.9	1.5	1	0	0
Timp1	Tissue inhibitor of metalloproteinase 1	C21-steroid hormone biosynthesis	59.8	5.7	2.4	1	0	0
Timp3	Tissue inhibitor of metalloproteinase 3	Induction of apoptosis	1.4	0.9	1.0	1	0	0
Tmod1	Tropomodulin 1	Actin filament organization	1.8	1.2	1.8	1	0	1
Tnfaip6	Tumor necrosis factor alpha induced protein 6	NA	3.1	1.3	2.6	1	0	1
Trh	Thyrotropin releasing hormone	Cell–cell signaling	29.6	1.4	3.6	1	0	1
Vapa	Vesicle-associated membrane protein, associated protein a	Regulation of I-kB kinase/NF-kB cascade	1.3	1.1	1.2	1	0	1
Vcam1	Vascular cell adhesion molecule 1	Cell adhesion	0.8	1.9	1.5	0	1	1
Vgf	VGF nerve growth factor inducible	Neuron differentiation	6.1	1.1	2.0	1	0	1

'1' and '−1' indicate significant up- and downregulated respectively, at $p < 0.05$.

considered to have antiepileptogenic properties (e.g., *Npy*, *Trh*, *Sst*, *Gal*), several others are proepileptogenic (e.g., *Bdnf*, *Penk*, *Crh*).

Interestingly, cell adhesion (e.g., increased *Vcam1*, various Cd antigens, *Itgb*, *Lgals3*, *Ptprf*, *Raph1*, *Sdc4*) is recognized as an upregulated process during this chronic phase, particularly in CA3. Cell adhesion proteins can promote adhesion and entrance of leukocytes into the brain. In this respect, it is interesting to note that *Cd244*, which is known to be regulated by lymphocytes and monocytes, is significantly upregulated in the chronic phase (**Figure 2(b)**), suggesting that the adaptive immune response could also play a role during this later phase. That the adaptive immune system can play a role in temporal lobe epilepsy is supported by the fact that Cd8-positive lymphocytes are still present in tissue from patients with hippocampal sclerosis. Several proteolytic and complement system-related genes are also still activated in this phase. For example, *Mmp9*, a metalloprotease that is associated with BBB disruption, can indirectly play a proepileptogenic role via entrance of blood proteins through a leaky BBB. *C3*, a complement protein, eventually can lead to formation of the membrane attack complex. Several inhibitors of proteases (e.g., *A2m*) remained strongly upregulated at the same level as during the latent period in both CA3 and EC regions, indicating a continued challenge to counteract the proteolytic activities of Mmps and other proteases.

Genes Associated with Growth, Neuronal Plasticity, Glutamate, and GABA

Epilepsy is often associated with overactivation of genes/proteins that are involved in learning and memory. However, in our study, 'synaptic plasticity' emerged as a significant downregulated process during the acute phase. Further, in the brain regions where cell death and inflammation are prominent – such as in CA3 and EC – the *calcium- and calmodulin-dependent protein kinase activity* (*CamKII*) emerged as a downregulated function during all three phases studied. Absence, or a downregulation, of CaMKII has been related to hyperexcitability and epilepsy. Contrary to what has been reported after long-term potentiation (LTP), there is a decrease in expression of genes related to glutamate and GABA signaling (mainly receptor subunits); 'synaptic transmission' (as reflected mainly in K, Na, Ca channel subunits) also appears to be a downregulated process in the acute and latent phase. These changes probably contribute to an imbalance between GABA and glutamate transmission, and subsequently to instability of the involved networks. Gene expression studies in these global (CA3 and EC) networks, have revealed that, in particular, genes for GABA-receptor subunits involved in tonic inhibition ($\alpha 5$ and δ) are downregulated. In the chronic phase, GABA signaling related gene expression is largely normalized, and there is even a tendency toward overcompensation; compared with control expression, some genes show significantly increased expression – for example, the vesicular inhibitory transporter Viaat (in EC) and the potassium chloride cotransporter *Slc12a2* (Nkcc1; in both CA3 and EC). The latter transporter is also markedly upregulated in subicular neurons of TLE patients. It is thought that the effects of such upregulation are proepileptogenic, since upregulation of Nkcc1 will lead to increased intracellular Cl^- accumulation and consequently to more depolarizing actions of GABA (instead of its more 'normal' hyperpolarizing actions).

The upregulation of the gene encoding Bdnf may be particularly relevant for epileptogenesis, since the mechanism leading to the shift of the membrane equilibrium potential for Cl^- (in hippocampal cells), associated with seizures, may be mediated by an increase in expression of Bdnf. Moreover, there is evidence that Bdnf may alter inhibitory processing in the brain by regulating the balance between phasic and tonic inhibition. We must be aware, however, that the GABA-receptor subunits can be differentially regulated and expressed, depending on the epileptic region under investigation. For instance, there is ample evidence that opposite regulation of receptor subunit expression can exist in CA1 versus dentate gyrus. Therefore, therapeutic strategies aimed at receptors (or any other target) can be region- and even cell-specific, as recently shown in a gene therapy study after pilocarpine-induced SE in rat.

Despite these apparent dissimilarities in gene expression between seizures and LTP, there are also interesting similarities if one looks at individual genes. For instance, as in studies related to learning and memory, many IEGs (*Fos*, *Jun*, *Homer*, *Arc*) are acutely induced, and also in some cases also overexpressed (e.g., Egr1), during the chronic phase of epileptogenesis. Moreover, genes related to growth – such as *Bdnf*, *Vgf*, *Gfra1*, *Tgfbi*, *Tieg*, *Nid67*, *Agpt2*, *Sox11*, and *DcamKII* – are also commonly induced. Some of these genes are associated with morphogenesis and sprouting (*Bdnf*, *Vgf*), others to gliogenesis (*Sox11*), neurogenesis (*DcamKII*, *Nid67*, *Gfra1*, *Pafah1*), and angiogenesis (*Ptgs2*, *Agpt2*). Expression of many of these genes is increased again in the chronic phase (**Figure 3**).

Future Directions-Hypothesis and Potential Targets

From the gene-expression changes that occur in the course of epileptogenesis (as described), we can extract the following picture: The overwhelming acute inflammatory response is reflected in a general and time-dependent activation of multiple genes that code proteases, cytokines, and other inflammatory substances (e.g., cox-2). Expression of these genes can alter the dynamics of

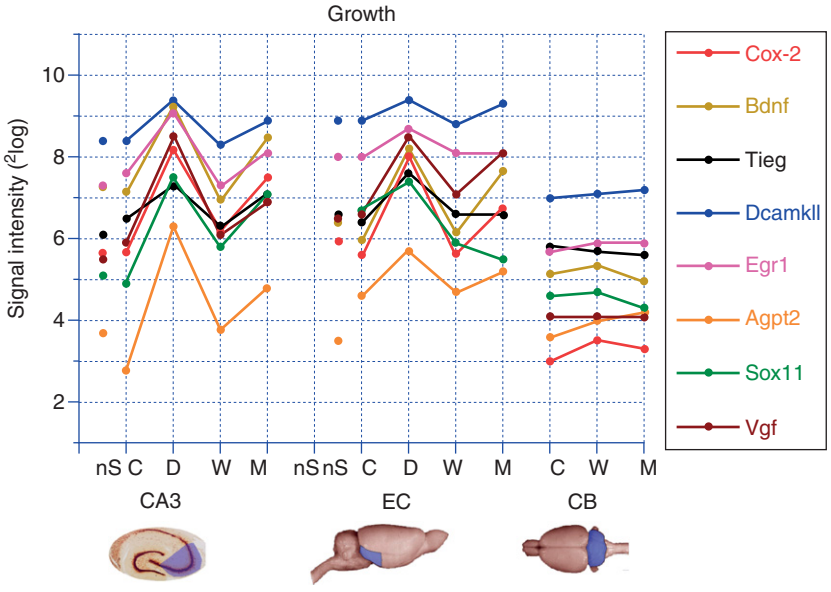


Figure 3 Examples of genes that are significantly activated in CA3 during the chronic phase and that are associated with 'growth.' Note that many of the same genes are also activated in EC but not in cerebellum (CB) and that gene expression in stimulated rats that did not exhibit SE after stimulation (nS, 3–5 months after stimulation) were almost similar to controls.

the neuronal networks, for example, by potentiating glutamate release from neuronal and glial sources. Some overactivated genes (e.g., cox-2, complement and Mmp9) may not only cause parenchymal inflammation but also inflammation of endothelial cells, leading to leakage of the BBB – a feature that can still be present during the chronic phase. Via leakage of blood proteins (e.g., albumin), and via entrance of leukocytes, inflammation will be sustained and will further destabilize neuronal networks and contribute to progression of epilepsy. From this scenario it can be speculated that antiinflammatory treatment, at least in the initial phases when treatment could be directed toward stopping inflammation at the BBB, could help to prevent further progression of the disease.

The microarray studies aforementioned have provided a few suggestions regarding specific targets that may be explored at specific time points during epileptogenesis in order to counteract the epileptogenic process. The relevance of several of these targets (e.g., caspases, metalloproteases, cox-2, complement proteins, cell adhesion proteins) has been, or is presently being tested in animal models for ischemic insults or neurodegenerative diseases (such as multiple sclerosis). Their relevance for epilepsy requires further investigation. Other strategies are also suggested by these gene-expression studies – for example, inhibiting proteins that are induced by seizures and that have proepileptogenic actions by amplifying the actions of glutamate (such as Bdnf and Cox-2) or affecting K^+ channels (Crh). Nkcc1, which regulates intracellular chloride levels and thereby GABA transmitter action, is a potentially interesting target, especially in the chronic phase of epileptogenesis. Genes for GABA-receptor subunits related to tonic inhibition are among the most persistently downregulated, and could be a target for therapy in any phase of epileptogenesis.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; Gene Expression in Immature and Mature Hippocampus After Status Epilepticus; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Seizures**: Seizure-Induced Gene Expression; **Temporal Lobe Epilepsy**: Functional Genomic Analysis in Temporal Lobe Epilepsy.

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Role of Transporter Proteins and Lipid Peroxidation in Epileptogenesis

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Introduction

An essential component of neuron transmission at glutamatergic and GABAergic synapses is removal of the transmitter from the synaptic cleft. Several types of cDNAs encoding high-affinity glutamate transporters have been cloned, including GLAST, GLT, EAAC-1. These glutamate transporters are found in the forebrain. We have examined the regulation of these glutamate transporter proteins in the context of epileptogenesis.

Disruption of inhibition mediated by GABA is one cause for neuronal hyperexcitability leading to convulsive seizures. Extracellular GABA concentration is regulated by specific Na^+-dependent transporter proteins on presynaptic terminals and glial cells. Three subtypes of GABA transporters, designated GAT-1, GAT-2, and GAT-3, have been identified in the rat brain. These transporter proteins modify extracellular GABA concentrations. In earlier studies, we have observed an enhancement of glutamatergic excitatory synaptic transmission efficacy in a spontaneous seizure model, and hypothesized that that enhancement was due to changes in the levels of GABA transporter proteins. We subsequently performed a series of experiments, using quantitative Western blotting, with antibodies specific to individual GABA transporters (GAT-1 and GAT-3) to measure GABA transporter levels (and glutamate levels) during the epileptogenic process.

Background

Models of Epileptogenesis and Transporter Proteins

Repetitive microinjection of glutamate into the amygdala causes seizure generation similar to that found in both kindling and the $FeCl_3$-induced epilepsy model. This observation suggests that the enhancement of excitatory neurotransmitter levels (especially of glutamate) within the limbic system has a critical role in seizure generation. Recent reports suggest that efficacy of long-lasting excitatory synaptic transmission in the kindling model is closely related to extracellular glutamate concentration, glutamate-mediated responses mediated via NMDA receptors, and reduction of GABA-mediated inhibition. However, the molecular and cellular events responsible for these changes remain unknown.

We wondered if enhanced glutamatergic excitatory synaptic transmission in spontaneous seizure models is associated with changes in the levels of glutamate transporter proteins. We performed a series of experiments, using quantitative Western blotting, with antibodies specific to the individual glutamate transporters to test this hypothesis, and to determine if glutamate transporter levels changed over time – reflecting the process of epileptogenesis. We examined the levels of these proteins at various times after $FeCl_3$ injection into the amygdala and correlated those data with the development of chronic seizures.

Experiments using knockdown and knockout of glutamate transporter genes in mice suggest that these proteins are important in epilepsy. Amygdala kindling causes diminished production of GLAST within the piriform cortex and amygdala of kindled rats. This kindling-induced change in GLAST within piriform cortex could result in an increase in extracellular glutamate that would result in increased regional neuronal excitability. This pattern of diminished GLAST production in kindling is identical to the changes in transporter proteins we observed in iron-injected animals with chronic seizures. In contrast to these results from kindling and iron injection models, animals with kainic acid-induced seizures show increased GLAST mRNA in the acute seizure

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phase (mainly in area CA3), as well as a modest increase in the expression of GLT-1. Of note, we have also seen the same pattern of changes in GLAST and GLT-1 protein during the acute phase (for 5 days) following iron injections.

In tissue obtained during resections of temporal lobes of patients with epilepsy, investigators have found that GLAST protein is reduced compared with the levels seen in control tissue, but observed no significant difference in GLT-1 protein levels. This pattern of transporter change is similar to the pattern we found in rats at 30 days after $FeCl_3$ injection. During the 30 days of our experiment, the level of protein expression of the three types of glutamate transporters changed in a time-dependent manner, with acute elevations in expression followed by collapse in GLAST at +30 days (while all other proteins returned to baseline). In contrast to changes in the glial glutamate transporters that are thought to have a neuroprotective function, neuronal EAAC-1 provides glutamate needed for GABA synthesis. Immunocytochemical studies show colocalization of EAAC-1 on the soma of GABAergic neurons, particularly in the cerebellum. It is tempting to speculate that neuronal glutamate transport may play a significant role in GABA formation, since GABA synthesis depends on uptake on glutamate as a metabolic precursor, with subsequent decarboylation by glutamic acid decarboxylase to form GABA. Introduction of antisense oligonucletides to EAAC-1, with subsequent impaired production of that transporter protein, has led to concomitant decreases in GABA levels, thus linking EAAC-1 with GABA production. Conversely, upregulation of EAAC-1 might be seen as a compensatory response to seizures, with the goal of increasing GABA synthesis in response to excessive glutamate levels.

Methods

In our laboratory, we have combined continuous, online microdialysis and molecular methods to correlate changes in the extracellular concentration of transmitters with alterations in the molecular regulation of transporter proteins. Detailed methods can be found in our previous publications (see suggested readings by Ueda *et al.* and by Willmore *et al.*).

$FeCl_3$-Induced Seizures

Sprague-Dawley rats, weighing between 250 and 280 g, are used for our studies. Surgical procedures are performed, using pentobarbital sodium as an anesthetic (37.5 mg kg^{-1}, i.p.). All experiments are reviewed and approved by the Animal Welfare Committee of our Health Science Center.

Microdialysis and recording from rats are performed in animals prepared with stereotaxic placement of a 22-gauge guide cannula, with the lumen occluded with a stylet and the end positioned just above the left amygdala. We prepare the cannula with a bare-tipped insulated wire affixed to the lip of the guide cannula orifice. After positioning the guide cannula, the stylet is removed and replaced with a fused silica tube (0.075 mm ID, 0.15 mm OD), attached to a microinjection pump (CMA Type 100), which is then lowered into the left amygdala. Animals are injected with 1.5 µl of 100 nmol $FeCl_3 \cdot 6H_2O$, diluted with distilled water, at the rate of 1 µl min^{-1} by microinfusion pump. Our control animals are injected with an equal volume of 0.9% NaCl with pH adjusted with hydrochloric acid to 2.2, to equal the pH of the ferric chloride solution. During injection, and for the following 30 min, we keep animals under continuous EEG recording. Following this recording period, the cannula is removed and animals are allowed to recover.

Animals are killed at designated times – 60 min, 5 days, 15 days, and 30 days – after amygdala injection. After sacrifice, we remove both the left and the right hippocampi, place the tissue in ice-cold PBS buffer, and homogenize the tissue (Polytron PT 3000, Brinkmann) for membrane extraction and immunoblotting.

Immunoblotting and Quantitative Densitometry

To extract a crude synaptic membrane fraction from the hippocampus, we use the method of Danbolt *et al.* Briefly, each hippocampus is homogenized in 1 ml ice-cold 0.32 mol mannitol containing 1 mmol EDTA (pH 7.4), and centrifuged at 1000 *g*<?tfqs- for 10 min at 4 °C. The supernatant is then centrifuged at 27 000 *g*<?tfqs- for 20 min at 4 °C, and the pellet is suspended in 1 ml of hypotonic 1 mmol Tris–HCl containing 1 mmol EDTA. This suspension is centrifuged at 27 000 *g*<?tfqs- for 20 min at 4 °C. The pellet is resuspended in 1 ml of resuspension buffer (100 mmol PBS, pH 7.4, 5 mmol Tris–HCl, 1 mmol $MgSO_4$, 0.5 mmol EDTA, 1% glycerol) and centrifuged at 27 000 *g*<?tfqs- for 20 min at 4 °C. The final pellet is resuspended in 200 µl of resuspension buffer, and is stored at −80 °C. Protein concentrations are determined by the Bio-Rad DC protein assay, using bovine serum albumin as the standard.

Samples of total protein (30 µg each) are solubilized by adding an equal volume of 4% SDS, 20% glycerol, and 125 mmol Tris–HCl, pH 6.8, and boiling them for 5 min. To obtain resolution of both small and large proteins, the samples are analyzed by discontinuous one-dimensional Tris–SDS-PAGE (4% stacking gel and 10% separating gel), using the method of Schagger and Von Jagow. Standard proteins for SDS-PAGE are as follows: phosphorylase (97 kDa), bovine serum albumin (67 kDa), ovalbumin

(43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa), α-lactalbumin (11.4 kDa), and myoglobin residues 1–153(17.2 kDa), residues 1–131 (14.6 kDa).

Proteins from the gel are transferred by electroblotting to 0.2-mm nitrocellulose membrane (Amersham). The nitrocellulose membrane is blocked for 10 min in 10% nonfat milk in PBS buffer containing 1% Tween 20 (PBS-T). Following this washing, the blot is incubated for 2 h in anti-GLT-1, prepared as 0.33 mg ml^{-1} of rabbit affinity-purified antibody raised against the 16 amino acid synthetic peptide RMHDSHLSSEEPKHRN 17–32, corresponding to part of the amino terminus (Alpha Diagnostic Inc, USA). The anti-GLAST, a 0.33 mg ml^{-1} antibody raised against the 15 amino acid synthetic peptide NRDVEMGNSVIEENE, 504–518, corresponds to part of the carboxy terminus, reported by Storck *et al.* (Alpha Diagnostic Inc, USA). The anti-EAAC-1 contains 0.33 mg ml^{-1} of antibody raised against the 14 amino acid synthetic peptide DKSDTISFTQTSQF, 510–523, corresponding to part of the carboxy terminus, reported by Kanai *et al.* (Alpha Diagnostic Inc., USA). These antibodies are suspended in PBS-T containing 0.67% blocking agent. The blot is then washed with PBS-T, incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 20 min (1:3000, Chemicon), washed again, and processed for immunoreactivity using enhanced chemiluminescence (Amersham). The blot that is incubated with anti-EAAC-1 is incubated concomitantly with anti-β-tubulin (1:1000, Sigma), using horseradish peroxidase-conjugated goat anti-mouse IgG as secondary antibody for 20 min (1:3000, Chemicon). Densitometry of the immunoblots is used to quantify the changes in expression levels of each transporter protein on the blot. Each blot contains a range of standards of membrane fractions from normal rat brain (7.5–60 μg) that were used to generate a standard curve. The linearity of the relationship between optical density and protein concentration is verified on each film. The transferred blotted membrane fractions are stained with Ponceau-S to verify that each lane is loaded with equivalent amounts of protein. The blots are normalized to β-tubulin for densitometry.

Results

We have found increases in the level of each glutamate transporter at 5 days post-injection. At 15 and 30 days, only GLAST shows downregulation. Increase in EAAC-1 levels continues to 30 days and is seen bilaterally. In contrast, GLT-1 returns to control level at 30 days, bilaterally.

GLAST

We have measured transporter levels in the hippocampus at 60 min after injection of ferric chloride into the amygdala. Measurements were repeated at 5 days post-injection (when spontaneous seizure had their onset) and again at 15 and 30 days after injection. At these later time points, rats injected with ferric chloride have spontaneous, stage 4 seizure behavior. None of the control animals had seizures. Western blotting reveals no change in the levels of GLAST 60 min after injection. Upregulation of GLAST, bilaterally, is observed by 5 days after injection, but at 15 and 30 days after injection GLAST protein immunoreactivity is reduced to 50% of control in both ipsilateral and contralateral hippocampi.

EAAC-1

In contrast to the pattern of GLAST protein, EAAC-1 immunoreactivity is markedly increased at 5 days after injection, particularly contralateral to the injection site. By 15 and 30 days after injection, when experimental rats were experiencing spontaneous seizures, EAAC-1 protein level remained elevated bilaterally.

GLT-1

Immunoreactivity of GLT-1 followed a pattern similar to EAAC-1, with the most pronounced effects of seizures observed in hippocampal regions contralateral to the injection site. However, by 30 days after injection, no significant increases in GLT-1 protein (compared with controls) were detected.

Conclusions and Future Goals

Reuptake of extracellular glutamate has a neuroprotective effect. However, glutamate transporters are capable of moving glutamate in the reverse direction (into the extracellular space), in response to driving forces such as ion gradients or membrane potentials. Reverse transport is thought to result in elevation of extracellular glutamate concentrations following seizures. Thus, an increase in the number of transporter proteins – as may occur associated with seizures – could enhance glutamate efflux and contribute to neuronal hyperexcitability and seizures.

We have been concerned about the initiation of brain injury responses that result in epileptogenesis, and have focused on the role transporter dysfunction as it may explain the collapse of glutamate regulation across various chronic models of epilepsy (amygdala injection of iron salts, kainic acid injection, electrical and PTZ kindling). We continue to evaluate the impact of various antiepileptic drugs on the entire system of transporter regulation, with the belief that such medications may have mechanisms of action that have an impact on the molecular mechanisms of epileptogenesis rather than

affection membrane and synaptic processes that are symptomatic treatments.

See also: **Cell Damage/Excitotoxicity**: Molecular Mechanisms of Cell Death After Seizures; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Post-Traumatic Epilepsy**: Traumatic Hemorrhage and Iron-Induced Epileptogenesis; **Transporters**: Glutamate Transporters in Epilepsy.

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Specific Gene Expression Before and After Seizure in Epileptic EL Mice

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Introduction

In EL mice, ictogenesis can be first seen at about 10 weeks of age. Epileptogenesis, in contrast, is established through the experience of repetitive seizures during development, starting about 20 weeks of age. It has been suggested that an 'abnormal neural plasticity' may play a major role in the establishment of ictogenesis and epileptogenesis, and therefore, can be a potential target for the development of new pharmacotherapies for epilepsy. For example, it has been reported that constitutive neuronal nitric oxide synthetase (n-NOS) and endotherial NOS (e-NOS) are both necessary for the establishment of long-term potentiation (LTP) phenomena. Abnormal LTP-like neural plasticity, and therefore abnormal NOS expression, are likely to play the key roles in the pathogenesis of epileptic disorders. In addition, anti- and proapoptotic factors, as well as neurotrophic factors, have recently been suggested to play crucial roles not only in 'normal' cell development and survival, but also in epileptogenesis.

Continuous immediate early gene (IEG) expression and abnormal γ-aminobutyric acid-ergic (GABAergic) function may also be involved in epileptogenesis in EL mice. It is likely that transiently expressed IEGs are involved in ictogenesis. Further, DNA fragmentation may be associated with the formation of epileptogenesis; in the brain of EL mice, DNA fragmentation without cell loss was observed in the parietal cortex and hippocampus, structures that play the major role in the seizure initiation and generalization (respectively) in this model. The susceptibility of hippocampal neurons to DNA fragmentation increases after experiencing repetitive seizures during development, probably due to a change in the balance between protective (Bcl-2, Bcl-X_L) mechanism and proapoptotic (Bax) pathway inactivation. Drugs that can suppress free-radical generation and protect brain – such as allopurinol (ALP) – may be promising in new antiepileptic agents.

Neurotrophic factors, such as brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3), and fibroblast growth factor-2 (FGF-2), may also play a role in

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Neurotrophic factors, such as brain-derived nerve growth factor (BDNF), neurotrophin-3 (NT-3), and fibroblast growth factor-2 (FGF-2), may also play a role in

ictogenesis and epileptogenesis during brain development by promoting abnormal synaptic plasticity. There is abundant evidence of neurotrophic factor expression during the early developmental stages. Another developmental feature studied in EL mice has been cell-cycle phenomena, and particularly how altered cyclins and the corresponding cyclin-dependent kinases (CDKs) are related to the cell proliferation during epileptogenesis. Developmental changes in cyclin and CDK families were examined during cell cycle. As compared with the control DDY mice, we found that the EL mouse shows an upregulation of cell-cycle-specific cyclins or CDKs during early development, suggesting that reentry into cell cycle is promoted prior to the beginning of seizures – perhaps to the presence of high levels of neurotrophic factors.

Background

IEG Expression

Rapid genomic responses to neural activation may play a critical role in long-term synaptic plasticity. Recent work has strengthened this idea by demonstrating that neurotransmitter-induced long-term synaptic plasticity requires RNA and protein synthesis during a critical time window immediately following stimulation. These studies prompted the suggestion that genes, such as *c-fos*, that are rapidly induced by cell-surface-receptor-mediated stimulation by growth factors or neurotransmitters might be involved in the early genomic response underlying long-term synaptic plasticity. Activation of IEGs occur by increased transcription and is thought to represent the primary genomic response to stimulation because it does not require protein synthesis. Several IEGs code for transcription factors. These include members of the *fos* family, which dimerize to form the transcription regulatory complex AP1, as well as *zif*, which is a member of a family of proteins characterized by their DNA-binding domains.

During EL mouse development, IEG expression shifts from one site to another, and the three-dimensional expression area gradually expands, suggesting a change in the regional sites involved in epileptogenesis. These studies also suggest that during development, as well as during repetitive seizures, frequent expression of IEGs and synthesis of Fos and Zif proteins might facilitate synaptic conductivity involved in epileptogenesis. We found that IEG expression after seizures is not related to the seizure pattern per se, but rather to the seizure history, seizure threshold, and developmental state of the EL mouse. Even in the interictal period, EL mice with long seizure histories and very low seizure thresholds demonstrate IEG expression continuously. The IEG expression site is the hippocampal CA1 region, which is the final terminal of various inputs from other areas of the limbic system. We have proposed that the continuous expression of IEGs might play a different role from that of transiently expressed IEGs. During development, as well as through repetitive seizures, the *zif* expression site shifts gradually from the primary input system of the hippocampus (i.e., the pyriform cortex, the entorhinal cortex, and the dentate gyrus) to the hippocampal CA1. We found the *zif* expression site was initially localized to parietal cortex, and was associated with ictogenesis. As *zif* expression became universal, epileptogenesis was established (with weak expression all over the cortex).

NOS Abnormalities

Nitric oxide (NO) is associated with a variety of normal brain functions, including synaptic plasticity. In addition, NO, identified as an Endotherial Relaxing Factor (EDRF), is a potent vasodilator, regulating cerebral blood flow in basal conditions and during certain types of neuronal activation, including seizures. It is also involved in glutamate neurotoxicity. In the central nervous system, NO is synthesized from L-arginine by a Ca^{2+}-calmodulin-dependent NOS that is activated mainly by the stimulation of NMDA receptors. In association with these distinct functions of NO, three isoforms of NOS have been identified: n-NOS, e-NOS, and i-NOS form.

NO has been identified as a source of free-radical oxidants with special relevance to pathological conditions in the brain, since reactive nitrogen intermediates could damage DNA. However, it remains to be seen how seizure activities are regulated by NO in EL mice during development. n-NOS and i-NOS are major and minor components, respectively, of the NO system in EL mice. e-NOS, however, appears to play a minimal role despite the fact that e-NOS appears to be responsible for the NO that mediates an increase in local cerebral blood flow during focal seizures. One might wonder whether the relatively low concentration of e-NOS in EL mouse brain may contribute to the anoxia, and subsequent damage, in those active neurons associated with the epileptic focus complex. NO has been proposed as a retrograde messenger for sustaining LTP, a phenomenon thought to be involved in learning and memory function. Recently, Kandel's group carried out an elegant study in which mice that lacked either n-NOS or e-NOS were shown to have essentially normal LTP, whereas mice deficient in both n-NOS and e-NOS exhibited substantially decreased LTP. This result suggests that both n-NOS and e-NOS are essential for neural plasticity. The process of development of seizure propensity in EL mice has been referred to as 'abnormal plasticity,' based on electroencephalogram studies, biochemical analysis of neurotransmitters, mitochondrial function, and gene expressions. By analogy with the neural plasticity in normal brains, both n-NOS and e-NOS may participate in this abnormal plasticity and further epileptogenesis in EL mice. Given that i-NOS

is more conspicuous than e-NOS, it is also conceivable that excess i-NOS (and subsequent increase in harmful antimicrobial NO) and the relatively lower concentration e-NOS (and subsequent decrease in NO identified as EDRF) may work together to contribute to a complex epileptic focus and ictogenesis.

DNA Fragmentation

An attempt was made to identify the region of EL brain targeted by free radicals as measured by DNA fragmentation. In EL[s] mice, which have experienced seizure induction since the age of postnatal 5 weeks and shown to have a low seizure threshold, DNA fragmentation was detected in the hippocampal CA1 and the parietal cortex (using in situ terminal transferase-mediated dUTP Nick labeling with the aid of alkaliphosphatase and peroxidase). DNA fragmentation was also detected in EL[ns] – animals that never experienced seizure induction and shown to have high seizure threshold. However, the DNA fragmentation in EL[ns] mice was localized to the parietal cortex, and was less intense compared with that of EL[s] mice. EL mice also showed low Cu,Zn-super oxide dysmutase (Cu,Zn-SOD) activities than did control DDY mice. These findings suggest that abnormalities in the region-specific Cu,Zn-SOD isoenzyme activity, which is localized in mitochondria, might produce spatially specific free radicals, leading to DNA fragmentation – a process that might contribute to epileptogenesis.

Proapoptotic and Antiapoptotic Factors

We have hypothesized that excess free radicals might produce DNA fragmentation. The possible free-radical candidates are superoxide and superoxynitrite, a result of decreased Cu,Zn-SOD and increased i-NOS in the brain of EL mice. Ordinarily DNA fragmentation leads to apoptosis. Although IEG expression is usually nonspecific, the expression of localized and continuous IEG expression suggests that some protein cascades occur in situ.

The Bcl family of proteins, and neurotrophic factors, are closely related to cell loss and cell survival as well as to epileptogenesis. We have therefore analyzed, developmentally, their expression in the parietal cortex (seizure initiation site) and in the hippocampus (seizure generalization site) of EL mouse brain. Cell death regulatory genes of the Bcl-2 and caspase families may play a role in ongoing neuropathologic processes in human epilepsy, and offer novel targets as an adjunct to anticonvulsant therapy. The Bcl-2 family of proteins play a crucial role in the regulation of cell death in many eukaryotic systems. The Bcl-2 gene was initially isolated from the t chromosomal translocation found in human B-cell follicular lymphomas, and was subsequently shown to repress cell death triggered by a diverse array of stimuli. However, biochemical process by which Bcl-2 regulates cell death is poorly understood. Bcl-2 family gene products are critical to the integration of cell death stimuli that target the mitochondria. Bcl-X_L has been shown to promote cell survival through the regulation of mitochondrial homeostasis. Bax has been shown to redistribute from the cytosol to the mitochondria during apoptosis; overexpression of Bax can accelerate cell death.

Bcl-2, Bcl-XL, and Bax are apoptosis regulatory proteins, which are variably expressed in brain tissue and are known to be involved in the abnormal plasticity during epileptogenesis. Seizure-induced neuronal damage may involve both excitotoxic and apoptotic (programmed cell death) mechanisms. The expression of proapoptotic Bax and antiapoptotic Bcl-2 are associated with apoptotic phenomena. The ratio of Bax/Bcl-2 expression is increased in the hippocampus by seizures, suggesting that apoptosis of hippocampal inhibitory interneurons may lead to disinhibition in the hippocampus and increased seizure susceptibility.

Neurotrophic Factors

Epileptiform activation can regulate the expression of neurotrophins, and increases or decreases in neurotrophins can affect both epileptogenesis and seizure-related axonal growth. Epileptic seizures increase the expression of BDNF in the hippocampus. Since this neurotrophin exerts modulatory effects on neuronal excitability in this structure, it may play an important role in hippocampal epileptogenesis. Several studies suggest that BDNF can exacerbate seizure development during status epilepticus (SE) and subsequent epileptogenesis in the adult brain. However, there is also evidence for the protective effect of BDNF. To study this controversy, we have studied the developmental changes of expression of neurotrophic factors in EL mouse brain. We found that various neurotrophic factors were over-expressed during the development of EL mice. BDNF showed a biphasic over-expression during 8–12 weeks postnatal, and also around 20 weeks of age – which covers the late period of epileptogenesis. NT-3 (a member of the neurotrophin family of neurotrophic factors important for cell survival, axonal growth, and neuronal plasticity) showed a peak expression from 8 to 12 weeks old, also corresponding to the early period of epileptogenesis. Finally, FGF-2 showed a linear increase in expression with age, and reached a maximum level at 24 weeks (covering the late period of epileptogenesis). Although seizure activity itself also induces expression neurotrophic factor mRNAs and proteins, it is worth noting that the level of NT-3 and BDNF in the hippocampus of EL mice show a significant increase in earlier developmental periods, before the animals exhibit frequent seizures. The high levels of trophic

factors could thus facilitate the induction of cell division-related processes and promote epileptogenesis.

Cell-Cycle Reentry

In proliferating cells, the cell cycle consists of four phases. Gap1 (G_1) is the interval between mitosis and DNA replication that is characterized by cell growth. The transition that occurs at the restriction point (R) in G_1 commits the cell to the proliferative cell cycle. If the conditions that signal this transition are not present, the cells stop the cell cycle and enter G_0, a nonproliferative phase during which growth, differentiation, and apoptosis occur. Replication of DNA occurs during the synthesis (S) phase, which is followed by a second gap phase (G_2) during which growth and preparation for cell division occurs. Mitosis and the production of two daughter cells occur in M phase. A family of cyclins act as regulatory subunits for CDKs that regulate passage through the four phases of the cycle. Altered cyclins, and the corresponding CDK (cyclin D/CDK-4, cyclin E/CDK-2, cyclin A/CDK-2, cyclin A/CDK-1, cyclin B/CDK-1), are related to abnormalities in cell proliferation during early stages of brain development, and may be related to epileptogenesis.

Cytokines or Inflammatory Agents

A variety of cytokines (IL-1b, IL-6, and interleukin 1 receptor antagonist (IL-ra)) are upregulated in the cerebrospinal fluid (CSF) in human temporal lobe epilepsy. IL-1b shows proconvulsant effects (intrahippocampal injection) in the kainate model, although the role of IL-1b in epileptogenesis remains unclear. IL-1b and IL-ra may play a role in altering neuronal network excitability and the associated events. In addition, IL-1ra, IL-6, and tumor necrotizing factor-a (TNF-a) show neuroprotective and anticonvulsant actions at low concentrations, apparently through blocking IL-1r-mediated activity.

Methods

EL Mouse

The EL mouse is an inbred mutant strain that has been used as an animal model of secondarily generalized seizures. The mode of inheritance is autosomal dominant and the penetration rate is 100%. EL mice will show the first seizures at about 10 weeks of age and show frequent seizures after 20 weeks. Several lines of evidence indicate that seizure discharges are initiated in the parietal cortex, and then spread through the hippocampus. These findings have been substantiated by the histochemical and biochemical analyses of glucose utilization and study of inhibitory neurotransmission mediated by GABA. The development of the epileptogenic zone – which mainly consists of the parietal cortex (seizure initiation site) and the hippocampus (seizure propagation and generalization site) – has been hypothesized to be key to epileptogenesis in EL mice.

In Situ Hybrydization and TUNEL Method

IEG (*c-fos* and *zif*) expression in EL mice was developmentally analyzed by in situ hybridization by using ^{35}S. DNA fragmentation was detected by in situ terminal transferase-mediated dUTP Nick end labeling (TUNEL method).

Preparation of Tissues for Immunoblotting Analysis

Immunoblotting analysis was carried for three isoforms of NOS (n-NOS, e-NOS, and i-NOS), proapoptotic Bax, antiapoptotic Bcl-2 and Bcl-X_L, neurotrophic factors (BDNF, NT-3, and FGF-2), cell-cycle reentry proteins (cyclin A, B, D, E, and CDK-1,-2,-4), and cytokines (IL-1a, IL-1b, TNF-a, IL-6, interleukin 1 receptor (IL-1r), and IL-1ra) in EL mice at 4, 6, 8, 10, 12, 15, 19, and 23 weeks of age ($n = 6$ animals per age group). DDY/TIP mice (background strain of EL) at 10 and 20 weeks were used as controls. After decapitation, brains were removed and the hippocampus was dissected out. Homogenates were prepared with 20 mM Tris–HCl buffer (pH 8.0). Just before use, homogenates (with dye for 15% SDS-PAGE gels) were boiled for 3 min and centrifuged for 5 min at 3000 rpm.

Seizures were induced in EL mice once every week (once every 7 days), starting at 5 weeks of age. Cytokines (IL-1a, IL-1b, IL-6, IL-1r, IL-1ra, and TNF-a) were examined by Western blotting in the parietal cortices and the hippocampus). In 15-week-old EL mice, which show seizures once a week regularly, these cytokines were similarly determined 5 min, 2 h, 4 h, 11 h, 24 h, 3 days, and 6 days after the last seizure.

Immunoblotting Procedures and Data Analysis

For the immunoblot analysis, 10% mouse brain homogenates (total protein: 30.0–31.0 μg) were separated by 15% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA), blocked and then incubated with primary antibody. After washing, the membrane was incubated with rabbit anti-goat, goat anti-rabbit, or rabbit anti-rat conjugated with horseradish peroxidase (1:10 000) (Sigma), which was followed by a Western blot-chemiluminescence reagent (DuPont NEN, Boston, USA). The luminescence of the membrane was detected using Hyperfilm-ECL (Amersham, Little Chalfont, UK).

The data were semiquantitatively analyzed by using the NIH Image software.

Preparation and Procedure of Labeling Brdu

EL mice of 5, 10, and 20 weeks of age were used ($n = 6$ per age group); DDY/TIP mice at 5, 10, and 20 weeks of age were used as controls. BrdU ($50\,mg\,kg^{-1}$) or vehicle was injected intraperitoneally. One hour after injection, mice were sacrificed and decapitated, brains were removed and frozen in ethanol on dry ice, and then mounted on the cryostat. Thin sections (4–5 μm thickness) were mounted on poly-L-lysine coated glass slides. These sections were further fixed in the ethanol fixative containing 15 mM glycine, at −20 °C, for an hour. Then sections were stained using the 5-bromo-2′-deoxy-uridine Labeling and Detection Kit II purchased from Roche (Penzberg, Germany). Seven sections from each mouse (covering the hippocampus at rostral–caudal distances from bregma −1.7, 1.8, 1.9, 2.0, 2.1, 2.2, and 2.3 mm distance) were obtained. Total positive cells were counted according to layers and statistically compared by t-test. All differences were considered significant at $P < 0.05$.

Recent Results

It has been reported that limbic seizures rapidly induce cytokines (IL-1a, IL-1b, IL-6, TNF-a, IL-1r, and IL-1ra) and related inflammatory mediators. It remains to be seen, however, whether cytokines contribute to the transition from interictal to ictal state. Our recent study was designed to address this issue using EL mice. In the parietal cortex and the hippocampus of EL mice, a significant increase in the level of cytokines was observed when compared with control animals at comparable stages during development. Cytokines were increased predominantly before animals experienced frequent seizures. In the hippocampus of EL mice, at the age of 15 weeks when they show seizures once a week, cytokine levels were highest 6 days after the last seizure. In contrast, peak levels of cytokines were observed 2 h after seizure in the parietal cortex. Cytokines were upregulated just before seizures in the hippocampus, or just after seizures in the parietal cortex.

In the seizure initiation site – the parietal cortex (Pcx), the expression of IL-1a gradually increased and reached a peak level at the age of 10 weeks, the time when the first seizure is seen. In the hippocampus (Hipp), the expression of IL-1a showed a sharp peak at 8 weeks, or just before the first seizure. The expression of IL-1b reached peak level at the age of 10 weeks in Pcx or at the age of 10–12 weeks in Hipp. The IL-6 monomer was expressed most highly at the age of 10 weeks, whereas the dimer was highly expressed at the age of 10 weeks in Pcx and 12 weeks in Hipp. The expression of TNF-a showed a relatively high level from 8 to 15 weeks of age in both Pcx and Hipp. In the case of IL-1r, the expression in Pcx showed a sharp peak at the age of 6–8 weeks, whereas the expression in Hipp showed a maximum peak at the age of 8 weeks and was maintained at a relatively high level during frequent

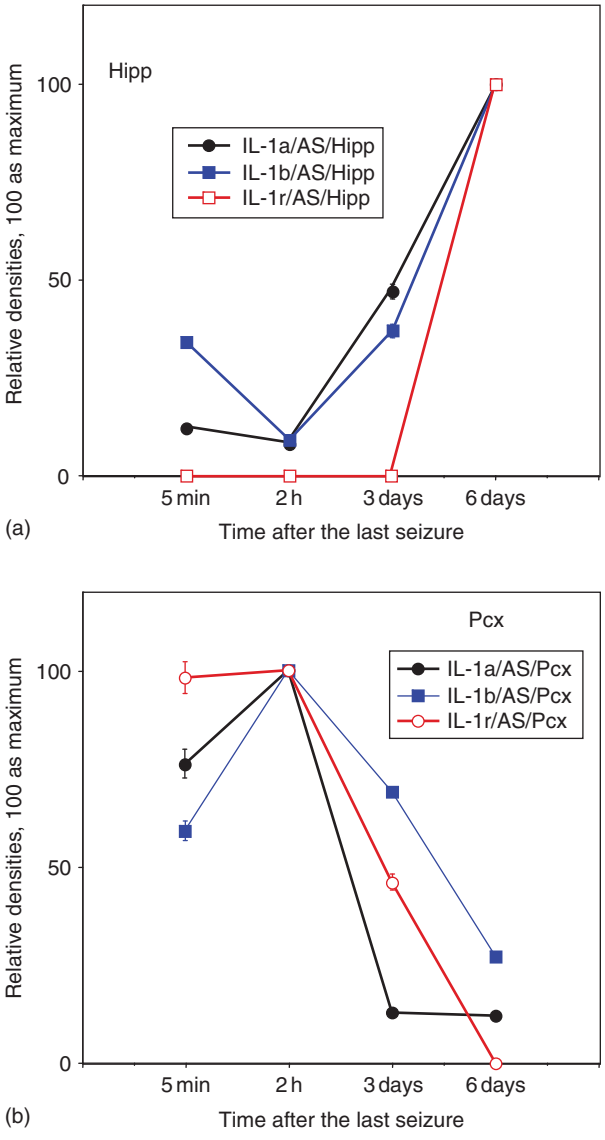


Figure 1 (a) Expression of cytokines after the last seizure in the hippocampus (Hipp), in a 15-week-old EL mouse that exhibited a seizure once a week associated with cage changing. Relative expression of IL-1a,-1b, and-1r. Mean + SEM. The physiological correlates of these changes are as follows: 5 min post-seizure – the EEG has recovered to a pattern similar to that seen before the seizure; 2 h – the absolutely seizure refractory period is over; 3 days – developmental interictal samples were normal; 6 days – just before the next seizure. (b) Expression of cytokines after the last seizure in the parietal cortex (Pcx), using a 15-week-old EL mouse that exhibited seizure once a week associated with cage changing.

seizures. IL-1ra showed a relatively high expression in both Pcx and Hipp from the early ages, from a time before the mice exhibited the first seizure to the later stage of frequent seizures; its peak level was seen at the age of 10 weeks.

In the seizure propagation site (hippocampus), cytokines were expressed at relatively low levels immediately after seizures (**Figure 1(a)**), but were expressed most strongly just before seizures. These results suggest that the upregulation of cytokines in EL mice is associated with triggering of the ictogenesis, rather than as a consequence of frequent seizures. In contrast, the seizure initiation site – Pcx – showed a maximum expression of cytokines immediately after seizures, with expression then gradually decreasing (**Figure 1(b)**).

Cytokines are also induced in forebrain regions where seizures originate and spread in kainate and bicuculline models. Their syntheses in neurons are high when seizure activity is advanced in these models.

Future Goals

The role of cytokines in the seizure initiation site and seizure propagation and generalization site may be different. Several reports have described the upregulation of cytokines after the seizures. However, in the commonly used chemical models of epilepsy or epileptogenesis, it is impossible to determine cytokine expression levels just before a seizure. The mutant EL mouse appears to be a useful model of epilepsy to investigate the transition from the preictal or interictal to the ictal state during development. Our studies suggest that proinflammatory cytokines are increased progressively and periodically in association with both brain development and with seizure activity. A periodic increase of cytokines just prior to a seizure episode may play a role in triggering the ictal activity. That is, alteration in region-specific cytokines may induce ictal activities. Future studies should examine whether inflammatory cytokines work together with other neuronal factors during epileptogenesis, and in the transition from the interictal to the ictal state.

See also: **Cell Damage/Excitotoxicity**: Molecular Mechanisms of Cell Death After Seizures; Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Epileptogenesis**: Gene Expression in Immature and Mature Hippocampus After Status Epilepticus; Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Inflammation**: Inflammatory Responses and Cytokines; **Ketogenic Diet**: Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies; **Models**: Model Characterization in Relationship to Human Disorders; **Neurotrophic Factors**: Fibroblast Growth Factor-2; Role of BDNF in Animal Models of Epilepsy; The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy; **Temporal Lobe Epilepsy**: Functional Genomic Analysis in Temporal Lobe Epilepsy.

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Surrogate Markers for Epileptogenesis

N M E A Hayward, K M A Kurkinen, O Gröhn, and A Pitkänen, University of Kuopio, Kuopio, Finland

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Introduction

The term 'epileptogenesis' refers to the development of an epileptic disorder, and implies abnormal neuronal reorganization occurring over a long period of time following a specific cerebral insult. One of the greatest challenges in epilepsy research is to prevent epileptogenesis in patients at risk or at least to modify the disease process in such a way that the epilepsy that develops is milder or easier to treat. This goal not only requires novel treatment strategies based on understanding the basic mechanisms of epileptogenesis, but also relies on new methodologies to study the epileptogenic process, to follow treatment effects, and to predict outcomes in individual subjects.

Neuronal reorganization during epileptogenesis is composed of a large number of neurobiological changes, including neurodegeneration, neurogenesis, axonal sprouting, axonal damage, gliogenesis, angiogenesis, dendritic plasticity, reorganization of the extracellular matrix, and changes in cellular membranes. All these changes require spatiotemporally orchestrated patterns of gene expression. In principle, these molecular and cellular alterations can serve as targets for the development of bio- and surrogate markers for epileptogenesis.

A biomarker can be defined as a characteristic that is objectively measured and evaluated as an indicator of a normal biological process, pathogenic process or pharmacological response to a therapeutic intervention. A surrogate marker is an indirect measure of disease progression. In a recent NINDS sponsored workshop 'Biomarkers for Epileptogenesis', the three major technological areas that were identified as the most promising in the search for bio- or surrogate markers of epileptogenesis were: imaging, electrophysiology, and 'omics' (e.g., genomics, proteomics, metabolomics).

Here we summarize the strategies and current data available using magnetic resonance imaging (MRI) and proteomics in the search for surrogate markers of epileptogenesis.

MRI-Related Surrogate Markers of Epileptogenesis

Background

MRI is one of the most versatile tools for in vivo visualization of the brain. MRI relies on a physical phenomenon of atomic nuclei called nuclear magnetic resonance (NMR). To create an image, we utilize the NMR signals of hydrogen nuclei in water molecules. The nature of these signals depends on multiple factors conferred by the tissue environment, for example, the local quantity and mobility of water. It is important to note that approximately 60% of an adult's body is water and much of this water lies in soft tissues. This feature of biological tissue composition results in a high signal density for MRI; when this signal is spatially localized (with the help of controllable magnetic field gradients within the MRI scanner), high resolution images with high signal-to-noise ratios are achievable. Typically, small animal MRI has a resolution in the order of 100 μm, which can be optimized to 50 μm using the most advanced hardware and measurement techniques. This resolution is quite remarkable, especially when one considers that the thickness of the granule cell layer in the rat hippocampus is about 50–100 μm. Therefore, neurobiological changes in even a small group of cells in the brain can lead to distinct, measurable changes in the black–white contrast captured within our MR images.

Tissue alterations during epileptogenesis (such as neuronal cell death, gliosis, or axonal alterations) can affect the volume of extracellular space and dynamics of water movement. Importantly, MRI contrast is dependent on the concentration of water in the sample, but is also determined by the nature of the dynamic behavior of water within the timescale of an MRI measurement (milliseconds to seconds). These water dynamics affect the relaxation times T2 (transversal) and T1 (longitudinal) that determine how hydrogen nuclei return toward equilibrium after excitation by a radio frequency pulse. By modifying the NMR excitation and acquisition method, image contrast can be optimized to observe a certain

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parameter, such as water diffusion or blood flow. Tricks to accentuate the contrast include employing contrast agents that modify water relaxation in tissue and/or blood. Contrast agents normally contain paramagnetic substances (such as Gd^{3+} and Mn^{2+}) that predominantly shorten T1 relaxation times to create hyperintensity in T1 weighted imaging. Alternatively, superparamagnetic iron oxide (Fe^{3+}) particles can be utilized to produce negative contrast in T2 weighted imaging.

Perhaps the greatest asset of using MRI in epileptogenesis studies is that it provides safe and noninvasive visualization of the brain. Consequently, MRI can be performed early during the disease process without causing too much burden for the patient, and the pattern and severity of damage can be reassessed and correlated with the long-term functional outcome on an individual basis. Appreciation of interindividual variability in findings makes it possible to identify an array of quantitative MRI changes in each subject that predict poor or favorable outcomes. Moreover, the physical principles of MRI make it suitable for both experimental and clinical studies of epilepsy. The strong magnetic fields involved pose no known threat to humans or animals, and the electromagnetic pulses are within the low energy, radio frequency range, just like those of the airwaves used in broadcasting and communications. Since these low energy pulses have excellent tissue penetration without any hazardous ionizing radiation, MRI can safely be repeated throughout prolonged studies.

Methods and Results

MRI studies predict outcome in animal models of epileptogenesis

In rat studies, epileptogenesis can be triggered by genetically disrupting cortical development, or by introducing a brain insult such as stroke or traumatic brain injury (TBI). However, most of the recent work relies on chemically or electrically induced status epilepticus (SE) (see Further Reading section). MRI studies in these models have aimed at identifying early structural alterations that would correlate with the later risk of seizure (i.e., lowered seizure threshold) or occurrence of spontaneous seizures. Some studies have also used MRI to monitor the effects of antiepileptic compounds on SE and the severity of its consequent structural lesion.

Recent studies have drawn correlations between T2 (see **Figure 1**) or diffusion MRI changes and the risk of epileptogenesis. For example, T2 prolongation in the piriform and entorhinal cortices, seen as early as twenty four hours post-SE in rats, was associated with a higher risk of epileptogenesis later in life. A newer technique, diffusion weighted imaging, can identify lesion development before detection is feasible by T2 weighted imaging. The diffusion MRI measurement is sensitive to small changes in water diffusion, which correlate with the early stages of lesion formation, when there is a decrease in normal diffusion values. Follow-up studies in chemically or electrically induced SE models have demonstrated that delayed secondary changes in both T2 and water diffusion may also develop weeks after SE, taking place either before or during the appearance of spontaneous recurrent seizures. Whether the development of secondary alterations would provide a specific and sensitive surrogate for the appearance of spontaneous seizures after SE requires further exploration.

Interestingly, in a model of posttraumatic epilepsy (PTE), a recent study demonstrated that diffusion in the hippocampus increases between the first and third month post trauma and remains elevated during the remaining 8 months of study. This increased diffusion correlates with a lowered seizure threshold in these animals. Notably, a diffusion decrease (as early as 3 h post insult) and a diffusion increase 23 days after injury were both associated with hyperexcitablity seen in EEG 11 months post injury.

A few studies have attempted to monitor the effects of antiepileptic drugs (AEDs) on SE and consequent structural damage. For example, administration of diazepam 15 min after induction of SE with pilocarpine normalized the diffusion change present during the course of SE, and consequently alleviated the neurodegeneration when assessed 2 weeks later. Similarly, topiramate administration during SE has been reported to alleviate T2 prolongation after SE, and this MRI result was associated with a mild neuroprotective effect.

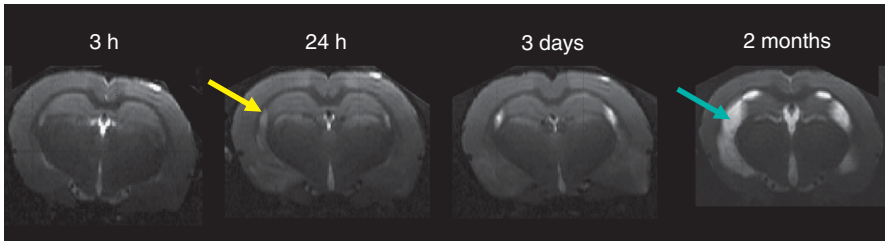


Figure 1 T2 weighted MR images of a rat brain following kainic acid (KA) induced status epilepticus (SE). Images were acquired using a 4.7 T MRI scanner. Images show the course of T2 weighted signal changes after SE. The yellow arrow highlights an edematic lesion forming 24 h after KA injection. The lesion develops into atrophy after 2 months (blue arrow). Images courtesy of Riikka Immonen.

What are the physiological translations of these MRI results? That is, can we already pinpoint the neurobiological substrates that predict epileptogenesis based on MRI information? The interpretation of physiological correlates of MRI relaxation and diffusion changes can be challenging. MRI relaxation and diffusion measurements probe the mobility and interactions of water molecules. In the complex environment inside epileptogenic tissue, it is feasible that many of the physiological changes mentioned earlier are ongoing concurrently. Each will impact on relaxation and diffusion of water to a variable extent. Thus, we cannot unequivocally link MRI changes to any one of the neurobiological processes in a progressive pathology. Nonetheless, it is still possible to use detailed findings from these MRI data to build a surrogate marker – for example, for severity and extent of cytotoxic edema in the brain. In this specific example, an initial diffusion decrease occurs as the extracellular water enters the intracellular space, where diffusion is more restricted. (Actually, net diffusion decrease is a result of multiple factors, including changes in diffusivity of the intracellular and extracellular spaces and extracellular tortuosity – see Further Reading section.) Conversely, a diffusion increase occurs when the cellular barriers that restrict water movements are lost. This happens in regions of substantial cell death, and thus is a common characteristic of most epileptogenic brain insults. Regarding T2 relaxation time changes, a T2 increase is well associated with vasogenic edema, in which there is, by definition, more free water than in healthy tissue. Since free water content positively correlates with T2 relaxation time, we have the basis for another surrogate marker. Importantly, such pathological explanations for these MRI findings from animal studies are normally supported by histological analyses.

A caveat regarding the use of various models in surrogate marker development revolves around the fact that some findings can be model-specific. For example, observation of an early epileptogenic T2 increase in the pilocarpine-induced SE paradigm has been challenged by reports that T2 actually decreases within the first 12 hours after SE was induced with an organophosphate, soman. Clearly, results from different animal models can sometimes be conflicting; thus, the interpretation of MRI findings must be made cautiously and without dependence on single studies or models.

Towards more specific imaging of cellular alterations in epileptogenesis

Since relaxation and diffusion based MRI can describe so many physiological processes which can often be difficult to discriminate, some more specific MRI techniques have recently begun to evolve. One such example, manganese enhanced MRI (MEMRI), has been applied to SE-induced epileptogenesis involving mossy fiber sprouting. Mossy fiber sprouting is present in the dentate gyrus of the hippocampus both in experimental models and human epilepsy. Its precise role in the onset and progression of epilepsy is debated. However, several studies have shown that it is present in the hippocampus, not only after the emergence of epilepsy but also before the appearance of spontaneous seizures. Therefore, mossy fiber sprouting has become an attractive candidate for imaging as a surrogate marker for epileptogenesis. Mossy fibers are extensions of the axons of granule cells in the dentate gyrus. These are extremely difficult to visualize by MRI in animals unless manganese ions (Mn^{2+}) are employed as a paramagnetic contrast agent. After both systemic injection and injection into the entorhinal cortex during epileptogenesis, Mn^{2+} translocates from the terminals of the perforant pathway to the axons of granule cells – that is, to the mossy fibers. This specific labeling brings them enhanced visibility in T1 weighted imaging, enabling the extent of mossy fiber sprouting to be visualized in a region-specific manner. Systemic administration of Mn^{2+} makes the method simple to use in a preclinical setting.

The relatively new technique of diffusion tensor imaging (DTI) is beginning to help complete the epileptogenesis puzzle. This method relies on the principle that water diffuses with the same directionality as that of axons within white matter tracts. The measurement is sensitive to unidirectional diffusion (see **Figure 2**); thus, nerve bundles, and possibly even mossy fibers, could be visualized with greater accuracy and resolution.

Much of this article is focused on structural MRI studies, yet functional magnetic resonance imaging (fMRI) has been used to characterize neuronal activation patterns in seizures. This technique relies on blood oxygenation level dependent (BOLD) contrast, which is derived from a hemodynamic response coupled with neural activity. As brain neurons are activated, their energetic demands create a transient regional increase in blood flow. This event increases the local oxyhemoglobin concentration, and therefore decreases the deoxyhemoglobin concentration at the activated site. As deoxyhemoglobin is paramagnetic, an NMR signal intensity increase occurs around active neurons. fMRI in animal models of epilepsy promises to be a valuable tool for describing the functional characteristics of the epileptogenic brain, and provides an attractive technique for following brain recovery processes and functional improvement in relation to epileptogenesis.

We must continue our efforts to determine whether these new techniques can also help assess the efficacy and safety of antiepileptogenic treatments. Our ability to interpret MRI data will undoubtedly improve as both the MRI technology and our animal models improve. Fortunately, most new or enhanced MRI techniques developed in the laboratory can be applied to clinical MRI scanners in hospitals, facilitating the translational goals of our research.

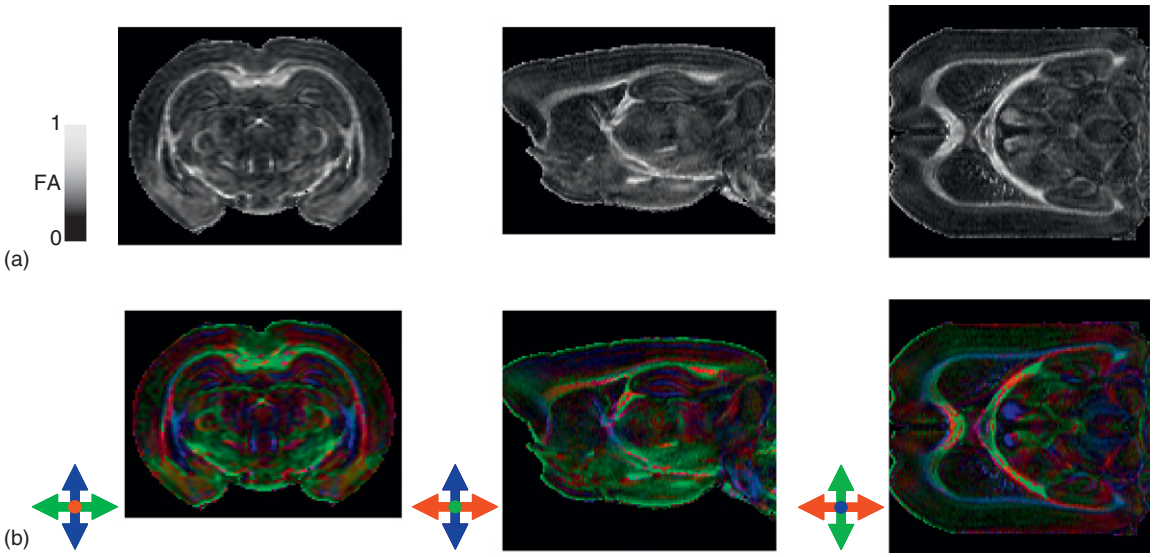


Figure 2 (a) Fractional anisotropy maps (FA) and (b) color-coded fractional anisotropy maps of a healthy rat brain acquired *ex vivo* using a 9.4 T MRI scanner by diffusion tensor imaging (DTI). Coronal, saggital, and horizontal views are presented (from left to right). Fractional anisotropy positively correlates with unidirectional water diffusion, thus white matter tracts have the greatest FA intensity (a). The color-coded maps (b) describe the principle diffusion direction in three dimensions. Maps courtesy of Teemu Laitinen.

Proteomic Approaches to Discovery of Surrogate Markers for Epileptogenesis

Background

The goal of proteomics research is to identify, characterize, and quantify proteins in a biological system. It has been proposed that about 20 000 genes in the genome can encode more than 500 000 proteins. From these proteins, a few thousand can typically be identified at one time using proteomic methods. Despite this 'limited view' of the entire proteome currently available, proteomic techniques can detect minute changes in a given proteome profile, and thus it is possible to identify pathological processes in preclinical investigations or at an early clinical stage of a disease.

Methods

The gel-based two-dimensional (2D) difference gel electrophoresis (2D DIGE) and the mass spectrometry-based isobaric tags for relative and absolute quantification (iTRAQ) are two proteomic methodologies that provide qualitative and quantitative information. These are used for the discovery of epileptogenesis-specific surrogate markers from CSF and/or serum/plasma.

2D difference gel electrophoresis

2D DIGE is the method of choice when complex protein mixtures are to be analyzed (Figure 3). It is a much more sensitive method than classical 2D gel electrophoresis.

2D DIGE is based on labeling of each protein sample of interest (control, treated, and pooled internal standard) with a different fluorophore (Cy3, Cy5, or Cy2) that binds covalently with the epsilon amino group of lysine residues. A clear benefit of the 2D DIGE system is that all three protein samples (Cy3-, Cy5-, and Cy2-labeled) can be separated on the same 2D gel. For example, CSF protein samples from control and epileptic populations are labeled with Cy3 or Cy5 dye, respectively. Next CyDye-labeled protein samples and a pooled internal standard (Cy2) (created by mixing aliquots of all samples to be analyzed) are mixed together and focused according to their isoelectric point on an IPG strip, then separated according to their molecular weight by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After the 2D run, the gel is scanned using an imaging analyzer, and data obtained from the scanner is processed with DeCyder software (GE Healthcare). DeCyder is a dedicated image analysis software package that utilizes a proprietary codetection algorithm (max triple detection) that allows automatic detection, background subtraction, quantification, normalization, and internal matching of fluorescence images. The use of an internal standard efficiently eliminates gel-to-gel variation and improves the accuracy of quantification between samples from different gels, allowing detection of small differences in protein level, since quantitative analysis is based on relative intensities of fluorescing labels at specific protein spots or labeled standard protein spots. A significant advantage of the DIGE system is that it covers a dynamic detection range of 3–5 orders of magnitude.

After DeCyder software data analysis, the protein spots of interest are picked up from the gel and processed for mass spectrometry (MS) analysis. Each protein spot is

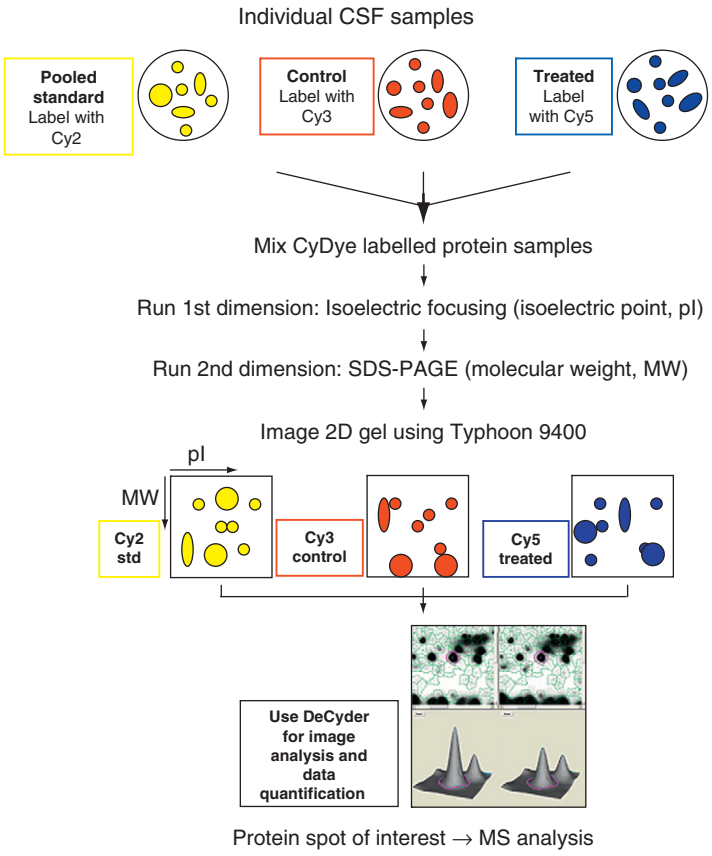


Figure 3 Two-dimensional difference gel electrophoresis (2D DIGE). Experimental design for labeling each individual cerebrospinal fluid (CSF) protein sample with fluorescent CyDye (Cy2, internal standard; Cy3, control; Cy5, treated) and separation of the labeled protein mixture in the first dimension run according to isoelectric point (pI), in which proteins' net charge is zero, and next in second dimension run on SDS-PAGE gel, in which proteins are separated according to their molecular weight (MW). Next, the protein 2D gel is imaged using a Typhoon 9400 laser scanner and data are analyzed with DeCyder software. Finally, a protein spot of interest is picked from the 2D gel and the protein is identified with mass spectrometry (MS).

separately in-gel digested with trypsin protease. Then, proteolytic trypsin digested peptide mixtures derived from a single protein spot are examined by mass spectrometry (Nano-LC QStar or MALDI-TOF/TOF). Finally, the Mascot algorithm is utilized for large-scale protein identification by searching a sequence database using mass spectrometry data.

Isobaric tags for relative and absolute quantification

Mass spectrometry based iTRAQ utilizes up to eight tag reagents, which bind covalently to the amino-terminus of the peptide (Figure 4).

First each control/treated CSF protein sample is separately digested with a proteolytic enzyme. After digestion, peptides are mixed with a specific iTRAQ tag reagent. Next, iTRAQ-labeled peptides are combined and fractionated using isoelectric focusing. Fractionated iTRAQ-labeled peptides are extracted from the IPG strip and processed for mass spectrometry analysis. Ionization of an iTRAQ-labeled peptide leads to fragmentation of the precursor ion using tandem mass spectrometry (MS/MS) and separation of the tag fragment from the peptide. On the spectrum, those fragmented iTRAQ-tag signature ions will show up from 113 to 119 m/z and 121 m/z, in a 'quiet' spectral region. Comparing the relative intensities of each of the tagged peaks with ProteinPilot 2.0.1 software (Applied Biosystems) provides quantitative analysis of the iTRAQ-labeled peptides.

It is important to note that high-abundance proteins should be removed from a complex protein mixture before iTRAQ-labeling, because those high-abundance proteins mask other low-abundance proteins and thus prevent their detection in mass spectrometry analysis. For example, CSF contains much albumin and IgG, and therefore it is beneficial to deplete those proteins from CSF using an albumin and IgG removal kit. This step will highly improve the detection of low-abundance proteins. Another option is to perform prefractionation of the protein sample, prior to iTRAQ labeling, by centrifugation. Also, antibody-based affinity depletion kits that remove

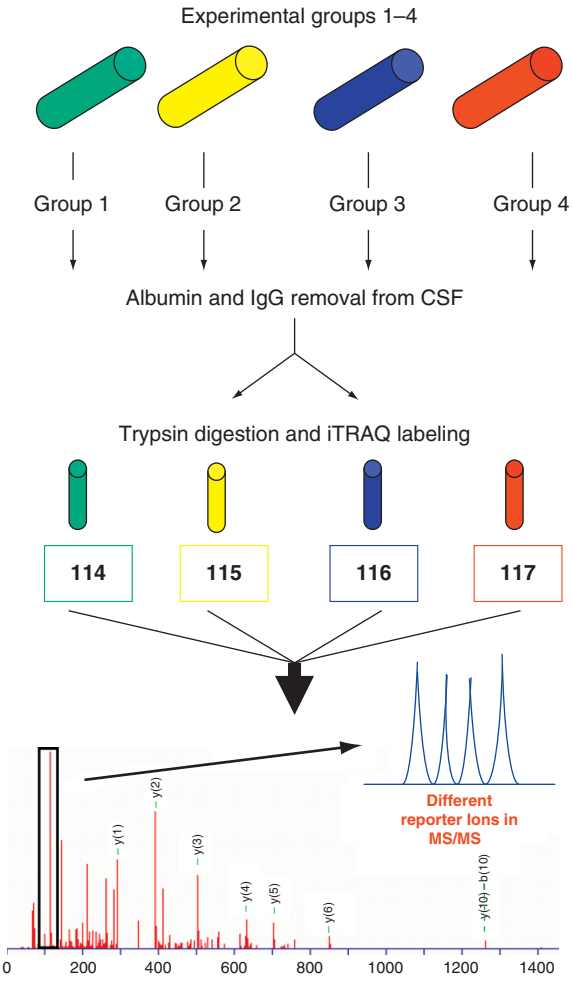


Figure 4 Isobaric tags for relative and absolute quantification (iTRAQ). Experimental scheme for the discovery of surrogate markers for epileptogenesis from rat cerebrospinal fluid (CSF) using mass spectrometry-based iTRAQ methodology. First, high-abundance proteins, such as albumin and IgG, are removed from CSF. Next, proteins are digested with trypsin and trypsin generated peptides are labeled with a specific iTRAQ tag (e.g., 114, 115, 116, and 117). Then iTRAQ-labeled peptides are fractionated with a cation exchange column or isoelectric focusing and analyzed with tandem mass spectrometry (MS/MS). Data can be processed, e.g., with ProteinPilotTM 2.0.1 software (Applied Biosystems).

the 6–12 most abundant plasma proteins and enrich low-abundant proteins are commercially available.

Results – Proteomics Studies During Epileptogenesis

To date, proteomic methods have not been utilized for the discovery of biomarkers for epileptogenesis from CSF or plasma/serum. Published epilepsy articles, which have exploited proteomic approaches in biomarker discovery, have mainly focused on finding tissue specific, epilepsy related protein alterations in the hippocampus and cerebral cortex at early time points. For example, putative biomarkers found in the rat hippocampus at acute (12 h) and later (72 h – latent period) phases in a pilocarpine induced TLE model can be linked to cytoskeletal modifications, altered synaptic function, mitochondrial dysfunction, changed ion channels, and chaperone functions. In turn, when absence seizures were induced by gamma-butyrolactone in mice, researchers concluded that 15 out of 16 thalamic proteins were down-regulated in the group of absence seizure-induced mice. Those down-regulated proteins belonged in the five functionally different groups of cytoskeleton rearrangement, neuroprotection, neurotransmitter secretion, calcium binding, and metabolism. These tissue-derived results provide a hint for developing therapeutic interventions for epilepsy. However, to carry out such studies currently requires surgical operations – so this approach does not provide an easy and noninvasive means of obtaining a useful biomarker.

What does the Future Hold?

It is likely that the use of one technology is not going to provide the sensitivity and specificity that are required for a surrogate marker to be reliably used in the clinic. A more realistic approach is exemplified in the Alzheimer's disease research field, where ongoing studies combine data from several technological platforms – such as MRI and proteomics – to optimize the prediction of outcome. It is likely that the development of surrogate markers for epileptogenesis will require multidisciplinary approaches, especially given the variability of spatiotemporal progression of epileptogenesis after brain injuries (e.g., traumatic brain injury, stroke, infection).

See also: **Imaging**: Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Functional MRI in Basic Epilepsy Research; Seizure-Related Acute MRI Changes; Structural Imaging in Epilepsy; Structural Magnetic Resonance Imaging of Epilepsy; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Post-Traumatic Epilepsy**: Posttraumatic Models; **Seizures**: Seizure-Induced Gene Expression.

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G

GABA

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Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy

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Introduction

GABA is the key inhibitory transmitter in the central nervous system (CNS). Synaptically released GABA activates specific receptors in the membrane; the resulting increase in permeability alters the neuron's membrane potential and excitability. Early electrophysiological data established two types of GABA-mediated synaptic events in central neurons, with different pharmacology and ionic mechanisms. The early inhibitory postsynaptic potential (IPSP) mediated by $GABA_A$ receptors ($IPSP_A$, time to peak about 20 ms) is sensitive to bicuculline, the prototype $GABA_A$ receptor antagonist, i.e is mediated by $GABA_A$ receptors. The late IPSP ($IPSP_B$, time to peak about 150 ms) is sensitive to established antagonists for $GABA_B$ receptors (saclofen, CGP 35348), i.e. is mediated by $GABA_B$ receptors. The receptors mediating these responses have been cloned revealing two molecularly distinct families of receptors.

The $GABA_A$ receptors are pentameric arrangements of subunits, each containing four transmembrane amino-acid helices, essentially forming a cluster of transmembranal protein columns enclosing a central pore. Binding of GABA alters the conformation of the protein, and the resulting change in the central pore allows small anions to flow down their electrochemical gradients. To date, 18 different $GABA_A$-receptor subunits have been cloned. These subunits can be grouped according to sequence homology into 6 α, 4 β, 4 γ, 1 δ, 1ε, and 2 ρ subunits. The stoichiometric composition of subunits governs the pharmacological and biophysical properties of a given $GABA_A$ receptor. Thus, a plethora of distinct $GABA_A$ receptors is present in the mammalian CNS. The composition of $GABA_A$ receptors exhibits regional specificity and developmental changes. It is also subject to activity-dependent alterations.

Functional $GABA_B$ receptors consist of a dimer of heptahelical subunits in the membrane, containing no pathway for the flow of ions; that is, $GABA_B$ is a metabotropic receptor. The extracellular domain of each subunit assumes a structure resembling half a sphere (or 'venus

flytrap'). The intracellular domains of the two subunits form coils adjacent to each other. Binding of GABA to the inside of one subunit's half sphere is conveyed to the inside coil; through a coil–coil interaction, the binding of G-protein to the other coil is enabled. The G-protein in turn interacts with two main effector systems, Ca^{2+} and K^+ channels. For example, a $GABA_B$ receptor-mediated increase in potassium conductance underlies the $IPSP_B$, observed in hippocampal and neocortical pyramidal neurons. A $GABA_B$ receptor-mediated decrease in Ca^{2+} currents, originally observed in dorsal root ganglion cells, also occurs in CNS presynaptic terminals, thereby modulating (for example) the release of GABA in a negative feedback manner. The dimerization of subunits appears to be unique to $GABA_B$ receptors, but other metabotropic receptors share – through different G-proteins – the same effectors. In fact, occlusion experiments using pairs of agonists for metabotropic receptors have demonstrated that binding of two agonists can occur simultaneously at distinct receptors, and can be blocked by different (receptor-specific) antagonists; yet, the combined application of agonists causes no additive effects. The divergence of a given metabotropic receptor to different effectors, together with the convergence of different receptors to the same effector, may enable a complex integration of signaling via various metabotropic receptors.

In the rodent neocortex, blockade of $GABA_A$ receptors (by, e.g., penicillin or bicuculline) inevitably induces paroxysmal depolarization shifts (PDSs) characterized by a large depolarization (about 25 mV), with a barrage of action potentials (APs) atop. These PDSs are regarded as the cellular correlate of the interictal spike of EEG recordings from the epileptogenic cortex. A brief summary of PDS properties produced in this way is instructive for a comparison with the synaptic responses in human epileptogenic cortex (as described later). These PDSs do not gradually increase in amplitude as stimulus intensity rises, but rather occur in an essentially 'all or none' fashion. Moreover, the peak amplitudes of the APs riding on the large depolarization are attenuated at the peak of the PDS and recover as the slow depolarization decays; the resulting troughs in the AP peaks probably is due to a slowed recovery from inactivation of sodium channels. Not surprisingly, drugs augmenting the efficacy of $GABA_A$ receptors (e.g., benzodiazepines) are established anticonvulsants. The data from experiments of $GABA_A$ receptor blockade and facilitation support the concept that a disturbance of $GABA_A$ receptor-mediated inhibition might contribute to hyperexcitability in human cortex. However, detailed patch-clamp analysis of $GABA_A$ receptor-mediated events has revealed no differences between epileptogenic human cortex and normal rat neocortex.

The role of $GABA_B$ receptors in focal epilepsies is less clear. Antagonists of $GABA_B$ receptors have minimal effects on normal excitatory synaptic responses but do prolong the duration of bicuculline-induced PDSs and increase their frequency. Interestingly, $GABA_B$-receptor agonists are also proconvulsant in the neocortex. Most likely, the tonic inhibition mediated by $GABA_B$-receptor agonists is overridden in the neocortex by the depressant effect of $GABA_B$ agonists on GABA release, which in turn attenuates $GABA_A$-mediated inhibition.

Background

Ionic Mechanism of $GABA_A$ Receptor-Mediated Inhibition

The proper functioning of GABAergic inhibition depends not only on the adequate release and the appropriate density of intact $GABA_A$ receptors, but also on the prevailing ionic gradient. This gradient is governed by the distribution of two ion species: the chloride gradient, which is maintained at a more negative level (in adult CNS neurons) than that of the resting membrane potential by the operation of a potassium-coupled chloride outward transport (a neuron-specific isoform of these transporters (KCC2) has been cloned), and the bicarbonate gradient ($GABA_A$-receptor channels are partially permeable to bicarbonate ions with a permeability ratio chloride:bicarbonate of 1:0.2). This small bicarbonate permeability has a marked effect on the reversal potential of $GABA_A$ inhibition, because the bicarbonate gradient across the neuronal membrane is (due to the rapid diffusion of CO_2) governed by the proton gradient, which has an equilibrium potential of about −10 mV. Previous estimates indicate that the partial permeability for bicarbonate, together with the bicarbonate equilibrium potential, causes a difference of about 15 mV between the chloride equilibrium potential and the $GABA_A$ equilibrium potential.

Previous studies on rat hippocampal neurons, as well as on neocortical neurons in human epileptogenic tissue, have demonstrated depolarizing $GABA_A$ responses. A key question concerns the mechanism of this depolarizing $GABA_A$ inhibition. The latter may be due to a reduced outward transport of chloride via KCC2, as occurs in early postnatal stages. The consequence, a shift of the chloride reversal potential (from about −85 mV) toward passive distribution (governed by the resting membrane potential, near −70 mV), may cause a depolarizing $GABA_A$ response because of the partial bicarbonate permeability. Alternative explanations include the following possibilities. First, aberrant $GABA_A$ receptors are expressed during the development of epilepsy and exhibit a different relative bicarbonate permeability, thereby causing the depolarizing $GABA_A$ reversal potential despite unaltered chloride outward transport and bicarbonate gradient. Second, there may be a change in the bicarbonate gradient per se. And third, there may be an upregulation of inwardly directed sodium–potassium-coupled chloride transporters (e.g., NKCC1).

Alterations in $GABA_B$ Receptor-Mediated Inhibition

The role of $GABA_B$ receptors in the initiation and spread of epileptiform activity is unclear. Part of the difficulty in resolving a possible role of $GABA_B$ receptor in epilepsy, apart from the lack of subtype-specific agonists and antagonists, stems from their presence at both postsynaptic and presynaptic sites. Changes in postsynaptic receptors would have different consequences on neuronal excitability than would changes at the presynaptic GABA or glutamatergic terminals.

In the healthy neocortex, synaptic responses evoked by single stimuli are only marginally affected by a blockade of $GABA_B$ receptors. Two possible explanations exist for this observation: First, the $IPSP_B$ is relatively weaker than the $IPSP_A$ and hence the reduction of $IPSP_B$ in the presence of intact $GABA_A$ inhibition has relatively little effect. And, second, the time course is so slow (a peak near 150 ms) that the $IPSP_B$ overlaps only slightly with a normal, brief excitatory postsynaptic potential (EPSP). The $IPSP_A$, by contrast, shortens the EPSP directly. Only when the $IPSP_A$ is attenuated and the resulting PDS lasts for more than 100 ms, is activity seen to be markedly curtailed by the $IPSP_B$. Of particular interest are presynaptic $GABA_B$ receptors that cause a frequency-dependent depression of both $IPSP_A$ and $IPSP_B$ by exerting a negative feedback of GABA on release. The attenuation of $GABA_A$ inhibition exerted through presynaptic $GABA_B$ receptors may therefore contribute to the initiation of epileptiform activity. Concurrently, however, presynaptic $GABA_B$ receptors at excitatory terminals would also reduce excitatory transmission; thus, the net effect of this presynaptic $GABA_B$ influence depends (at least in part) upon the diffusional spread of released GABA to these two sites.

A virtual absence of $GABA_B$ receptor-mediated effects has been demonstrated in the hippocampus in an animal model of temporal lobe epilepsy (self-sustained limbic status epilepticus). Neither $IPSP_B$ nor paired-pulse depression of $IPSP_A$, an index for presynaptic $GABA_B$ receptors, were detected, suggesting that $GABA_B$ receptors are impaired. This finding seems difficult to integrate with the notion that a use-dependent attenuation of $GABA_A$ inhibition contributes to epileptiform activity; according to that notion, robust, frequency-independent inhibition should limit hyperexcitability. However, $GABA_{B1}$-receptor knockout mice present with spontaneous seizures, thus providing some evidence that a lack of $GABA_B$ receptors may indeed contribute to epilepsy.

Methods

All methods have been described previously. In brief, slices from human cortical tissue are cut with a vibratome and stored in beakers containing artificial cerebrospinal fluid (ACSF) equilibrated with carbogen. Individual slices are transferred to a submersion-type chamber, where intracellular recordings with sharp microelectrodes are carried out. The chamber is continuously perfused with ACSF at constant temperature (32 °C). Electrodes are usually filled with a solution containing 1 M potassium acetate and 1 mM KCl (titrated to pH 7.2). In some experiments, electrodes are filled with 1 M KCl solution. Typically we recorded neurons in layer 2 or 3, using standardized protocols to facilitate comparison between the different tissues. Synaptic responses are evoked with stimulus intensities between 2 and 20 V (100 or 200 μs, 2 V increments) in control condition and during application of various drugs. We routinely measured current–voltage relationships with current pulses of incrementing amplitudes (−0.5 to about +1 nA; duration, 600 ms) to evaluate firing properties and neuronal input resistance. A second series of current injections is applied while synaptic responses are elicited with a constant stimulus (usually 12–16 V) to estimate reversal potentials and synaptic conductances. The temporal properties of synaptic responses are evaluated with a paired-pulse paradigm – i.e., two identical stimuli applied at incrementing intervals (50–1400 ms in 50 ms increments, every 10 s). The kinetics of chloride extrusion are evaluated by measuring the amplitudes of synaptic responses following cessation of current injections (0.5, 1, and 2 nA for 0.5, 1, and 2 min) and fitting the recovery with an exponential function.

Recent Results

Alterations in $GABA_A$ Receptor-Mediated Inhibition

Synaptic potentials elicited in human cortical neurons exhibit some similarities to bicuculline-induced PDSs of rodent cortex. In particular, they usually exhibit a steep increase in amplitude with increasing stimulus intensities, in some cases even exhibiting almost 'all or none' behavior. Yet, synaptic responses are, in most cases, devoid of the barrage of APs on top of the slow synaptic depolarization. Therefore, I refer to these synaptic responses in human epileptogenic tissue as DSs. DSs exhibit strong voltage dependence; i.e., they increase with membrane hyperpolarization and decrease with depolarization. In some neurons, the peak of the DS attains the same potential, regardless of the cell's membrane potential preceding the DS, i.e. during the peak of the DS membrane potential is essentially clamped to a certain value. Since the DS peak amplitude also exceeds the threshold for current-induced generation of APs, I assume that the DSs represent a state of such high conductance that it precludes the initiation of APs.

To delineate the receptor mechanisms, we applied D-APV (*N*-methyl-D-aspartate (NMDA)-type glutamate receptor antagonists, usually 20 μM) alone and together with CNQX (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid/kainate-type receptor antagonist, usually 10 μM). D-APV alone caused a slight depression (about 10%) in the amplitudes of the DSs; this result contrasts with earlier observations indicating a marked depression of synaptic responses by D-APV in human epileptogenic cortex. The reported lack of correlation between the magnitude of NMDA receptor-mediated responses and the severity of epilepsy is consistent with our data. The combined application of D-APV and CNQX reduced the DSs to a great extent, but a depolarizing synaptic component remained in some of the neurons. Application of bicuculline (50 μM) eliminated the remaining synaptic depolarization, suggesting that this component of the response is mediated by $GABA_A$ receptors. Quantification of the reversal potential of the remaining component revealed a less-negative value compared with rat cortical neurons (but with a fairly large standard deviation). Further analysis of the data revealed a clear dichotomy between reversal potentials; about half of the human cortical neurons exhibited reversal potentials indistinguishable from those observed in rat cortex, and another half exhibited a reversal potential close to −55 mV. This less-negative reversal potential would suggest a chloride gradient governed by passive distribution near the resting potential together with the partial bicarbonate permeability of the $GABA_A$ channels.

Most recently, I have begun to evaluate the ionic mechanisms for these depolarizing $GABA_A$ responses in human epileptogenic neurons, using intracellular chloride injections to ascertain the methods, parallel experiments were carried out in rat cortical neurons. Recordings from rat neurons with KCl electrodes shifts the $IPSP_A$ peak toward depolarization, with the response merging with the EPSP. In fact, this response looked strikingly similar to those recorded under normal conditions (K-acetate filled electrode) in some of the neurons from human epileptogenic tissues. Chloride loading by current injection resulted in a further increase of the synaptic component. On cessation of the injection current, the amplitudes of the synaptic responses recovered with a time constant of about 10 s, as previously shown in guinea-pig cortex. To reduce ambiguities concerning the kinetics of chloride extrusion, we also evaluated the recovery kinetics of synaptic responses in the presence of CNQX, D-APV, and CGP 55845A (a $GABA_B$-receptor antagonist acting at both pre- and postsynaptic sites). Under these conditions, the time course of recovery from chloride injections was on average similar to the standard conditions (near 10 s).

In human neurons, the time constant of recovery was much larger, on average about 20 s, for both control conditions and in the presence of CNQX, D-APV, and CGP 55845A. Interestingly, 2 of 10 human cortical neurons investigated to date have exhibited a time constant of recovery close to rat neurons (8 s).

Taken together, these data indicate that $GABA_A$ receptor-mediated inhibition is impaired in some neurons of human epileptogenic cortex. However, unlike the bicuculline-model in the rodent cortex, the receptors are unaltered. Instead, the basis for a $GABA_A$ receptor-mediated depolariziation is the altered contribution is the altered ionic gradient. The pathophysiologically altered ionic gradient of $GABA_A$ inhibition in about half of the neurons is probably due to impaired net outward chloride transport as initially reported. A depolarizing $GABA_A$ response generating a high conductance state at the peak would account for the large DS depolarization devoid of APs. This high conductance also suggests that GABA release and the $GABA_A$ receptors are essentially unaltered, as previously shown with patch-clamp recordings in human neurons. However, the crucial change, an altered ionic gradient, would have been undetectable with whole-cell patch-clamp recordings, because the filling solution of the electrodes rapidly equilibrates with the cell's interior and thus determines the intracellular concentration of ions.

$GABA_B$ Receptor-Mediated Inhibition in Human Epileptogenic Cortex

Previous data suggested that the $GABA_B$ receptor-mediated postsynaptic inhibition is rather small in the human epileptogenic cortex. In more recent experiments, we evaluated the properties of $GABA_B$ receptor-mediated inhibition in a larger number of tissue samples and quantified inhibition with established methods. On average, the postsynaptic $GABA_B$ receptor-mediated inhibition was significantly smaller in epileptogenic tissue (about 5 nS) than in human neurons from tumor resections without history of epilepsy (17 nS). To evaluate possible differences in the magnitude of $IPSP_B$ conductance between various clinical groups, we segregated the data according to the leading clinical symptom into tissue from: (1) TLE with Ammon's horn sclerosis, (2) TLE without Ammon's horn sclerosis, (3) tumors with epilepsy, (4) cortical dysplasia, (5) lesion with epilepsy, (6) frontal lobe with epilepsy, and (7) tumors without epilepsy. The $IPSP_B$ conductances in neurons from the groups 1–6 were significantly smaller than those in neurons from non-epileptogenic human tissues but not significantly different from each other. Although there were in each group a few neurons (around 5%) that exhibited fairly large $IPSP_B$ conductances, the data generally indicate a marked depression of $GABA_B$ receptor-mediated conductance in the majority of neurons.

Of course, this apparent conductance also depends on the amount of GABA released and hence the low conductance may be due to insufficient GABA release.

We therefore applied the established $GABA_B$ receptor agonist baclofen at 2 μM, close to the concentration yielding half-maximal effects in the rat neocortex (1 μM), and determined the induced conductance in both human and rat neurons. On average, the baclofen-induced conductance was about half of that induced by synaptic stimulation in both rat and human neurons. We considered the possibility that the average values may be misleading because some neurons may have 'extrasynaptic' $GABA_B$ receptors not accessible by synaptically released GABA, while others may have a reduced release. We therefore compared the conductance increase during the $IPSP_B$ with the conductance increase induced by exogenous baclofen. Plotting the data revealed a very good correlation: Human neurons exhibiting a small $IPSP_B$ conductance also exhibited minimal responses to baclofen. Conversely, neurons with a high $IPSP_B$ conductance also demonstrated large baclofen-induced conductance. On average, the slope was ~2, indicating that in each neuron the $IPSP_B$ conductance was about twice as large as the baclofen-induced conductance. This result indicates that the small $IPSP_B$ conductance is not due to insufficient GABA release, but rather to impairments in postsynaptic receptor function or density. That is, if the small and variable $IPSP_B$ conductances were due to a variation of release, a constant conductance should have been observable, due to the constant level of exogenous baclofen. These data provide functional evidence for a reduced function or density of $GABA_B$ receptors in human epileptogenic cortex, and are consistent with immunohistochemical data from human epileptogenic hippocampus, revealing marginal immunosignals for $GABA_B$ receptors, except in a few neurons. The latter cells would correspond to our finding of a small percentage of neurons in human epileptogenic tissues with large $IPSP_B$ conductance.

To evaluate presynaptic $GABA_B$ receptor-mediated effects, we employed a paired-pulse paradigm in human neurons, and in rat cortical neurons treated with bicuculline (to induce a defined hyperexcitability). In the human cortical neurons, paired stimulation of DSs yielded constant amplitudes at all interstimulus intervals. This constancy was at odds with the pronounced failure of bicuculline-induced PDSs of rat cortical neurons at interstimulus intervals up to about 1 s. At these intervals, a second PDS was virtually abolished and only a small EPSP remained, yielding an average depression of PDS amplitude by 75% (at 200 ms) in rat neurons. We tested our hypothesis that this well-established phenomenon was mediated by $GABA_B$ receptors by applying the $GABA_B$-receptor antagonist CGP 55845A, known to also be effective at presynaptic sites in the cortex. CGP 55845A eliminated the paired-pulse depression of the PDS; PDSs were virtually constant at all interstimulus intervals. Comparison of bicuculline-induced PDSs in rat cortical neurons with the DSs present in human epileptogenic neurons is hampered for several reasons (e.g., amounts of transmitter released) and we therefore also tested the effects of bicuculline in human neurons. Bicuculline converted the DS responses into PDSs, and paired-pulse stimulation revealed failures of PDSs in some neurons, as was the case in rat neurons. However, in other neurons the bicuculline-induced PDSs were only slightly attenuated with interstimulus intervals of, for example, 200 ms. The depression of a second PDS at 200 ms averaged 25% in human neurons, significantly less than that seen in rat neurons. The small attenuation of bicuculline-induced PDSs in human cortical neurons was also antagonized by CGP 55845A.

Our data indicate that both pre- and postsynaptic $GABA_B$ receptors are impaired in the human epileptogenic neocortex. The transient failure of bicuculline-induced PDSs in the rat cortex, eliminated by the $GABA_B$-receptor antagonist CGP 55845A, suggest that $GABA_B$ receptors normally limit the maximal frequency of PDSs. The frequency-independent DSs seen in human cortical neurons from epileptogenic tissues therefore imply that impaired $GABA_B$ receptors may be a cause of seizure activity. This conclusion complements existing data that decreased $GABA_B$ function may be a consequence of epilepsy. Together, the data indicate that deficits of $GABA_B$-receptor function can be both cause and consequence, and thus may form a vicious circle.

Future Goals

The depolarizing $IPSP_A$ in about half of the neurons of human epileptogenic cortex may account for some of the hyperexcitability seen in this tissue. These depolarizing responses probably contribute to the steep increase of synaptic responses with increasing stimulus intensities. This relationship has two conceivable consequences: first, there is a shift of the half-maximal stimulus intensity (i.e., any input will produce a larger synaptic potential); and second, there is a steeper slope of the input–output curve (i.e., any increase in input would cause a supranormal increase of synaptic responses). It remains to be tested whether the alteration of input–output relations correlate with clinical parameters of the patients (e.g., seizure frequency).

The slow recovery of $IPSP_A$ in human cortical neurons, following chloride injections indicates an impeded chloride outward transport. This observation leads to several important questions: which route of chloride flux is impaired, and can this mechanism account for a chloride equilibrium below resting membrane potential? Voltage-activated chloride channels, for instance, may contribute to chloride efflux when chloride is artificially elevated by the injection (if the channels are substantially activated near resting potential), yet such channels cannot easily account for a chloride equilibrium potential below the

resting membrane potential. Nevertheless, characterization of these currents in human and rat neurons is planned. Also downregulation of NKCC1 (usually believed to mediate inward chloride transport) may slow the recovery from injection if it proceeds in a backward fashion at elevated intracellular chloride levels. An upregulation of NKCC1 might shift the balance between inward and outward chloride transport rates and cause depolarizing $GABA_A$ responses – as in hippocampal granule cells – but whether such upregulation could impede chloride efflux is unclear. To further evaluate the mechanisms of disturbed chloride homeostasis in human cortical neurons, we will evaluate the effects of available blockers of chloride fluxes on steady-state levels under normal conditions and on the kinetic of recovery from chloride loading. Together with staining of the recorded neurons (to obtain morphological characterization) and immunohistochemistry, we might resolve the mechanism of depolarizing $GABA_A$ responses.

The reduced efficacy of pre- and postsynaptic $GABA_B$ receptors suggested by our data has two implications pertinent to hyperexcitability. First, the reduced efficacy of presynaptic $GABA_B$ receptors may contribute to larger synaptic responses per se. If tonically activated by ambient GABA, they normally might attenuate the release of GABA and glutamate. Second, the attenuated $GABA_B$-receptor effects have profound consequences for temporal properties of synaptic responses. Previous data suggested that depolarizing $GABA_A$ responses mediate interictal activity of human epileptogenic hippocampus. A similar spontaneous synaptic activity near 1 Hz was observed in some of the cortical neurons reported in this article as well. The $IPSP_A$ conductance decreases rapidly by about 80%, when stimulus frequency is increased from 0.1 to 1 Hz, due to activation of presynaptic $GABA_B$ receptors. Thus, for GABA-depolarizing events to occur spontaneously at frequencies near 1 Hz, not only a depolarizing $GABA_A$ response but also an impaired $GABA_B$-receptor function must be present.

I had previously assumed that the frequency-dependent depression of inhibition via presynaptic $GABA_B$ receptors might be a crucial mechanism for the initiation and spread of epileptiform activity. At first glance, this prediction seems difficult to equate with the present data, indicating impaired presynaptic $GABA_B$ receptors in human epileptogenic cortex. However, the concept of $GABA_B$ participation in seizure initiation and spread may still be valid, if different properties of focal and perifocal tissues are considered. Our data are probably from focal epileptogenic tissue. A disinhibition via presynaptic $GABA_B$ receptors is very unlikely in this case; instead, impaired $GABA_B$ receptors allow repetitive $GABA_A$-depolarizing events to contribute crucially to interictal activity. But in perifocal tissue, containing normally functioning $GABA_B$ receptors, the interictal activity may, at sufficiently high frequency, cause a use-dependent depression of inhibition and facilitate recruitment of larger arrays of neurons. Conceivably, that disinhibition may cause the transition from a small subclinical focus to clinically detectable focal activity.

Acknowledgements

Some of the $GABA_B$-related experiments were done by L. Spilker during her PhD work. I am most grateful to Dr. T.-N. Lehmann for providing the human tissues, to Prof. H.-J. Meencke and his team at the Epilepsiezentrum Berlin–Brandenburg for their care for the patients and their interest in my work, and to Dr. S. Gigout for helpful suggestions on the manuscript.

See also: **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations; **Pharmacoresistance**: Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue; **Pyramidal Cells**: Intrinsic Mechanisms Contributing to Hyperexcitability of Human Epileptogenic Cortex; **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

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Differential Alterations of $GABA_A$ Receptors in Epilepsy

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Introduction

$GABA_A$ receptors mediate most of the fast synaptic inhibitory transmission in mammalian brain. They belong to the family of ligand-gated ion channels and form multimeric GABA-gated channels selectively permeable to Cl^- and HCO_3^- ions. They are encoded by a large family of subunit genes (α1–6, β1–3, γ1–3, δ, ε, π, θ, ρ1–3), whose differential assembly into receptor complexes gives rise to multiple $GABA_A$ receptor subtypes differing in functional and pharmacological properties. $GABA_A$ receptors are a major target for drugs used clinically for the treatment of anxiety, sleep disorders, muscle spasms, and epilepsy. $GABA_A$ receptors are widely expressed in the CNS, and influence all major brain functions by controlling excitability of postsynaptic neurons and synchronization of neuronal networks.

The convulsant effects of $GABA_A$ receptor antagonists, and conversely the clinically relevant antiepileptic action of classical benzodiazepines such as diazepam, led to the concept that epileptic seizures reflect an imbalance between excitatory and inhibitory transmission in the brain. As a corollary, a deficit in $GABA_A$ receptor-mediated transmission is often suspected as an underlying cause of recurrent seizures.

However, unlike acute drug effects which occur in an intact system, drug effects during epileptogenesis and associated with recurrent seizures in chronic epilepsy likely reflect pathological disturbances that may have multiple origins. Furthermore, the simple view that GABAergic transmission acts like a break to prevent overexcitation of neuronal circuits has been challenged by the highly sophisticated anatomical and functional organization of GABAergic interneurons in cerebral cortex. Furthermore, in epileptic tissue, neuronal networks undergo extensive rewiring that considerably changes the function of interneurons and their control over pyramidal cells. Therefore, the concept of imbalance between inhibitory and excitatory GABAergic/glutamatergic transmission has to be revised, to integrate the fact that the role of GABAergic transmission in epilepsy is much more complex than suggested by simple pharmacological experiments.

This article will address two major issues about the involvement of $GABA_A$ receptors in epilepsy. First, we will discuss the relevance of tonic inhibition mediated by extrasynaptic receptors for the control of neuronal excitability. Second, we will review the recent results indicating that alterations in $GABA_A$ receptor assembly, subunit composition, trafficking, and posttranslational modification can be altered in experimental models of epilepsy and potentially also in patients with epilepsy.

Background

$GABA_A$ receptor-mediated transmission is essential for fine tuning of neuronal networks and generation of rhythmic, synchronized neuronal activities. Conceptually, both a decrease and an increase in GABAergic tone in cortical circuits can facilitate seizure initiation and propagation. In the first case, reduced inhibition might favor hyperexcitability; in the second case, enhanced inhibition might result in hypersynchronization and generation of rhythmic discharges. To establish whether an alteration in $GABA_A$ receptors is linked with epileptogenesis and/or recurrent seizures in chronic epilepsy, one has to consider several mechanisms that altogether determine $GABA_A$ receptor function:

1. Regulation of $GABA_A$ receptor assembly, trafficking, synaptic clustering, and cell-surface mobility. All these processes might be disturbed by mutations in $GABA_A$ receptor subunits and/or associated proteins, which might underlie familial forms of epilepsy. Collectively,

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1. Regulation of $GABA_A$ receptor assembly, trafficking, synaptic clustering, and cell-surface mobility. All these processes might be disturbed by mutations in $GABA_A$ receptor subunits and/or associated proteins, which might underlie familial forms of epilepsy. Collectively,

mechanisms regulating $GABA_A$ receptor synaptic targeting, clustering, and cell-surface mobility are responsible for short- and long-term regulation of the number of receptor molecules available for synaptic transmission.
2. Regulation of receptor properties by posttranslational modification, with phosphorylation being one of the major covalent modifiers. Increasing evidence indicates that chemical modification affects receptor trafficking and cell surface expression, as well as intrinsic functions of the ligand-gated ion channel.
3. Regulation of subunit expression, at the transcriptional and translational level. This mechanism determines the abundance and subunit composition of $GABA_A$ receptors in a given cell type or brain region, and is known to be of relevance for alterations of physiological network function (such as hormonal fluctuations during the ovarian cycle and after delivery), as well as for pathophysiological conditions, including neurological diseases and drug abuse.

GABAergic synaptic transmission is regulated also by modifications affecting presynaptic function (regulation of GABA synthesis, storage, and reuptake; modulation of GABA release probability; etc.) and $GABA_B$ receptors, but these aspects are outside the scope of the present review.

Studies of $GABA_A$ receptors in epilepsy have focused on two major diseases, namely absence epilepsy and temporal lobe epilepsy (TLE). In both cases, alterations of $GABA_A$ receptor expression, pharmacology, and functional properties have been studied in detail over many years. Recent results highlighting novel features will be discussed in more detail in the subsections 'Phasic–Tonic Inhibition' and '$GABA_A$ Receptor Alterations in Epilepsy.' Importantly, changes observed in experimental models need to be compared to alterations taking place in the brain of TLE patients. The availability of tissue resected at surgery from patients with intractable seizures represents an invaluable source for studying the pathophysiology of the disorder. Conversely, the functional consequences of mutations associated with familial forms of epilepsies are not readily amenable for analysis in patients, but can be investigated in recombinant expression systems and in transgenic mouse models. A close link between the clinic and experimental research is, therefore, a prerequisite for successful studies of the mechanisms of epilepsy.

Methods

Experimentally, absence seizures can be genetically determined (GAERS, WAG/Rij rats) or induced pharmacologically; for example, by treatment with a cholesterol biosynthesis inhibitor, AY-9944. TLE is mimicked by induction of a prolonged status epilepticus in mice or rats (either upon repetitive electrical stimulation of sensitive regions of the temporal lobe or by injection of a convulsant, such as kainic acid or pilocarpine), which is followed in most cases by the occurrence of spontaneous recurrent seizures. Kindling, either electrical or chemical, is also used to model TLE in rodents, with the major difference that the animals do not present with recurrent seizures. Despite their usefulness, these models of TLE are limited by the fact that experimental animals have generalized seizures (unlike patients with TLE), and epileptogenesis frequently is accompanied by patterns of neuronal damage, notably in the extrahippocampal regions, that are not observed clinically.

An alternative model of TLE was introduced, in which chronic partial seizures are induced in adult mice upon unilateral injection of kainic acid into the dorsal hippocampus. These mice have a nonconvulsive status epilepticus that subsides spontaneously and is followed by a latent period of about 2 weeks prior to the onset of spontaneous partial seizures. During the latent period and initial chronic phase, extensive neuronal damage occurs in the CA1 region, accompanied by a strong astrogliosis and, most remarkably, a prominent dispersion of dentate gyrus granule cells. The latter feature is reminiscent of the granule cell dispersion occurring in a subset of patients with TLE. All in vivo models are used for a wide range of investigations, including molecular biology, biochemistry, histology, electrophysiology, and behavior. Alterations of $GABA_A$ receptor assembly, localization, and function can be related to distinct stages of epilepsy and can be investigated further by pharmacology and/or genetic manipulation.

Tissue resected at surgery from patients with intractable seizures likewise is amenable to histological, electrophysiological, or biochemical analysis. Thus, the expression of $GABA_A$ receptor subunit genes can be analyzed by in situ hybridization, providing insight into their regional and cellular distribution, as well as relative level of expression. More specific information about the subcellular localization of receptor subunits can be obtained with immunohistochemistry using subunit-specific antibodies. This method cannot distinguish, however, between intracellular and cell-surface proteins and provides no information about 'functional' receptors, which are a heteromeric protein complex located at synaptic sites. Radioligand binding studies, including autoradiography, allows the investigator to distinguish functional receptors (which bind the radioligand), but have limited spatial resolution and do not distinguish among $GABA_A$ receptor subtypes, which differ in subunit composition. For studies of $GABA_A$ receptors, the high affinity and specificity of benzodiazepine site ligands (e.g., ^{3}H-flumazenil) make them very well suited for autoradiography. The major limitation of these studies in tissue obtained from surgery is the lack of appropriate 'control' tissue. Among potential 'control,' alternatives are apparently healthy tissue

resected along with the diseased tissue, resections derived from tumor-related epilepsies, postmortem brain tissue from autopsy, or brain tissue from another mammalian species.

Recent Results

Phasic–Tonic Inhibition

Phasic and tonic neurotransmission are used to discriminate between the short, spatially restricted action of transmitters activating postsynaptic receptors, and the continuous activation of receptors localized peri- or extrasynaptically by transmitter spillover into the extracellular space. Although tonic GABAergic inhibition has been largely overlooked in the past, it might play a major role in modulating neuronal activity, given that most of the total charge transported by ligand-gated ion channels occurs via tonic transmission (due to the prolonged duration of tonic transmission compared to the short channel opening times associated with postsynaptic receptors).

The subunit composition of $GABA_A$ receptors appears to be a major determinant of phasic and tonic GABAergic transmission. The γ2 subunit, which is present in the vast majority of $GABA_A$ receptor subtypes (along with α and β subunit variants), is required for clustering of the receptor at postsynaptic sites. Multiple γ2-containing $GABA_A$ receptor subtypes (also containing α1-, α2-, α3-, and, in part, α5-$GABA_A$ receptor subunits) indeed are clustered postsynaptically in various neuronal populations. A major additional property of the γ2 subunit is to confer diazepam sensitivity to receptor complexes containing these α subunit variants. It is important to note, however, that $GABA_A$ receptors containing the γ2 subunit are not necessarily confined to postsynaptic sites. For instance, most α5-containing $GABA_A$ receptors are extrasynaptic, contribute to tonic inhibition modulated by diazepam, and regulate the excitability of pyramidal cells in neocortex and hippocampus. Consequently, postsynaptic and extrasynaptic receptors formed with the γ2 subunit are diazepam-sensitive and contribute to the pharmacological profile of classical benzodiazepine-site agonists.

Receptors containing the δ subunit, in contrast to the γ2 subunit, appear to be excluded from postsynaptic sites. The δ subunit is associated mainly with the α4 subunit – e.g., in thalamus and dentate gyrus – or the α6 subunit in cerebellar granule cells. These receptors are diazepam-insensitive but are selectively modulated by GABA agonists such as gaboxadol and muscimol as well as neurosteroids, pointing to a possible novel target for drug therapy. Importantly, $GABA_A$ receptors containing the α4 and/or δ subunit exhibit unique functional properties that may contribute to epileptogenesis and recurrent seizures upon altered expression, as occurs in TLE. δ Subunit-null mice exhibit enhanced sensitivity to pentylenetetrazol-induced seizures, but it is not established whether this sensitivity reflects a reduced tonic inhibition in thalamocortical circuits or a reduced availability of binding sites for endogenous neurosteroids with anticonvulsant activity.

Ectopic overexpression of the α6 subunit has been used to assess the functional and pharmacological significance of enhanced tonic inhibition. Such transgenic mice overexpress α1/α6/β/γ2-$GABA_A$ receptors and exhibit a five-fold increase in tonic inhibition in CA1 pyramidal cells. Behaviorally, these mice are essentially normal, but are more sensitive than wild type to the convulsant effects of $GABA_A$ receptor antagonists, suggesting an overall decrease in GABAergic synaptic strength.

$GABA_A$ Receptor Alteration in Epilepsy

Changes in subunit composition

Alterations in $GABA_A$ receptor subunit expression and composition in epilepsy are well documented in human and in animal models. Given the heteromeric structure of $GABA_A$ receptor subtypes, a change in expression of a given subunit is often taken as evidence for a change in subunit composition of $GABA_A$ receptors present in this cell or brain area. However, since the complete $GABA_A$ receptor subtype repertoire of neurons in most brain regions is not yet established, these interpretations need to be treated with caution.

It should be emphasized, furthermore, that many of the studies focusing on transcriptional alterations have not used quantitative methods and did not address the issue whether gene transcription or mRNA half-life were changed. Furthermore, the relevance of these changes for the function of $GABA_A$ receptors is difficult to assess in most studies; the major impact on function of receptor localization, targeting, and regulation must be assessed, in addition to the mere abundance of a given subunit mRNA. There are very few studies, indeed, which have demonstrated a direct correlation between altered $GABA_A$ receptor subunit gene transcription and a corresponding functional alteration.

Changes in $GABA_A$ receptor subunit protein levels are also well documented in the hippocampal formation and neocortex of patients with partial seizures, as well as in a variety of TLE models – mainly using immunohistochemistry. Both a decrease and an increase in specific subunits have been reported. There are some discrepancies among studies, but it is not clear whether they relate to methodological differences in the analysis or are due to differences among the models. Importantly, besides a reduction of $GABA_A$ receptor subunits in regions affected by neurodegeneration, a subunit-specific *increase* has been reported in many studies, notably in the dentate gyrus. It is not known whether this increase leads to abnormal hypersynchronization of neuronal networks or represents a compensatory reaction to protect against recurrent seizures.

Recent experimental studies extend previous work by demonstrating a major contribution of extrasynaptic $GABA_A$ receptors to the changes in inhibitory function that might underlie epileptogenesis and occurrence of chronic recurrent seizures. For example, in the mouse pilocarpine model of TLE, a profound decrease in δ subunit immunoreactivity was observed, correlating with a redistribution of the γ2 subunit from synaptic to perisynaptic sites, where it assembles with the α4 subunit (which is normally associated with the δ subunit). A downregulation of the α5 subunit also occurs in CA1 pyramidal cells of pilocarpine-treated rats, resulting in a loss of diazepam-sensitive tonic inhibition seen upon blockade of GABA reuptake. Despite this change, tonic inhibition is enhanced in pyramidal cells, suggesting compensatory upregulation of other extrasynaptic $GABA_A$ receptors, possibly containing the α4 subunit.

Taken together, changes in $GABA_A$ receptor subunit expression and subsynaptic localization, as occurring in TLE and in experimental models, are open for interpretation. First, it is not established whether they reflect changes in connectivity, for example, due to axonal sprouting and synaptogenesis in response to the selective loss of specific populations of interneurons, or changes in the nature of inhibition received by a given cell type, as determined by the distinct functional properties of specific $GABA_A$ receptor subtypes. The reduction in synaptic receptors in favor of extrasynaptic receptors, as discussed in the previous paragraph, might represent an example of functional reorganization. However, in the pilocarpine model, it has been suggested that perisomatic inhibition of CA1 pyramidal cells is increased, whereas dendritic inhibition is decreased as a consequence of differential cell loss and circuit reorganization. Second, neurogenesis should be considered as an additional factor likely to play an important role in the long-term maintenance of synaptic circuits in the dentate gyrus. The effects of epileptogenesis and recurrent seizures on neurogenesis are still debated. Some reports suggest that seizures enhance neurogenesis, but disturb the migration of new born cells, which ultimately from abnormal circuits. Other reports suggest, on the contrary, that neurogenesis might be suppressed in TLE, notably when the subgranular zone of the dentate gyrus is disrupted due to granule cell dispersion. More information will be required before we understand how $GABA_A$ receptor-mediated transmission is adjusted in epileptic dentate gyrus.

Region-specific changes in $GABA_A$ receptor function and expression have also been reported in models of absence epilepsy. In the pharmacological model of absence seizures induced by neonatal treatment with the cholesterol biosynthesis inhibitor AY-9944, a reduced expression of the α1 and γ2 subunit has been reported, with distinct sex differences and temporal profiles, correlating with the higher incidence of absence seizures in female rats. In the GAERS model of absence, $GABA_A$ receptor-mediated currents are altered selectively in the thalamic reticular nucleus (but not in ventrobasal complex or somatosensory cortex), with mIPSCs exhibiting enhanced amplitude and reduced decay kinetics. Such changes might be accounted for by expression of the β1 subunit. Finally, in WAG/Rij rats, a loss of $GABA_A$ receptor α3 subunit-immunoreactivity has been shown to occur without alteration in mRNA expression in the reticular thalamic nucleus, suggesting a local and highly specific deficit in $GABA_A$ receptor function as a possible cause of absence seizures in these mutant rats.

Changes in $GABA_A$ receptor phosphorylation

Although multiple $GABA_A$ receptors can be phosphorylated by various serine/threonine and tyrosine kinases in vitro and in vivo, the significance of this posttranslational modification for the regulation of $GABA_A$ receptor function and of network properties remains poorly understood. Available evidence indicates that phosphorylation might affect $GABA_A$ receptor channel function as well as trafficking to and from the cell surface and synaptic sites. The net effect of phosphorylation-associated changes can be an increase or a decrease in $GABA_A$ receptor function, depending on the subunit involved and on the balance between kinase and phosphatase activity. Of relevance for epilepsy is the recent report showing a deficit in endogenous $GABA_A$ receptor phosphorylation in tissue resected from patients with partial epilepsies, an abnormality which might favor seizure initiation and propagation. Importantly, in this study, the reduced extent of phosphorylation was not related to an overall decrease of $GABA_A$ receptors in the epileptic focus. Another approach to study potential deficits of $GABA_A$ receptor phosphorylation is to transplant membranes prepared from resected human brain tissue into oocytes. Functional $GABA_A$ receptors are inserted into the oocyte membrane within hours and their properties can be compared to $GABA_A$ receptors present in control, nonepileptic tissue. Such studies have revealed specific alterations in $GABA_A$ receptor phosphorylation selectively in 'epileptic' tissue, but the precise mechanisms underlying these alterations remain to be discovered.

Mutations affecting assembly and trafficking

An increasing number of mutations in $GABA_A$ receptor subunits have been associated with familial idiopathic epilepsies. In recombinant expression systems, these mutations typically affect single channel gating and/or cell surface availability of $GABA_A$ receptors. The precise mechanism underlying seizure generation in most of these cases remains ill-defined. The $GABA_A$ receptor γ2 subunit mutation R43Q associated with $GEFS^+$ has been reported to impair assembly and cell surface expression of $GABA_A$ receptors. The mutation causes an increase in intracortical excitability in patients (compared to

unaffected relatives). Intriguingly, the effect of the mutation was shown to be temperature-dependent, with cell surface expression being reduced in vitro at temperatures higher than 37 °C. However, since most $GEFS^+$ patients do not carry this mutation, such a mechanism alone is not sufficient for explaining the onset of seizures in $GEFS^+$. In fact, other studies have shown that the γ2(R43Q) mutation affects $GABA_A$ receptor cell surface trafficking and subunit composition independently of temperature. Another mutation, α1(A322D), associated with juvenile myoclonic epilepsy, is characterized by reduced subunit expression due to enhanced proteosomal degradation, probably a result of protein misfolding. Finally, mutations in the δ subunit (forming mainly extrasynaptic $GABA_A$ receptors) affect both channel kinetics and cell surface expression, and are associated with GEFS+.

Future Goals

The ultimate goals of experimental epilepsy studies are to better understand the pathophysiology of this disorder and to provide better therapeutic approaches than those available today. Since antiepileptic drugs do not 'cure' epilepsy but only suppress seizures, an ideal treatment strategy would be to prevent epileptogenesis, as least in acquired epilepsies. Since very little is known, yet, about the mechanisms of epileptogenesis, there is a crucial need for developing animal models that allow insight into the changes in neuronal circuits underlying emergence and recurrence of spontaneous seizures following a brain insult. The observation that prolonged seizure activity (status epilepticus) is required in experimental models of TLE for the emergence of recurrent seizures, after a latent phase of several days or weeks, was important for subsequent studies of epileptogenesis. A major question that remains open in relation to epileptogenesis is whether emergence of recurrent seizures at the end of the latent phase represents a failed attempt of the brain to compensate for the detrimental effects of the initial lesion (or status epilepticus) or whether it results from abnormal reorganization of neuronal circuits triggered by the initial lesion. There is no doubt that GABAergic transmission is profoundly affected during the latent phase. A proper answer to this question will hopefully provide guidance for the design of therapeutic strategies aimed at prolonging or curtailing the latent phase.

Regarding $GABA_A$ receptors, there are at least three main goals to achieve: (1) To further our understanding of the significance of $GABA_A$ receptor heterogeneity for proper brain function; (2) To investigate the mechanisms underlying the effects of mutations in idiopathic epilepsies. Here, the main stumbling block is the fact that different mutations can lead to similar phenotypes – or conversely, that a given mutation can generate different phenotypes across individuals; (3) To develop new drugs that take advantage of $GABA_A$ receptor heterogeneity to achieve better efficacy and reduced side-effects (compared to the drugs – e.g., benzodiazepine agonists – in current clinical use.

See also: **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; Molecular Pathology of Genetic Epilepsy Associated with $GABA_A$ Receptor Subunit Gene Mutations; Plasticity of GABAergic Systems during Epileptogenesis.

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GABA Excites Immature Neurons: Implications for the Epilepsies

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Introduction

In the adult brain, glutamatergic neurons and synapses provide most of the excitatory drive by means of receptor channel complexes permeable to cations. GABAergic neurons and synapses provide the inhibitory drive by means of receptor channel complexes permeable to anions and particularly chloride. Equilibrium between the operations of both synapse types is instrumental for avoiding seizures and toxicity. A wide range of agents that block GABA signaling induce seizures, and many agents that increase GABA signaling effectively block seizures or even reduce their long-term consequences. In addition, GABAergic neurons play a central role in the generation of behaviorally-relevant network oscillations by means of an extremely complex and diversified pattern of innervation of all the elements of the networks, including the somata and dendrites of principal cells and other GABAergic neurons. Thus, the operation of brain networks relies heavily on a time- and space-dependent activation of excitatory and inhibitory synaptic currents that enable the brain to integrate information and generate the appropriate response by means of network oscillations.

During brain construction, once neurones start their differentiation, they establish functional synapses and start generating signals and patterns that in turn influence the network wiring. The more the neuron matures, the more the genetic program is influenced by activity and signals generated by the neuron and its neighbors. This influence is particularly important in complex brains such as that of the human's, with its 10^{15} synapses. During network construction the neurons may discharge, so some mechanisms for maintaining equilibrium between excitation and inhibition is needed during the process; in this respect, the process is very different from, say, the construction of a car where the engine is ignited only when all the pieces have been put together. The basic question examined in this article is how the brain resolves that equilibrium issue in relation to GABA and glutamate signaling. If GABAergic inhibitory synapses are operative too early, then the process will be retarded because of inhibition and reduction of calcium influxes; if glutamate excitatory influences develop first, then the excitotoxicity may result in injury and lesions. The solution to this problem appears not to be a parallel and general equilibrated/titrated excitatory/inhibitory drive, but rather a unique developmental sequence with an earlier maturation of *excitatory* GABA synapses followed by a later maturation of glutamate synapses. This sequence prevents the possible deleterious sequelae of too early development of inhibitory GABA or excitatory glutamate systems, while still providing the calcium surge needed for maturation and neuronal growth.

These issues are of particular importance for understanding the epilepsies. For example, is the well-established higher incidence of seizures at an early age related to these features? Is it possible that a weaker inhibition, or an early unbalanced excitatory drive, fosters a lower threshold for seizure generation? Do certain developmental features, such as the immature states of neurons, synapses or networks, make the immature brain particularly prone to seizure generation? Answers to these questions would, of course, give important clues as to the means by which treatments should proceed. We shall focus here on the maturation of GABA and glutamate signaling, and evaluate the implications of the unexpected cascade of events initiated by these immature signalling processes on seizures on the developing brain.

Background

Key Findings

A higher $[cl^-]_i$ in immature neurons leads to excitatory actions of GABA (Figure 1)

In 1989, using old fashioned intracellular recording techniques, we recorded hundreds of hippocampal neurons in slices from young rats, and discovered a higher intracellular concentration of chloride ($[Cl^-]_i$) that disappeared progressively around the second week of post natal life. Consequent to the membrane depolarization thus induced by GABA actions at its receptor/channel complex, GABA excites immature neurons and generates sodium action potentials, calcium currents and removes the voltage-dependent Mg^{2+} blockade from NMDA channels in immature neurons, leading to a massive calcium influx. This result is contrary to that seen at more adult stages, where activation of GABA usually reduces calcium influx and the activation of NMDA receptors channels. This property of GABA activation in immature neurons has been confirmed in all brain structures and animal species investigated to date, suggesting that it has been conserved throughout evolution.

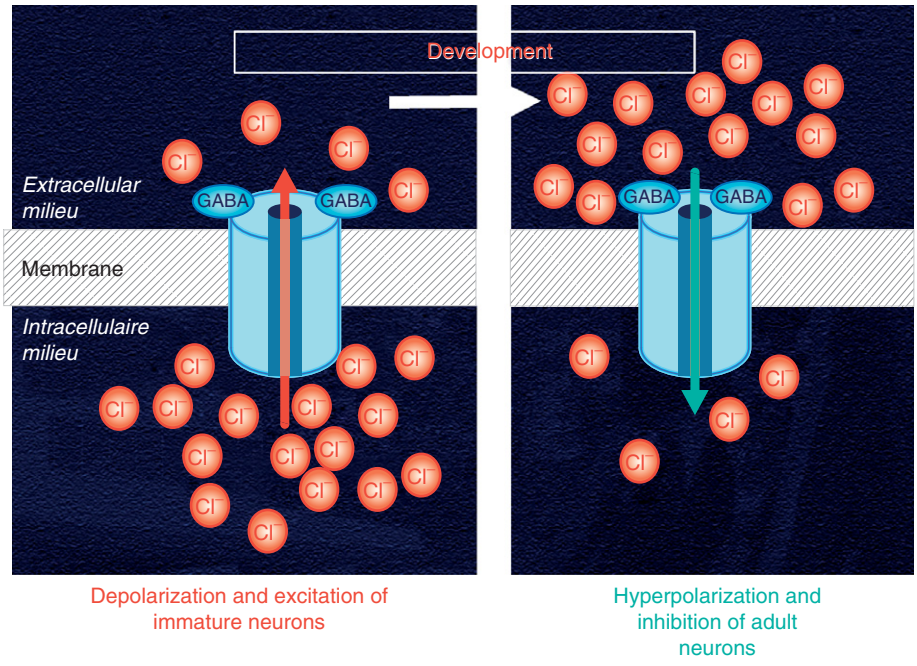


Figure 1 The developmental gradient of $[Cl^-]_i$ and excitatory actions of GABA. A higher intracellular concentration of GABA in immature neurons leads to depolarizing actions of GABA. This action is replaced with maturation and GABA resumes its adult actions.

Early excitatory actions of GABA provide the source of the calcium increase needed for neuronal growth and synapse formation, while avoiding toxic actions of glutamate. The higher concentration of chloride in immature neurons is due to an early expression of a chloride importer (NKCC1) AND a delayed maturation of a chloride exporter. This maturational difference has been observed in various experimental animals, and also in humans, suggesting that these mechanisms are also conserved. Interestingly, the immature chloride gradient has many functional implications, and probably is instrumental in the trophic roles of GABA in neuronal and network maturation. An example of that role is illustrated by the experiment performed by Medina and colleagues, who showed that early transfection of KCC2 in immature neurons (that have a high $[Cl^-]_i$) induces the expected change of the actions of GABA but also augments the formation of GABAergic synapses in the neurons that have shifted their chloride too early. Thus the excitatory actions of GABA, caused by high $[Cl^-]_i$ is an important signal in synapse formation.

The GABA-glutamate sequence: GABA comes first

Earlier studies had shown that GABAergic interneurons divide and mature before principal neurons. We also found that blockers of GABAergic receptors that trigger seizures in adults can block ongoing activity at an early stage. We then recorded and reconstructed a large number of pyramidal neurons and interneurons, and found that GABAergic synapses appear first on both cell types; neurons with few or no dendrites are silent, with no synapses; neurons with a small apical dendrite have only GABAergic postsynaptic currents (PSCs); and only neurons that have both large apical dendrites and basal dendrites have both GABAergic and glutamatergic synapses. The sequence occurs earlier in interneurons than in pyramidal cells; with over 80% of pyramidal cells and less than 20% of intereurons being silent at birth. In both cell types, GABAergic currents provide most of the activity at an early developmental stage.

GABA exerts a paracrinic actions even before synapses have been formed

Immature neurons express functional receptors prior to the formation of synapses. We showed that these neurons that bear receptors but no synapses act as sensors of extracellular GABA, as reflected by the large tonic currents generated by GABA receptor antagonists. In addition, extracellular electrical stimuli induce large slow currents mediated by a non-vesicular release of GABA – only at an early stage of development. This GABA release and the resultant currents have a functional significance, as blocking GABA receptors at this early stage reduces neuronal migration. The implications of this result are reflected by the observation that GABA-acting antiepileptic drugs slow neuronal migration and lead to heterotopic neuronal masses (see later text).

The immature hippocampal network generates a primitive universal pattern

During this earlier series of experiments, we also found that immature rat hippocampal neurons generate – until

the 2nd week postnatal – a pattern of discharge that we called Giant Depolarizing Potentials (GDPs). This pattern is the first synapse-driven pattern in the hippocampus, and consists of large polysynaptic events that provide most of the excitatory activity in these neurons (as long as a substantial proportion of GABAergic synapses are excitatory). GDPs are generated by the depolarizing effects of GABA. The depolarization also removes the voltage-dependent Mg^{2+} block from NMDA channels, thus facilitating the generation of large glutamatergic NMDA receptor-mediated synaptic currents that activate most neurons in the population. GDPs are primitive events that are replaced by behaviorally-relevant patterns. They enable co-activation of neurons that are in different developmental stages. Similar patterns have been observed in a wide range of structures and animal species.

Confirming this sequence in primate neurons in utero

In a parallel study, we performed the first recordings ever made in primate central neurons in utero. These recordings required a Caesarean surgery followed by slices production and determination of synaptic currents, evaluation of spine density, determination of the speed of axonal and dendritic growth, etc. We found a similar earlier formation of GABAergic synapses in the primate hippocampus as seen in the rat, as well as GDPs in both pyramidal neurons and interneurons. The hippocampal CA1 pyramidal neuron in primate has no synapses at mid-gestation, and more than 7000 synapses at birth (some 2 months later). This study provided a detailed description of the general developmental sequence in primate hippocampus, and showed that the basic principles described in immature rodents are valid in primates in utero.

Why have these sequences been kept throughout evolution?

We have suggested that this developmental sequence provides a solution to the need for preserving equilibrium between excitation and inhibition at all times. We hypothesize that the excitatory drive of GABA at an the early developmental stage is sufficient to augment calcium and stimulate neuronal growth – without endangering the integrity of immature cells since E_{rev} for chloride is 30–40 mV below zero (contrary to the much more depolarized E_{rev} for glutamate. In addition, the shunting actions of GABA prevent excessive hyperactivity (but see below). The GDPs, by means of their long-lasting duration, enable immature neurons (which are endowed with few synapses) to fire together with more adult neurons, and thus wire together. This scenario also promotes synaptic plasticity, since summation of currents is more likely to occur with long PSCs. Once the GABA shift occurs and a sufficient density of glutamate synapses has been formed, primitive patterns disappear and are replaced by the plethora of behaviorally-relevant patterns.

The importance of measuring adequately V_{rest} and E_{GABA}

The above observations raise the issue of the importance of adequately measuring the genuine resting membrane potential and reversal potential of GABA. Clearly, if recording methods modify resting membrane potential or $[Cl^-]_i$, the measurement of the excitatory actions of GABA will be invalid. Apparently excitatory actions of GABA have been reported with all possible recording techniques, including extracellular, intracellular, whole cell patch clamp and perforated patch recordings (permeable to cations but not to chloride and thus thought not to affect $[Cl^-]_i$). However, in a recent study, we measured V_{rest} by a non-invasive method. We found that with whole cell and gramicidin perforated-patch recordings, resting membrane potential was −44 mV at postnatal days P0-P2 and −67 mV at P13–15; in contrast, with single NMDA channels recordings, it was −77 mV at both ages. Therefore, V_{rest} is not altered during maturation, and the developmental decrease of the effects of GABA are genuinely due to alterations of $[Cl^-]_i$. More recently, we also measured single GABA channels in cell attached recordings, and confirmed this developmental reduction (from over 25 mM chloride to around 7 mM). These results suggest that invasive techniques, including perforated patch recordings, provide an inadequate estimation of V_{rest}. Computer simulations suggest that depolarized values of invasive recordings could be explained by a shunt through the seal contact between the pipette and membrane.

Generalization of theses features to other brain structures

Extensive studies made by several groups have essentially confirmed the chloride developmental shift in intracellular chloride concentration for most brain structures and animal species. The mechanism underlying these shifts have also been clarified and shown to depend on an early maturation of a chloride importer and a late maturation of a chloride exporter. To the best of our knowledge, there is to date no exception to the early depolarizing actions of GABA. Whether, GABA excites – i.e., generates action potentials – has been more controversial, since GABA also inhibits even when depolarizing because of its 'shunting' actions that tend to clamp V_{rest} at E_{GABA}. However, cell attached extracellular recordings show unambiguously that the depolarization provided by GABA – *at an early stage* – is above the threshold for action potential generation. GABAergic currents have also been shown in many systems to operate first (i.e., before glutamate currents); however, compelling evidence relying on neuronal reconstruction is lacking in most structures. Primitive patterns such as the GDPs have been reported in a wide

range of brain structures, and the GABA shift does play a central role in their generation and/or their termination. More problematic is the relevance of these patterns to the in vivo situation, since various patterns have been reported in pups in vivo; a comparison between the generating mechanisms in vivo and in vitro remains to be performed.

Implications for the epilepsies

As should be clear from the above discussion, immature neurons lack one of the main inhibitory systems characteristic of the adult brain. In addition, other major inhibitory transmitter-gated postsynaptic mechanisms – e.g., $GABA_B$ receptor and other G-protein-mediated activation of K^+ channels – are also late to develop. In contrast, pre-synaptic G-protein-mediated currents (activated via $GABA_B$, 5HT, or A1 receptors) operate at an early stage, suggesting that pre-synaptic inhibition is functional before postsynaptic inhibition. Therefore, during maturation, neurons lack postsynaptic transmitter-gated hyperpolarizing inhibition, and only voltage-gated K^+ currents operate. Given this situation, immature networks may be more prone to seizure generation because of the excitatory actions of GABA.

Studies devoted to estimating the contribution of GABA to neonatal seizures have, however, led to a more complex image. Using non-invasive extracellular field potential and cell-attached recordings in hippocampal slices, we have found a dual action of GABA (**Figure 2**): seizure-like events generated by a high K^+ milieu were reduced by the $GABA_A$ receptor antagonist bicuculline, but at the same time the amplitude of the population spikes was increased – suggesting that GABA exerts dual excitatory and inhibitory actions. Other observations suggest a direct contribution of excitatory actions of GABA to epileptogenesis, as reflected by the powerful antiepileptic actions of bumetanide – a blocker of chloride import that reduces the excitatory actions of GABA and seizures in the developing brain. Excitatory actions of GABA may play an important part in the generation of seizures by developing networks (working together with other factors, including the high input resistance of immature neurons). This possibility does raise a question about the widespread use of GABA facilitators – notably benzodiazepines – to treat early seizures. However, this topic deserves more direct investigation.

The excitatory effect of GABA is also illustrated by the powerful pro-convulsant actions of the cannabinoid agents. Since endogenous cannabinoids exert a negative feed back

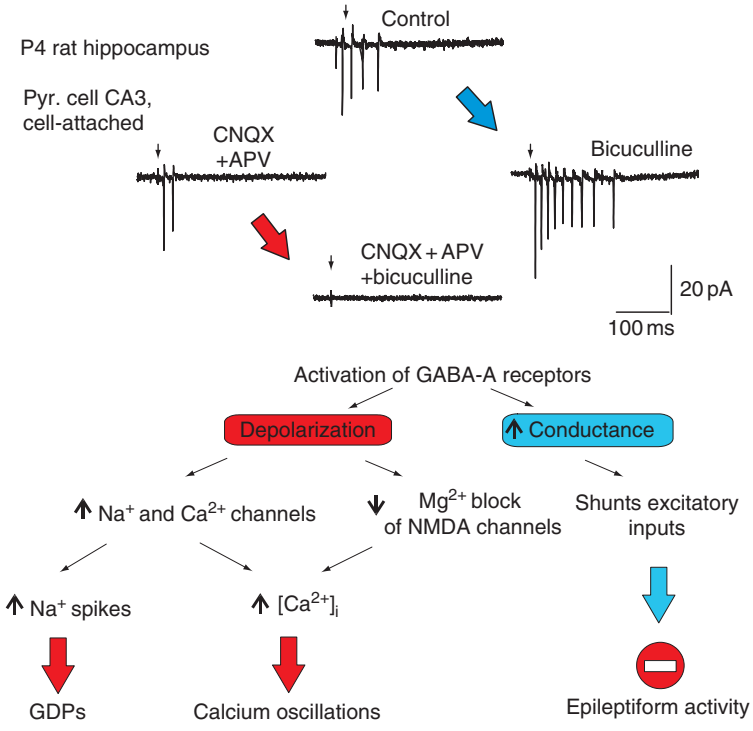


Figure 2 Dual actions of GABA. In an immature hippocampal neuron recorded in a cell attached configuration, ionotropic Glutamate receptor antagonists blocks many spikes generated by an electrical stimulus, however the remaining ones are blocked by a GABA receptor antagonist reflecting the genuine excitatory actions of GABA. In the bottom part, the dual actions of GABA are depicted with on the one hand the depolarizing actions of GABA generating action potentials and calcium currents as well removing the Magnesium block from NMDA channels and on the other, the shunting action of GABA exerting an inhibition that reduces the efficacy of glutamatergic synaptic currents.

on the release of GABA, they should act differently on adult and immature neurons. Blockade of cannabinoid type 1 (CB1) receptors leads to epileptic discharges during maturation, and conversely, activation of CB signaling reduces network activity in vivo and in vitro. Therefore, endocannabinoid signaling maintains population activity within a narrow physiological time window and controls the generation of the primitive GDPs pattern. Disturbing that delicate balance during pregnancy (in either direction) will affect neuronal activity, and blocking these CB1 receptors will contribute to seizure generation in the fetus.

Methods

These studies have used the entire panoply of recording, imaging and morphological techniques in vitro and in vivo. These methods include whole cell voltage and current clamp recordings, as well as the first single channel GABA and NMDA recordings to estimate correctly the resting membrane potential and reversal membrane potential for GABA. Fast imaging techniques were also used to measure alterations of calcium signals and activity in large number of neurons. In vivo studies used extra and intracellular and whole cell recordings in rat pups. A variety of anatomical and histological tools were used, including 3D neuronal reconstructions to determine the relation between neuronal activity and morphological properties.

Recent Results

Maternal Oxytocin Reduces $[Cl^-]_i$ before Delivery and Shifts Transiently GABA to Inhibition (Figure 3)

An interesting illustration of the excitation (E) to inhibition (I) shift in the actions of GABA was recently observed during a study aimed at determining the time course of changes of $[Cl^-]_i$ from embryonic to postnatal stages. We found an abrupt drop of $[Cl^-]_i$ immediately before and after delivery. This transient shift is associated with a transient inhibitory effect of GABA, and is mediated by the maternal hormone oxytocin (which is released to start labor). Thus, the release of maternal oxytociçne both triggers labor and shifts GABA effects from excitatory to inhibitory. The effects are most likely mediated by a reduction of the actions of NKCC1 that imports chloride into neurons. Indeed, whereas oxytocin receptors are present in utero, we did not find oxytocin positive neurons in fetal brain, and in vivo administration of an oxytocin receptor antagonist prevented the E-to-I shift. This mechanism has a neuroprotective action, since fetal neurons are more resistant to anoxo-ischemic episodes when oxytocin receptors are operative (not blocked). Blocking in situ oxytocine receptors also made neurons more readily blocked by anoxic episodes. Using a fast dynamic two-photon imaging process, we determined the sequential maturation of patterns from the embryonic to the post natal stage. We found that the earliest pattern present

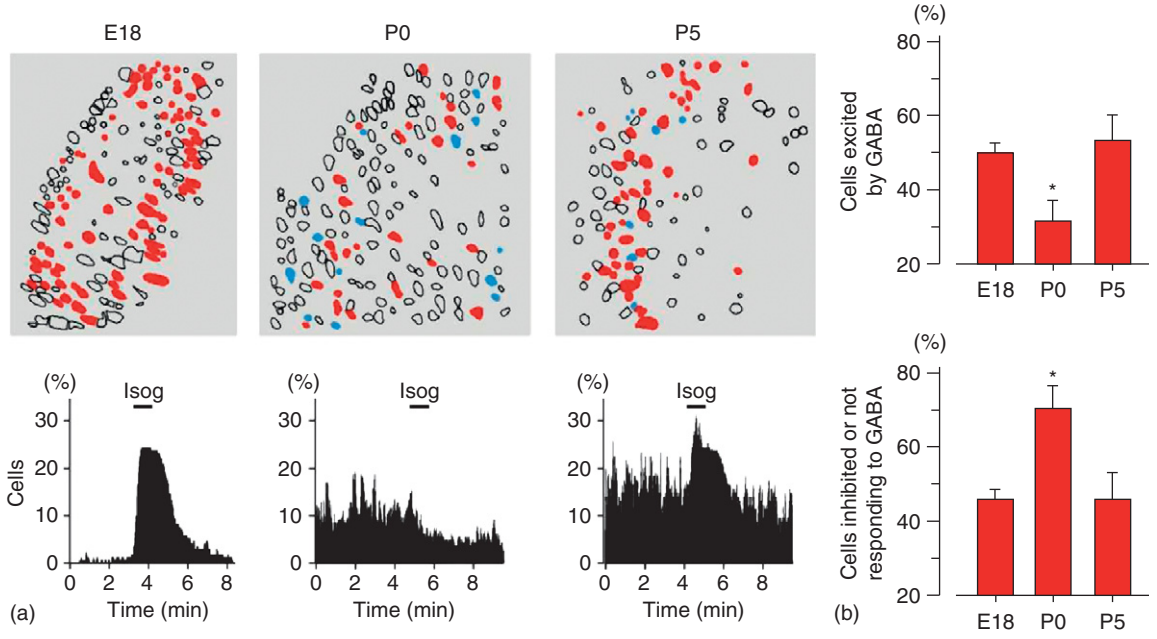


Figure 3 Dramatic shift of the actions of GABA before delivery. A GABA agonist – isoguvacine – excites immature neurons *in utero* (E18) and at P5 as shown in the dynamic imaging of calcium recorded by two photon imaging laser microscopy. In contrast immediately before delivery, the same agent inhibits neurons. The number of neurons excited and inhibited by isoguvacine at these developmental stages is shown at the right side of the figure.

(around delivery) is a calcium plateau oscillation mediated by voltage-gated calcium currents in a small population of neurons connected by gap junctions. This pattern is triggered also during delivery by oxytocine.

These data suggest that the fetus is informed that delivery is to commence, and is protected from labor-associated insults by the ocytocin hormone. This scenario has important clinical implications in relation to preterm deliveries. Indeed, the use of atosiban and other oxytocin receptor antagonist may remove an endogenous neuroprotective mechanism; agents that block peripheral but not central oxytocin receptors may be advantageous. Also, oxytocin agonists may offer interesting tools to reduce neuronal excitability at an early stage.

In Utero Administration of Some Antiepileptic Drugs Affects Neuronal Migration and Dispersion

Since immature neurons express receptors before functional synapses, it is important to examine the functional significance of these receptors – and the possible pathological implications of activating these receptors. In an earlier study, we found that GABA and NMDA receptor agonists generate currents in neurons endowed with no functional synaptic current. GABA – but not glutamate – receptor antagonists also generate a tonic current, suggesting an ongoing release of GABA. In later studies, we found that the lack of effects of glutamate receptor antagonists is due to an early efficient maturation of glutamate transporters. Indeed, glutamate transporters develop before GABA transporters, and if they are blocked, severe seizures are generated. To determine the functional consequences of these receptors and the currents that they generate in neurons that bear no synapses, we tested the effects of various agents on neuronal migration in an in vitro preparation. We found that several drugs – notably antiepileptic drugs that act on $GABA_A$ receptors – retard migration. Interestingly, in vivo administration in rats of some antiepileptic drugs, at concentrations that are similar to concentrations found in pregnant epileptic women during gestation, lead to abnormalities in neuronal and layer organizations. Such results support the relevance of these findings to human disorders in general, and specifically to the effects of antiepileptic regimens during gestation. These observations suggest that GABA-acting agents used during migration may cause migration disorders and heterotopic masses with their well known deleterious sequelae.

Future Goals

The developmental sequences described here have been conserved throughout evolution. These rules have important implications in relation to seizure generation and seizure sequelae. Clearly, major signalling cascades – including those initiated by GABA, but perhaps also by many other molecules – operate differently in neonates and adult. Thus, the development of therapeutic strategies for neonates can only rely on observations obtained in neonatal experimental models. Further, the widespread use of GABA enhancers in treating early epilepsies may not be appropriate – perhaps only partially effective (as some observations already suggest) and potentially pro-epileptic. An important goal of future research is to determine the interactions of GABA and various other molecules to better comprehend the roles of the excitatory and inhibitory (shunting) actions of GABA in neurones endowed with few synapses and poorly developed dendritic arbors.

A second goal of future research is to determine the roles of oxytocin (and other agents released during delivery) on protecting fetal neurons during the birth process form traumatic and anoxic insults. The fetal brain is endowed with oxytocin receptors but apparently not with capacity to synthesize and release the hormone. Oxytocin is known to exert a powerful protective actions on neuronal activity. Understanding the actions of these agents (including, in addition to oxytocin, vasopressine, and catecholamines) is important as it will provide a rationale for better understanding how GABA and chloride are regulated during these unique situations.

In a more general perspective, the developing brain is known to be relatively resistant to cell loss when compared with that of adults. Yet seizures and other insults do lead to long-lasting consequences, suggesting that contrary to adult networks, the pathological sequelae are produced by alterations of the developmental program rather than lesions and neuronal damage. During maturation, many essential processes are activity-dependent and thus may be affected by episodes of hyperactivity or excessive release of transmitters that act via their paracrine actions (even before synapses have been established). The pharmacopoeia of immature neurons must be completely revised in the process of incorporating these new vistas. Widely-used drugs that act on GABA receptors, that are used during gestation and delivery, may deserve more detailed scrutiny.

In sum, the developmental alterations of the actions of GABA provide a unique opportunity for studying how neurons develop and networks are formed, and for evaluating how these processes may be endangered by manipulations that have been validated in adult preparations but not in developing networks.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency

Oscillations; **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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http: www.inmednet.com.

$GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization

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Introduction

Florey and MacLennan were the first to suggest that 'factor I' was an inhibitory neurotransmitter; after some debate, this compound was identified as the amino acid GABA. Half a century later, we know that GABA released from interneuron terminals activates pre- and postsynaptic GABA receptors in forebrain neurons. These receptors include ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are receptor-operated channels with a large molecular diversity and a largely somatodendritic expression. Signals mediated metabotropically, by $GABA_B$ receptors, are slower and may be involved in opening or closing somatodendritic channels or in controlling transmitter release from excitatory and inhibitory terminals, via distinct G proteins.

A relation between GABA and seizures first emerged when convulsions were seen in infants fed with a formula that was accidentally deficient in pyridoxine (known also as vitamin B6). Pyridoxine is the coenzyme for the synthesis of GABA from glutamic acid via the enzyme glutamic acid decarboxylase. Moreover, shortly after its identification, GABA was found to prevent seizures, and several molecules that interfere with GABA synthesis and signaling were shown to induce convulsions. Work in the 1970s and early 1980s confirmed a function for GABA receptor-mediated mechanisms in controlling neuronal excitability and thus epileptiform synchronization. Later

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GABA$_A$ Receptor Plasticity in Epileptogenesis

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Introduction

It is clear from studies carried out in various animal models and retrospective studies from humans that an initial precipitating event such as status epilepticus (SE) can increase the risk of later development of epilepsy. The process of epilepsy development (epileptogenesis) is complex and multifactorial. Many laboratories, including our own, have focused on understanding the role of γ-aminobutyric acid (GABA) receptor plasticity in epileptogenesis. GABA is the major inhibitory neurotransmitter in the mature brain, and various drugs such as diazepam and barbiturates that enhance GABAergic inhibition are commonly used as antiepileptic drugs. Also, drugs that block GABAergic inhibition can induce seizures in animals, further supporting the potential importance of alterations in GABAergic transmission in epileptogenesis. In this article we will describe the changes that have been observed in GABA$_A$ receptor (GABAR) subunit expression following prolonged seizures in various animal models of epilepsy, and the role these changes may play in epileptogenesis. Also, we will highlight the differences in GABAR subunit expression that occur following SE in adult and developing animals.

Background

GABA is the major inhibitory neurotransmitter in the adult brain. Three types of GABA receptors, GABA$_A$, GABA$_B$, and GABA$_C$, are found in the mature central nervous system. GABA$_A$ and GABA$_C$ are ionotropic receptors, whereas GABA$_B$ is a metabotropic receptor. Most fast synaptic inhibition in the mature brain is mediated by GABARs, whereas slow inhibition is mediated by GABA$_B$ receptors. GABARs are composed of multiple subunits subtypes (α1–6, β1–3, γ1–3, δ, ε, π, θ, and ρ1–3) that form a pentameric anion-selective channel. The most common in vivo subunit composition is two α, two β, and one γ subunit. There is remarkable receptor heterogeneity, with subtype combinations varying in different brain regions, cell types, and during different times in ontogeny. Different subunit subtypes confer distinct functional and pharmacological properties to the receptors.

Several laboratories have studied the changes in GABAR subunit composition in human temporal lobe epilepsy (TLE) and in animal models of TLE. Houser and colleagues have shown that in the CA1 and CA2 areas of the hippocampus there are long-lasting reductions in the α5 subunit of GABARs in pilocarpine-treated animals that develop spontaneous seizures. The α5 subunit is primarily extrasynaptic and is thought to be involved in tonic inhibition in the CA1 area. Lower levels of α5 subunit may thus result in reduction of tonic inhibition. In the molecular layer of denate gyrus, levels of α1, α3, α4, α5, and γ2 increase and levels of δ decrease chronically following SE. In interneurons of dentate gyrus, however, α1 subunit levels decrease and δ subunit levels increase, suggesting that there are cell-specific changes within the same region of the hippocampus. Additional studies from the Houser group suggest that changes in GABA receptor function after SE may depend not only on the levels of different subunits within individual neuronal cell types, but also on the subcellular localization of different GABAR subunits within these cells. Using immunogold labeling, these investigators showed that the subcellular location of γ2 and δ subunits in molecular layer of dentate gyrus was changed in pilocarpine-treated epileptic mice; they found a decrease in δ and an increase in γ2 subunits at perisynaptic locations, and a decrease in γ2 labeling near the center of the synaptic contacts. The change in the location of these subunits correlated with an insensitivity of the tonic current to neurosteroids and reduced phasic inhibition of DGCs in pilocarpine-treated epileptic mice. This impairment in inhibition could play an important role in development of epilepsy in this mouse model of TLE.

Additional support for the role of GABAR alterations in epileptogenesis comes from genetic studies. Mutations in the GABAR subunit γ2 gene (GABRG2) are found in the families with generalized epilepsy with febrile seizures plus ($GEFS^+$) and childhood absence epilepsy with febrile seizures. The γ2 subunit plays important roles in receptor trafficking, clustering, and synaptic maintenance; for example, Macdonald and colleagues found that slight increases in temperature caused impaired trafficking and accelerated endocytosis of α1β2γ2 receptor containing mutant γ2 subunit but not wild-type α1β2γ2 receptor. This finding suggests that some genetic forms of febrile seizures might be associated with reduced inhibition related to temperature-induced internalization of GABARs from the cell surface.

It is known from clinical experience and numerous animal studies that the immature brain is more susceptible to induction of seizures than the mature brain. The increased seizure susceptibility is thought to result from a combination of factors at play in the immature brain, including enhanced excitation and reduced inhibition. Animal model studies also suggest that the outcomes of events such as SE occurring in early-life are different from those outcomes from SE occurring in adult life. For example, almost all animals that experience pilocarpine-induced SE in adulthood exhibit spontaneous seizures following a short latency, whereas rat pups exposed to pilocarpine-induced SE at postnatal day (P) 10 never develop epilepsy in later life. Similarly, there are age-dependent molecular and morphological changes following SE. Effects of early-life SE on GABAR plasticity are different from those found in animals exposed to SE in adulthood. Rocha and colleagues found short-term increases in benzodiazepine ([^{3}H] flunitrazepam) binding in brain regions such as dentate gyrus, amygdala, and piriform cortex after kainic acid-induced SE at P15. However, there was a reduction in benzodiazepine binding in the same brain regions after 35 days. These results were in contrast to what has been found in adult animals, where there was an initial reduction in benzodiazepine binding in various brain regions followed by an increase in benzodiazepine binding 40 days after kainic acid-induced SE. Using in situ hybridization and immunohistochemistry, Holopainen and colleagues found a reduction in GABAR α1 and α4 subunit levels and increases in α2, α3, β3, and γ2 subunit levels in CA1, CA3, and dentate gyrus regions of hippocampus 1 week following kainic acid-induced SE at P9. These studies suggest that developing brain responds to SE differently from mature brain.

Methods

Various animal models of epilepsy are available to study different forms of epilepsy. Our laboratory is interested in studying the mechanism of development of TLE following an initial insult such as SE. SE can be induced by chemoconvulsants such as kainic acid or pilocarpine, or by continuous electrical stimulation administered through intracerebral electrodes. In our laboratory we have used pilocarpine (for adult animals) or lithium–pilocarpine (for 10–20-days-old rat pups) to induce SE. Adult rats were first injected with 1 mg kg^{-1} body weight scopolamine to block the peripheral effect of acetylcholine activation and then, 30 min later, with 385 mg kg^{-1} pilocarpine. Rats that did not exhibit convulsive seizures within 1 h of pilocarpine injection were given a second dose of pilocarpine (192.5 mg kg^{-1}) to produce equivalence in seizures between animals. Diazepam (6 mg kg^{-1}) was administered at 1 h after the onset of SE to stop seizure activity and was administered again every 2 h (3 mg kg^{-1}) until rats stopped seizing. To induce SE in developing rats, rat pups were briefly separated from the mother and injected intraperitoneally (i.p.) with 3 mEq kg^{-1} body weight lithium chloride. Fourteen to 18 h after the lithium injection, pups were again separated from their mother and received i.p. injection with either 60 mg kg^{-1} pilocarpine (SE group) or 0.9% saline (control group). Rats were continuously video-EEG monitored to determine the occurrence of seizures.

To selectively study the change in GABARs subunit expression in dentate granule cells (DGCs), our laboratory has used a single-cell mRNA amplification technique that was modified from the original technique described by Eberwine. For studies of regional RNA expression, subfields of the hippocampus such as dentate gyrus, CA1, and CA3 were microdissected; RNA was isolated, reverse transcribed, and amplified, and then analyzed using reverse Northern blots. T7-(dT)24 primer and SuperScript II RNase H-reverse transcriptase were used for cDNA synthesis, and the AmpliScribe T7 High Yield Transcription kit was used for RNA amplification. The cRNA was purified using the RNeasy MinElute Cleanup kit protocol (Qiagen, Valencia, CA) according to the protocol of the manufacturer. [P 32]dCTP was incorporated into the radiolabeled cDNA probe using SuperScript II RNase H-reverse transcriptase and hybridized for 18 h against a slot blot containing GABR cDNAs (α1–6, β1–3, γ1–3, δ, ε, π, φ).

To determine whether changes observed in mRNA result in changes in protein levels, our laboratory has used Western blotting to quantitatively, and immunocytochemistry to qualitatively, determine the alteration in protein content in various brain regions. For Western blotting, tissue was homogenized in a buffer and homogenates were run on SDS/polyacrylamide gels and then transferred to nitrocellulose. Blots were blocked with 5% milk, incubated with primary antibody (against protein of interest), and then incubated with secondary antibody conjugated with horseradish peroxidase. Protein bands were developed using Super Signal West Pico chemiluminescent substrate

kit (Pierce, Rockford, IL) and quantified using NIH Image software (National Institutes of Health, Bethesda, MD). To carryout immunocytochemistry, brains were cut into thin sections and washed 2–3 times in phosphate buffered saline (PBS) containing 1% Triton. Brain sections were then incubated with goat serum to block nonspecific binding of the primary antibody. The sections were incubated at 4 °C for 48 h with primary antibody (against GABAR subunits), and after washing with PBS sections were incubated with secondary antibody at room temperature. Sections were washed with PBS and covered with mounting medium. Intensity of immunofluorescence was quantified using various methods such as confocal microscopy.

Recent Results

To selectively study changes in GABAR subunit expression and function in DGCs, our laboratory (in collaboration with Coulter and colleagues) has used single-cell mRNA amplification technique combined with whole cell patch-clamp. We found that levels of α1 subunit mRNA decreased whereas levels of α4 subunit increased, beginning 24 h after SE and continuing as the animals became epileptic. The change in subunit expression correlated with a decreased sensitivity to zolpidem augmentation and increased sensitivity to zinc inhibition of GABAR responses. Similar functional and subunit expression changes have been observed in DGCs isolated from surgically resected hippocampus from patients with intractable TLE. The changes in GABAR subunit expression and function in DGCs of epileptic animals precede the development of epilepsy, suggesting that these changes contribute to the epileptogenesis process.

A recent study from our laboratory supports the idea that these GABAR subunit alterations may be critical contributors to epilepsy development. We designed a viral vector that contains the promoter for the α4 subunit gene of the GABAR; this promoter drives the expression of an α1 subunit transgene and enhanced yellow fluorescence protein reporter gene. The viral vector was directly injected into dentate gyrus before SE induction. Following SE, activity of the α4 promoter is upregulated, resulting in enhanced α1 expression in DG. Rats were continuously video-EEG monitored to determine the latency for the development of spontaneous seizures. We found that α1 gene transfer resulted in a threefold increase in mean seizure-free time after SE and a reduction in the number of rats developing epilepsy in the first 4 weeks after SE.

In contrast to adult rats where we found an decrease in α1 subunit in DG following SE, rats that experience pilocarpine-induced SE at P10 show an increase in GABR α1 expression in dentate gyrus and enhanced type I benzodiazepine augmentation of GABA currents. Also, unlike adult animals that uniformly develop spontaneous seizures following SE, none of the rats that had SE at P10 developed epilepsy later in life. Similarly, rats that had experienced SE at P20 have increased GABAR α1 expression in dentate gyrus, and only two-third of the rats develop epilepsy; in the subpopulation that does develop epilepsy, the frequency and severity of the seizures that develop are much less and the latency much longer, than in adults exposed to SE. These changes in GABAR α1 subunit levels in rats that had SE at P20 occurred selectively in the dentate gyrus, preceded the onset of epilepsy, and were not model specific. Also, there was no change in any other GABAR subunits in rats exposed to SE at P20.

Future Goals

Increased neuronal activity associated with induced seizures results in a variety of changes in GABAR-mediated neurotransmission, some of which may contribute to the later development of epilepsy. Going forward, it will be critical to determine which of these changes actually contribute to epileptogenesis and which are adaptive. Also, as the majority of studies have described changes that occur after the development of seizures, additional studies examining receptor changes during the latent period – i.e., prior to onset of epilepsy – are needed. Further, as one of the major goals of epilepsy research is to prevent the development of epilepsy after an initial precipitating event, it will be essential to establish which of the changes that occur during the latent period directly contribute to development of spontaneous seizures. Similarly, identification of the factors resulting in susceptibility or resistance to development of epilepsy following an event such as SE is critical. These studies will require models that generate groups of animals that do and do not develop spontaneous seizures after the insult. One choice for such studies will be developmental animal models of epilepsy, using juvenile animals (P15–P25) where only a subset of exposed animals become epileptic. For example, in the lithium–pilocarpine-induced SE model at P20, 70% of the animals go on to develop spontaneous seizures following a long latency. This model therefore provides a good opportunity to study and compare the receptor activity over time, between the animals that develop epilepsy and those that do not. Finally, in chronically epileptic animals, the effects of spontaneous seizures themselves are difficult to differentiate from those primarily responsible for epileptogenesis. Therefore, a key goal for the future will be to identify a biomarker that can predict which animal will develop epilepsy following an injury, so as to permit us to differentiate critical mediators of epileptogenesis from the secondary effects of spontaneous seizures in susceptible animals.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations.

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Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy

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Introduction

Epilepsy is a disease of complex etiology and multiple molecular mechanisms contributing to its development. The development of temporal lobe epilepsy (TLE) often occurs after a traumatic event to the brain, such as head injury or prolonged seizure activity (status epilepticus, SE). After this initial event, a latent period ensues before recurrent spontaneous seizures (epilepsy) develop. Changes in the expression of multiple genes, including those that encode ligand- and voltage-gated ion channels, have been implicated in the process of epileptogenesis. Several growth or transcription factors are known to be activated by SE, including (but not limited to) brain-derived neurotrophic factor (BDNF), cAMP response element binding protein (CREB), inducible cAMP early repressor (ICER), and early growth response factors (EGRs). Results of multiple studies suggest that these factors regulate gene expression in a way that is pertinent to seizure-induced changes in brain function. This article will introduce each of these signaling elements and provide an overview of their possible roles in gene regulatory pathways that are critical for, or associated with, the epileptic condition. Recent work elucidating specific mechanisms that regulate changes in subunits that assemble to form the major inhibitory neurotransmitter receptor in the brain, the type A γ-aminobutyric acid receptor (GABAR), will then be discussed in detail. Major alterations in the expression of certain GABAR

Oscillations; **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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Relevant Website

http: www.inmednet.com.

$GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization

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Introduction

Florey and MacLennan were the first to suggest that 'factor I' was an inhibitory neurotransmitter; after some debate, this compound was identified as the amino acid GABA. Half a century later, we know that GABA released from interneuron terminals activates pre- and postsynaptic GABA receptors in forebrain neurons. These receptors include ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors. $GABA_A$ receptors are receptor-operated channels with a large molecular diversity and a largely somatodendritic expression. Signals mediated metabotropically, by $GABA_B$ receptors, are slower and may be involved in opening or closing somatodendritic channels or in controlling transmitter release from excitatory and inhibitory terminals, via distinct G proteins.

A relation between GABA and seizures first emerged when convulsions were seen in infants fed with a formula that was accidentally deficient in pyridoxine (known also as vitamin B6). Pyridoxine is the coenzyme for the synthesis of GABA from glutamic acid via the enzyme glutamic acid decarboxylase. Moreover, shortly after its identification, GABA was found to prevent seizures, and several molecules that interfere with GABA synthesis and signaling were shown to induce convulsions. Work in the 1970s and early 1980s confirmed a function for GABA receptor-mediated mechanisms in controlling neuronal excitability and thus epileptiform synchronization. Later

studies, however, challenged this view, showing that (i) epileptiform discharges occur when inhibition is preserved and even enhanced, and (ii) $GABA_A$ receptor-mediated mechanisms are at least partially preserved in animal models of temporal lobe epilepsy. More complex, at times ambiguous, roles for GABAergic signaling in epilepsy have been identified. In particular, recent evidence suggests that mechanisms involving $GABA_A$ receptors may assist, support, or shape epileptiform synchrony in limbic structures.

Background – $GABA_A$ Receptor-Mediated Functions

$GABA_A$ receptor activation opens channels that are permeable to chloride and to a lesser extent to bicarbonate. With a reversal potential ($E_{GABA\text{-}A}$) usually negative to neuronal resting potential, GABA release induces a fast inhibitory postsynaptic potential (IPSP), hyperpolarizing postsynaptic cells and thus suppressing firing. The inhibitory effect is also sustained by an increased membrane conductance responsible for the IPSP 'shunting effect'; consequently, inhibition – that is, a reduction in the likelihood that neurons will discharge action potentials – can also be achieved even without prominent hyperpolarization. Recent evidence indicates that $GABA_A$ receptors also mediate tonic inhibition, mediated by activation of extrasynaptically localized receptors with a distinct subunit composition that endows these receptors with a particularly high affinity for GABA (Semyanov *et al.*, 2004). $GABA_A$ receptors are also expressed at presynaptic sites in some brain regions (including the spinal cord – but their presynaptic existence in the forebrain remains to be proven), and act there to control transmitter release.

Finally, GABA can act as an excitatory transmitter under certain conditions. $GABA_A$ receptor activation depolarizes neurons when the E_{GABA} is positive to resting membrane potential – which may occur due to changes in transmembrane gradients of chloride or bicarbonate. If associated conductance changes are small enough to preclude an inhibitory shunting effect, excitation may result. This 'aberrant' behavior of GABA occurs in immature tissue before an adult chloride homeostasis is established, and also in adult tissue when GABAergic systems are strongly or repetitively activated.

Methods

Findings reported in **Figures 1 and 2** were collected from rat brain slices that were maintained in vitro under standard procedures, and were superfused with a medium containing 4-aminopyridine (4AP) or zero magnesium. Data for **Figure 3** derive from temporal lobe tissue obtained after operations at the CHU Pitié-Salpêtrière, Paris on patients with pharmacoresistant epilepsies of the temporal lobe (tissues with hippocampal sclerosis). All patients gave their informed written consent. Intracellular records were obtained with sharp electrodes filled with 2 M potassium acetate, and extracellular records were made from sharpened tungsten electrodes. Recorded cells were identified by intracellular biocytin staining (biocytin included at 1.6% in the intracellular electrode solution), developed with the avidin–biotin reaction using Avidin-Texas Red. The transporter KCC2 was detected immunohistochemically using standard techniques and a polyclonal antibody made by the laboratory of John Payne. Results shown in **Figure 4** are from human cortical tissue that was obtained during surgery for focal cortical dyspalsia at the Montreal Neurological Institute and Hospital, Montreal, QC. All patients gave their informed written consent. Field potential and extracellular potassium-selective records were obtained using standard techniques (see Further Reading).

Results

$GABA_A$ Receptors and Epileptiform Synchronization in the Limbic System

Blocking $GABA_A$ receptor-mediated inhibition induces epileptiform synchrony in cortical regions. Early data supporting this evidence were obtained in vivo from penicillin application to the neocortex or hippocampus. Later, the use of hippocampal slices opened the way toward work on underlying mechanisms. The activity, induced in vitro by $GABA_A$-receptor antagonists, is similar to the interictal discharges recorded from epileptic patients, consisting of short duration (a few hundred milliseconds) population events. Simulation studies have suggested that strong but sparse recurrent excitatory synapses within a single cortical area can explain the genesis of interictal-like synchrony. Electrophysiological studies confirmed this idea, and showed that suppressing inhibition induces synchrony by permitting transmission in existing – but previously not functional – polysynaptic recurrent pathways. Parallel in vivo studies demonstrated that the appearance of epileptiform activity during electrical stimulation is accompanied by failure of GABA-receptor-mediated increases in membrane conductances, and that the transition between nonconvulsive absence seizures and tonic–clonic discharges is associated with breakdown of intracortical inhibition. Thus, exogenous perturbation of inhibition induces epileptiform activity.

In contrast to this interictal-like discharge, prolonged epileptiform synchronization similar to electrographic seizures is only seen during experimental procedures

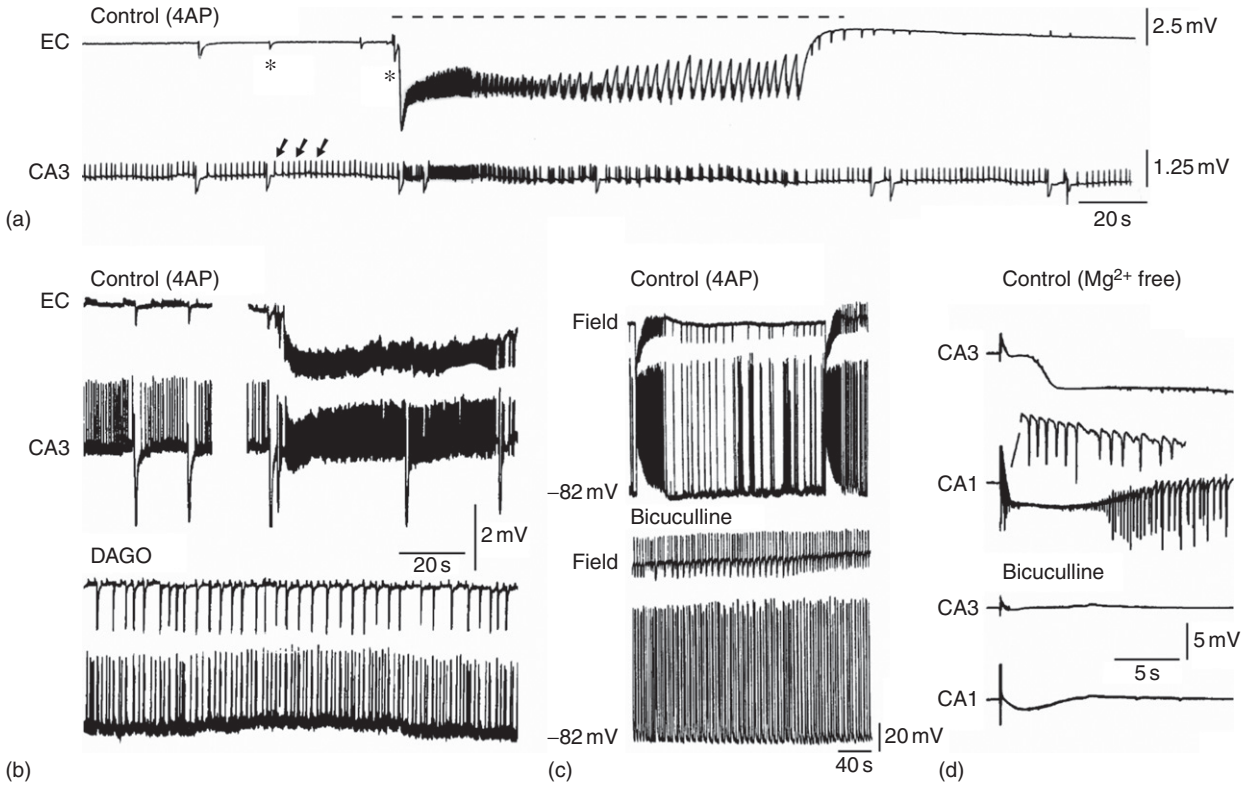


Figure 1 (a) Synchronous epileptiform activity recorded from a combined hippocampus-entorhinal cortex slice bathed with a medium containing 4AP. Note that 4AP induces the appearance of (i) fast interictal-like discharges at around 0.5 Hz (arrows), (ii) slow interictal events at 0.01–0.05 Hz (asterisks), and (iii) ictal discharge (interrupted line). (b) Effects induced by the μ-opioid receptor agonist (D-Ala-2-*N*-Me-Phe,Gly-ol)enkephalin (DAGO) on the synchronous activity recorded in the entorhinal cortex and CA3 area of the hippocampus during application of 4AP; note that DAGO blocks slow interictal events (which can be recognized by their negative-going polarity) and ictal discharge. (c, d) Effects similar to those seen with DAGO are reproduced by the $GABA_A$ receptor antagonist bicuculline on the ictal activity recorded from brain slices superfused with 4AP (c) or with Mg^{2+}-free medium (d), respectively. In (c), field potential and intracellular recordings were obtained from the entorhinal cortex, while field potential was recorded in (d) from the CA3 and CA1 areas of the hippocampus. Note that in the latter experiment the ictal response is preceded by oscillations (inset). Panels (a) and (b) are modified from Avoli M, Barbarosie M, Lucke A, Nagao T, Lopantsev V, and Köhling R (1996) Synchronous GABA-mediated potentials and epileptiform discharges in the rat limbic system in vitro. *Journal of Neuroscience* 16: 3912–3924; panel (c) is from Lopantsev V and Avoli M (1998) Participation of GABAA-mediated inhibition in ictal-like discharges in the rat entorhinal cortex. *Journal of Neurophysiology* 79: 352–360; panel (d) is from Köhling R, Vreugdenhil M, Bracci E, and Jefferys JG (2000) Ictal epileptiform activity is facilitated by hippocampal $GABA_A$ receptor-mediated oscillations. *Journal of Neuroscience* 20: 6820–6829.

that leave inhibition functional or even enhance it. These procedures include the application of 4AP or the removal of Mg^{2+} in acute slice preparations of the hippocampus and entorhinal cortex. Application of 4AP to hippocampal slices from young animals, or to slices including the entorhinal cortex in the adult, induces epileptiform interictal-like discharges presenting at 'fast' (~0.5 Hz, see **Figure 1(a)**, arrows) or rather slow events (0.01–0.05 Hz – see **Figure 1(a)**, asterisks), along with prolonged ictal-like discharges (see **Figure 1(a)**, thick line). While the fast interictal discharges are primarily mediated through excitatory synaptic transmission, both slow interictal-like and ictal, seizure-like events depend on GABAergic processes. In addition, these ictal-like discharges are immediately preceded (and thus presumably initiated) by slow potentials similar to the slow interictal events; they also show a reversal potential close to E_{Cl} and reflect bicarbonate-mediated $GABA_A$ receptor-dependent depolarizing discharge. Critically, ictal discharges – along with the slow interictal events – are blocked by preventing GABA release (e.g., by activating μ-opioid receptors (**Figure 1(b)**)) or by antagonists of the $GABA_A$ receptor (**Figure 1(c)**). These pharmacological maneuvers induce a pattern of robust interictal activity. As illustrated in **Figure 1(d)**, the ictal events that are recorded in hippocampal slices during removal of Mg^{2+} are also markedly reduced by application of the $GABA_A$ receptor antagonist bicuculline.

As illustrated in **Figure 2(a)**, the intracellular counterpart of ictal discharge onset during 4AP application is a long-lasting depolarization that resembles the depolarizations seen in isolation between ictal discharges (i.e., during interictal events) and becomes hyperpolarizing

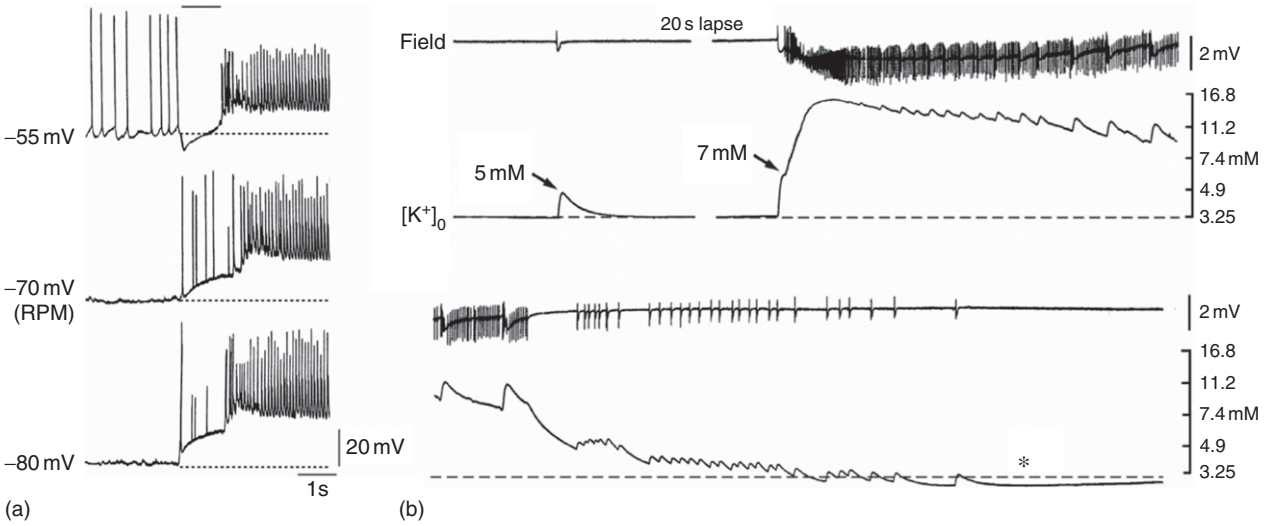


Figure 2 (a) Characteristics of the long-lasting depolarization occurring at ictal discharge onset in a neuron of the medial entorhinal cortex that was recorded with K-acetate-filled microelectrode. Note that when the neuronal membrane is depolarized by intracellular injection of steady positive current (−55 mV) the amplitude of the sustained ictal depolarization decreases while the initial long-lasting depolarization becomes hyperpolarizing as compared with the recording obtained at resting membrane potential (RMP, −70 mV). Note also that when the membrane is hyperpolarized by intracellular injection of steady negative current (−80 mV) both long-lasting depolarization and ictal depolarization increase in amplitude as compared with the sample obtained at RMP. (b) 4AP-induced synchronous activity recorded in the rat entorhinal cortex with simultaneous field potential and $[K^+]_o$ recordings. Note that the slow interictal event (arrow) is associated with a transient increase in $[K^+]_o$ up to around 5 mM while that preceding and apparently leading to ictal discharge initiation is accompanied by an increase of around 7 mM. Panels (a) and (b) are modified from Lopantsev V and Avoli M (1998) Participation of GABAA-mediated inhibition in ictal-like discharges in the rat entorhinal cortex. *Journal of Neurophysiology* 79: 352–360; Avoli M, Barbarosie M, Lucke A, Nagao T, Lopantsev V, and Köhling R (1996) Synchronous GABA-mediated potentials and epileptiform discharges in the rat limbic system in vitro. *Journal of Neuroscience* 16: 3912–3924.

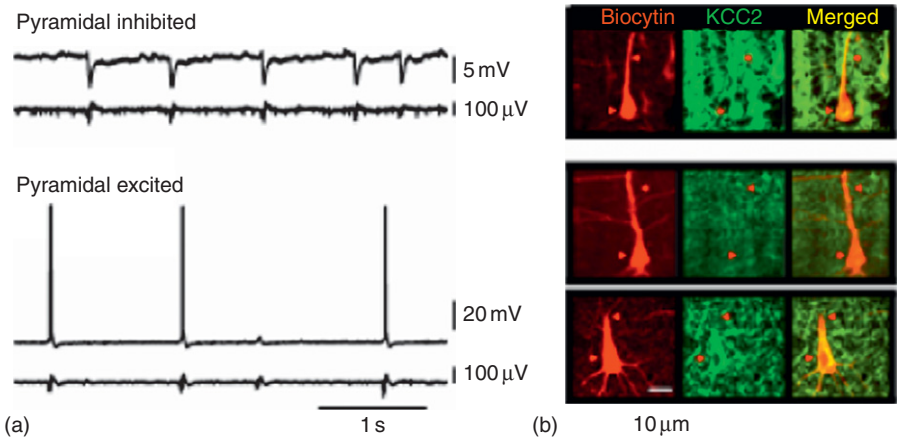


Figure 3 (a) Two different subicular pyramidal cell activities during spontaneous interictal bursts in slices from epileptic patients. Intracellular and extracellular records: In the upper traces the pyramidal cell is inhibited during interictal bursts. The lower traces show a pyramidal cell that is excited and often fires during interictal bursts. (b) Comparison of the expression of the K–Cl cotransporter, KCC2, and pyramidal cell activity during interictal bursts. Six out of six cells showing hyperpolarizing responses expressed KCC2. Four out of seven cells that fired during bursts did not express KCC2. KCC2 was detected in three out of seven cells that depolarized during interictal bursts. Recorded cells were identified by biocytin injection (red); KCC2 expression (green) was detected by immunohistochemistry; in the merged image biocytin and KCC2 expression appears yellow.

during steady injection of depolarizing current. Thus, under these experimental conditions, ictal depolarizations paradoxically originate from a GABAergic event. When GABA$_A$ receptors are activated at high frequency, internal Cl^- increases and a postsynaptic cell depolarization occurs due to the positive shift in reversal potential. Furthermore, the KCC2 transporter starts to operate in reverse, extruding K^+, which further depolarizes neighboring neurons. Application of exogenous GABA is known to increase external K^+ via GABA$_A$ receptor

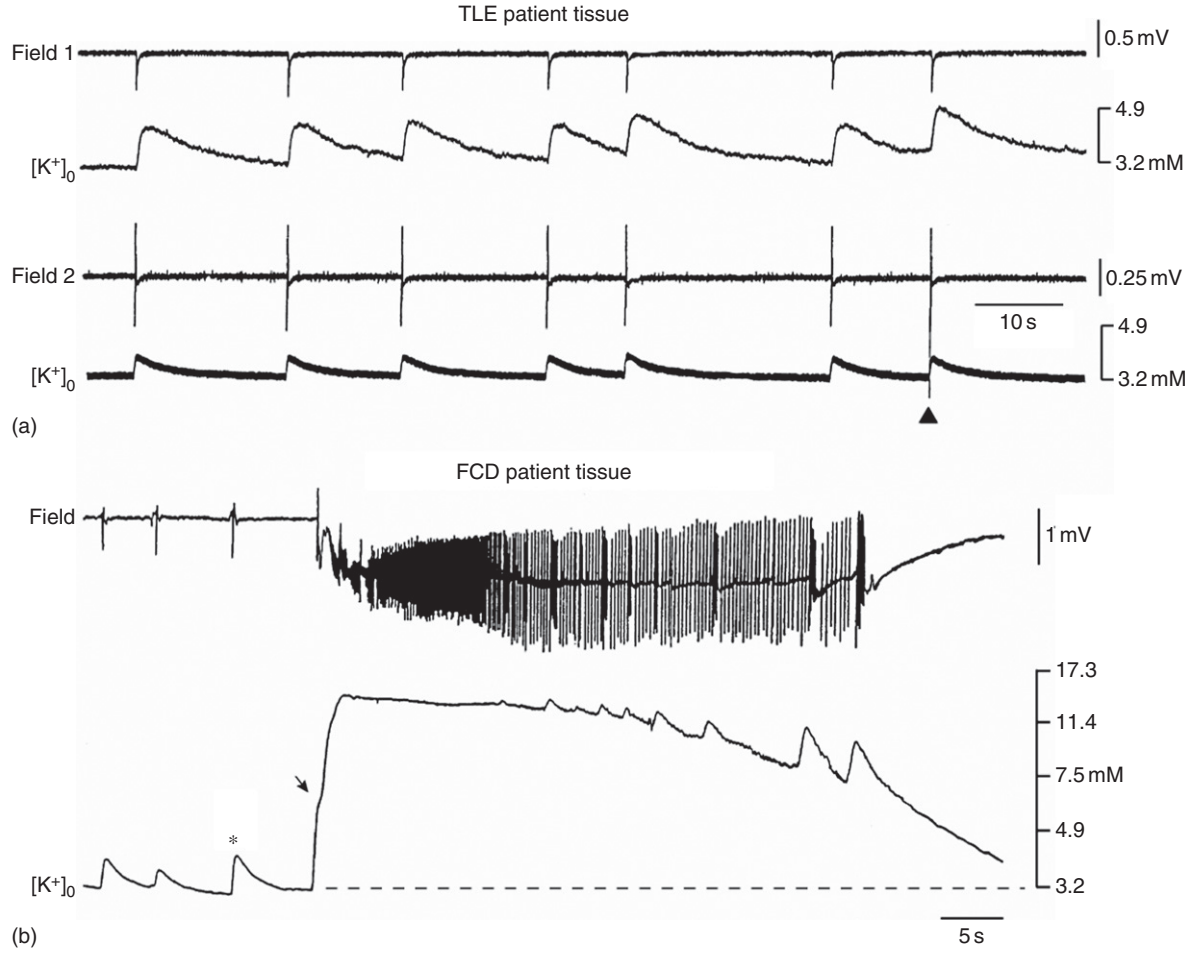


Figure 4 Synchronous activity induced by 4AP in neocortical slices obtained from TLE (i.e., presenting with no architectural anomaly) (a) and FCD patients (b). In both experiments field potential and $[K^+]_o$ recordings were obtained simultaneously at 1000–1500 μm from the pia. Note that only isolated field potential events can be recorded from the TLE neocortical tissue as well as that each of these events is associated with a transient increase in $[K^+]_o$ up to 4.4 mM. $[K^+]_o$ increases up to 4.5 mM accompanying the isolated negative field events (asterisk) but an elevation up to 6.4 mM characterizes the field potential leading to the ictal discharge onset (arrow). Panel (a) modified from Louvel J, Papatheodoropoulos C, Siniscalchi A, *et al*. (2001) GABA-Mediated synchronization in the human cortex: Elevations in extracellular potassium and presynaptic mechanisms. *Neuroscience* 105: 803–813; Panel (b) from D'Antuono M, Louvel J, Kohling R, *et al*. (2004) $GABA_A$ receptor-dependent synchronization leads to ictogenesis in the human dysplastic cortex. *Brain* 127: 1626–1640.

activation. An involvement of GABA-mediated K^+ efflux is supported by the increased external K^+, detected at the initiation of slow interictal events induced by 4AP in young rat hippocampus and rat or mouse adult entorhinal cortex (**Figure 2(b)**). The elevation in external K^+ during the slow event preceding an ictal discharge is consistently higher than that associated with a slow interictal event.

As already discussed, the interictal and ictal-type discharges induced by reducing external Mg^{2+} are similar to those initiated by exposing slices of hippocampus and entorhinal cortex to 4AP. They are also accompanied by depolarizing, bicarbonate-dependent GABA events. Depolarizing GABA-mediated events have been associated with gamma frequency oscillations (30–100 Hz). Inhibitory connections between GABAergic interneurons promote network oscillations at gamma frequencies, which may have a physiological role in learning and memory consolidation. Gamma oscillations may be synchronous over short and long distances. Long-range synchrony is presumably mediated via interneurons, which make long-range connections between hippocampal regions, or between hippocampus and other brain regions – particularly the entorhinal cortex. In the entorhinal cortex, gamma oscillations also appear to be mediated by direct excitation of inhibitory networks, although entorhinal gamma oscillations are blocked by antagonists of glutamate receptors while hippocampal oscillations are not. Both gamma oscillations and ictal events in the entorhinal cortex are blocked by $GABA_A$ receptor antagonists (**Figure 1(d)**).

Intriguingly, gamma frequency discharges may contribute to the emergence of an epileptic focus. In a model of an epileptic 'mirror focus' (based on two connected

hippocampi, one of which was treated with a convulsant), it was recently shown that independent mirror focus activity emerged in the untreated hippocampus only if it received activity in the gamma frequency range from the treated primary focus. More importantly, the emergent activity of the mirror focus was not dependent on glutamatergic signaling but was suppressed by $GABA_A$ receptor antagonists. The clinical observation that neocortical seizures are often initiated in zones that generate gamma oscillations supports the hypothesized role of gamma frequency oscillations as a marker for seizure initiation sites.

Involvement of $GABA_A$ Receptors in Animal Models of Temporal Lobe Epilepsy

A more direct approach to the role of GABA receptors in epileptic disorders is provided by animal models that mimic the spontaneous occurrence of epileptic seizures in temporal lobe epilepsies. As in patients, these models are characterized by Ammon's horn sclerosis together with synaptic reorganization, particularly in the dentate gyrus. While the ability of interneurons to release GABA is intact in most animal models of temporal lobe epilepsy, the function of GABA-mediated inhibition may be changed. Some studies have shown a reduction in $GABA_A$ receptor function in electrically kindled rats, while other studies suggest that a reemergence of ontogenetically immature GABA-receptor expression patterns may underlie epileptogenesis. Recent investigations using kainate and pilocarpine modes have described a reconfiguration of inhibitory circuits that produces an enhanced somatic inhibition, together with reduced GABAergic actions on the dendrites of CA1 pyramidal neurons. The increased somatic inhibition was ascribed to enhanced firing of somat-targeting interneurons, and the decreased dendritic inhibition to degeneration of interneurons producing dendritic input. Computational studies suggest that these differential changes in GABAergic circuits may contribute to the transition from interictal to fast ictal activity.

Changes in Basal Cl^- Homeostasis and Interictal-Like Activity

A different proepileptic alteration in GABAergic signaling has emerged from work on tissue obtained from surgery on patients with pharmacoresistant temporal lobe epilepsies. Several studies have shown that antagonists of $GABA_A$ receptors suppress synchronous population discharges generated by human epileptic tissue in vitro. This observation suggests that GABAergic signaling may contribute, together with glutamatergic transmission, to the generation of interictal-like synchrony. Cellular studies on human tissue slices revealed that the reversal potential for $GABA_A$-mediated synaptic events was depolarizing (with respect to resting potential) in a minority of pyramidal cells of the subiculum, the region in which the interictal synchrony was initiated. Such a depolarized reversal potential is suggestive of a basal change in Cl^- homeostasis. Recordings of IPSP reversal potential in pyramidal cells, together with immunostaining of the same cells, suggest that expression of KCC2 (a K^+–Cl^- cotransporter that contributes to the regulation of internal Cl^-) may be reduced in a subset of subicular pyramidal cells. In this cell group, GABA provides an excitatory drive, which combines with that transmitted via glutamatergic synapses to assure synchrony. In contrast, GABA effects on the majority of pyramidal cells is hyperpolarizing, thus limiting the duration of interictal discharges.

Depolarizing GABAergic responses in pyramidal cells of the epileptic subiculum, and the associated alterations in molecules controlling Cl^- homeostasis, 'recapitulate' events during early postnatal development in rodents. In rat, the Cl^- cotransporter KCC2 is expressed at about 10 days after birth, and until this time GABAergic signaling is depolarizing. Investigators have speculated that the partial denervation of subicular cells in epileptic temporal lobe, due to the sclerosis-induced loss of their presynaptic partners in the CA1 region, induces a return to an immature pattern of Cl^- homeostasis. That mechanisms initiated by glial cells might be involved is suggested by the observation that following damage to afferents in the spinal cord, BDNF liberated by microglia activates trkB receptors leading to a KCC2 downregulation and a depolarizing shift in GABAergic reversal potentials associated with pathological pain responses. Shorter term changes in KCC2 function might also be involved in the rapid kindling-like depolarizing shift of GABAergic responses, as seen in the establishment of a mirror focus in vitro.

$GABA_A$ Receptors and Epileptiform Synchronization in Human Focal Cortical Dysplasia

Taylor's type, focal cortical dysplasia (FCD) is characterized by a localized disruption of normal cortical lamination, with the presence of balloon cells and an excess of large aberrant neurons in the underlying white matter. Seizures in FCD patients are often medically intractable, which makes them candidates for neurosurgical interventions to resect the epileptogenic FCD area. During intraoperative electrocorticography, epileptic activity is often recorded from electrodes overlying the dysplastic cortical area. Immunocytochemical analysis of surgically resected human FCD tissue has revealed a decreased number of presumptive interneurons, but also suggests that the remaining interneurons provide an increased GABAergic innervation to principal cells. Slices of FCD tissue, exposed to 4AP, generate NMDA receptor-mediated ictal discharges as well as isolated, interictal-like synchronous potentials

largely dependant on $GABA_A$ receptor-mediated conductances. 4AP treatment of human neocortical tissue without obvious structural abnormalities induces only interictal-like potentials (**Figure 4**). These data support the view that epileptogenicity is a functional feature of FCD tissue. Furthermore, 4AP-induced activity in FCD slices resembles pre-excision ECoG records made from the dysplastic lesion, and appears to be a specific, sensitive indicator of FCD lesions.

As in the rodent limbic system, ictal-like activity in slices of human FCD tissue treated with 4AP is initiated (and presumably maintained) by mechanisms that depend on $GABA_A$ receptors. These ictal discharges are preceded by negative-going field events similar to those observed in isolation during the interictal period. However, the negative field potentials leading to ictal discharge onset are consistently of larger amplitude than those associated with interictal events, and are often followed by a secondary, slow negative field event from which ictal oscillations emerge. In FCD tissue slices, transient elevations in $[K^+]_o$ occur during slow 4AP-induced interictal events to levels consistently larger than those occurring during synchronous discharges recorded in non-FCD slices obtained from temporal lobe epileptic patients. Furthermore, the increases in $[K^+]_o$ associated with GABA receptor-mediated potentials preceding the onset of ictal discharges are larger than those associated with any field potentials observed during the interictal period. These findings from studies of FCD tissue are similar to those made in immature cortical slices, suggesting that the human dysplastic cortex may revert to – or rather retain – ontogenetically immature properties.

The role of GABA receptor-mediated synchronization in initiating ictal activity in FCD tissue is also supported by pharmacological manipulations that modify $GABA_A$ receptor function. $GABA_A$ receptor antagonism, or activation of μ-opioid receptors to block GABA release from interneuron terminals, suppresses both ictal discharges and GABA receptor-mediated interictal events in FCD slices, and recurrent interictal epileptiform activity reemerges. Conversely, ictal and interictal activity is enhanced when $GABA_A$ receptor function is potentiated with low doses of phenobarbital.

Conclusions and Directions for Future Research

While is there no doubt that blocking GABA receptor function leads to neuronal hyperexcitability and short-lasting epileptiform discharges, evidence obtained from several in vitro models of epileptiform discharge indicates that inhibition can also initiate and sustain ictal discharge depolarizations. It also appears that this mechanism rests on the ability of $GABA_A$ receptor-mediated conductances to depolarize, under specific circumstances, brain cells – thus causing sizeable elevations in $[K^+]_o$. The possibility that activation of $GABA_A$ receptors can initiate epileptic discharge goes beyond the experiments performed in normal animal tissue, as similar mechanisms have been identified in the subiculum of temporal lobe epilepsy patients as well as in human FCD tissue.

See also: **Absence Seizures**: Increased $GABA_A$ Receptor-Mediated Transmission in Typical Absence Seizures; Thalamocortical Synchronization and Absence Epilepsy; **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; GABA Excites Immature Neurons: Implications for the Epilepsies; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations; **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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GABA$_A$ Receptor Plasticity in Epileptogenesis

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Introduction

It is clear from studies carried out in various animal models and retrospective studies from humans that an initial precipitating event such as status epilepticus (SE) can increase the risk of later development of epilepsy. The process of epilepsy development (epileptogenesis) is complex and multifactorial. Many laboratories, including our own, have focused on understanding the role of γ-aminobutyric acid (GABA) receptor plasticity in epileptogenesis. GABA is the major inhibitory neurotransmitter in the mature brain, and various drugs such as diazepam and barbiturates that enhance GABAergic inhibition are commonly used as antiepileptic drugs. Also, drugs that block GABAergic inhibition can induce seizures in animals, further supporting the potential importance of alterations in GABAergic transmission in epileptogenesis. In this article we will describe the changes that have been observed in GABA$_A$ receptor (GABAR) subunit expression following prolonged seizures in various animal models of epilepsy, and the role these changes may play in epileptogenesis. Also, we will highlight the differences in GABAR subunit expression that occur following SE in adult and developing animals.

Background

GABA is the major inhibitory neurotransmitter in the adult brain. Three types of GABA receptors, GABA$_A$, GABA$_B$, and GABA$_C$, are found in the mature central nervous system. GABA$_A$ and GABA$_C$ are ionotropic receptors, whereas GABA$_B$ is a metabotropic receptor. Most fast synaptic inhibition in the mature brain is mediated by GABARs, whereas slow inhibition is mediated by GABA$_B$ receptors. GABARs are composed of multiple subunits subtypes (α1–6, β1–3, γ1–3, δ, ε, π, θ, and ρ1–3) that form a pentameric anion-selective channel. The most common in vivo subunit composition is two α, two β, and one γ subunit. There is remarkable receptor heterogeneity, with subtype combinations varying in different brain regions, cell types, and during different times in ontogeny. Different subunit subtypes confer distinct functional and pharmacological properties to the receptors.

Several laboratories have studied the changes in GABAR subunit composition in human temporal lobe epilepsy (TLE) and in animal models of TLE. Houser and colleagues have shown that in the CA1 and CA2 areas of the hippocampus there are long-lasting reductions in the α5 subunit of GABARs in pilocarpine-treated animals that develop spontaneous seizures. The α5 subunit is primarily extrasynaptic and is thought to be involved in tonic inhibition in the CA1 area. Lower levels of α5 subunit may thus result in reduction of tonic inhibition. In the molecular layer of denate gyrus, levels of α1, α3, α4, α5, and γ2 increase and levels of δ decrease chronically following SE. In interneurons of dentate gyrus, however, α1 subunit levels decrease and δ subunit levels increase, suggesting that there are cell-specific changes within the same region of the hippocampus. Additional studies from the Houser group suggest that changes in GABA receptor function after SE may depend not only on the levels of different subunits within individual neuronal cell types, but also on the subcellular localization of different GABAR subunits within these cells. Using immunogold labeling, these investigators showed that the subcellular location of γ2 and δ subunits in molecular layer of dentate gyrus was changed in pilocarpine-treated epileptic mice; they found a decrease in δ and an increase in γ2 subunits at perisynaptic locations, and a decrease in γ2 labeling near the center of the synaptic contacts. The change in the location of these subunits correlated with an insensitivity of the tonic current to neurosteroids and reduced phasic inhibition of DGCs in pilocarpine-treated epileptic mice. This impairment in inhibition could play an important role in development of epilepsy in this mouse model of TLE.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations.

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Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy

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Introduction

Epilepsy is a disease of complex etiology and multiple molecular mechanisms contributing to its development. The development of temporal lobe epilepsy (TLE) often occurs after a traumatic event to the brain, such as head injury or prolonged seizure activity (status epilepticus, SE). After this initial event, a latent period ensues before recurrent spontaneous seizures (epilepsy) develop. Changes in the expression of multiple genes, including those that encode ligand- and voltage-gated ion channels, have been implicated in the process of epileptogenesis. Several growth or transcription factors are known to be activated by SE, including (but not limited to) brain-derived neurotrophic factor (BDNF), cAMP response element binding protein (CREB), inducible cAMP early repressor (ICER), and early growth response factors (EGRs). Results of multiple studies suggest that these factors regulate gene expression in a way that is pertinent to seizure-induced changes in brain function. This article will introduce each of these signaling elements and provide an overview of their possible roles in gene regulatory pathways that are critical for, or associated with, the epileptic condition. Recent work elucidating specific mechanisms that regulate changes in subunits that assemble to form the major inhibitory neurotransmitter receptor in the brain, the type A γ-aminobutyric acid receptor (GABAR), will then be discussed in detail. Major alterations in the expression of certain GABAR

subunits are thought to contribute to epilepsy after SE in human TLE patients and in animal models of TLE. Specifically, α1 GABAR subunit mRNA levels decrease and α4 GABAR subunit mRNA levels increase by 24 h after SE, as measured in single dentate granule neurons of the rat hippocampus. These subunit changes are accompanied by a reduction in α1- and an increase in α4-containing synaptic (γ2-containing) GABARs in the dentate gyrus (DG) of the hippocampus. This change in receptor composition may result in impaired inhibition, making the brain more vulnerable to hyperexcitability.

Background

Signaling Proteins Involved in $GABA_A$ Receptor Subunit Regulation

Brain-derived neurotrophic factor

BDNF is one member of the mammalian neurotrophins (NTs). The classical view of NT function includes its effects on the growth and survival of neurons during development through the activation of Trk receptors. Recent studies suggest that the presence of NTs in the adult brain has a striking influence on neuronal excitability. Expression levels of BDNF and TrkB increase in kindling kainic acid (KA) and pilocarpine models of TLE. BDNF mRNA and protein levels are elevated in temporal lobe tissue from human epileptic brains.

Evidence that BDNF upregulation is associated with seizure activity suggests that BDNF may play a role in epileptogenesis. Lindvall and colleagues have shown that $BDNF^{+/-}$ mice show a marked reduction in the rate of kindling development and Scharfmann and colleagues have demonstrated that transgenic mice overexpressing BDNF have more severe seizures in response to KA. McNamara's group showed that intracerebroventricular administration of TrkB-Fc receptor bodies, to sequester endogenous BDNF, inhibits development of kindling, and that kindling development is impaired in conditional TrkB null mice. While evidence clearly points to a role of the TrkB receptor and its ligand (BDNF) in seizure susceptibility, the cellular and molecular mechanisms of BDNF/TrkB promotion of epileptogenesis remain to be fully elucidated. BDNF has been shown to both enhance excitatory synaptic transmission and reduce inhibitory transmission. The mechanisms underlying excitatory synaptic potentiation by BDNF is currently unclear, but could involve the facilitation of neurotransmitter release, phosphorylation of NMDA receptors, and direct effects on ion channels and/or conductances.

Enhanced excitatory neurotransmission by BDNF might also arise indirectly through alteration of the structure and function of GABAergic neurons. Several mechanisms have also been suggested regarding BDNF-induced modulation of inhibitory transmission, which include phosphorylation of the GABAR, alteration of GABAR trafficking, and regulation of subunit gene expression. For details, see suggested readings and discussion below.

Early growth response factors

A growing number of studies demonstrate an association between induction of immediate early genes (IEGs), such as Egrs, and seizure activity. Egrs are a family of four proteins (Egr1, 2, 3, and 4) that share nearly identical zinc finger DNA binding domains and bind to a common Egr response element consensus sequence (EREs), GCG T/GGG GCG. However, studies in Egr1-4-deficient animals illustrate quite divergent physiological roles for each protein. Baraban and colleagues have shown that there is sequential induction of Egr1 and Egr3 transcription factors after electroconvulsive shock (ECS) and gel shift assays demonstrate that Egr1 and Egr3 DNA binding activities follow the same pattern. These findings indicate that Egr1 and Egr3 act in concert to mediate early and late phases, respectively, of the transcriptional response regulated by their cognate response element and may play different regulatory roles.

Our laboratories have also shown induction of Egr family transcription factors after SE, with protein levels of Egr3 increasing in the DG of the hippocampus 24 h after pilocarpine-induced SE. The role that changes in Egrs plays in the process of epileptogenesis remains to be determined. One hypothesis is that Egrs may be critical for differential regulation of ligand-gated ion channel receptors that occurs in TLE patients and in several animal models of TLE. Egr1 has been shown to increase promoter activity and expression levels for several N-methyl-D-aspartate receptor subunits. Egr transcription factors may also play an important role in the regulation of different subunits of GABARs as discussed later.

CREB/ICER

The CREB is another key transcription factor that may mediate observed gene expression changes in epilepsy. CREB is a stimulus-induced bZIP transcription factor that is activated by phosphorylation at its Ser 133 site. Phosphorylated CREB (pCREB) dimerizes and binds to cAMP response element (CRE) motifs on promoters that contain the consensus sequence TGACGTCA. Along with its anchoring binding partner, CREB binding protein (CBP), pCREB upregulates transcription of target genes. Transcriptional regulation through CREB has been implicated in mechanisms of cell survival, plasticity, and learning and memory paradigms. Target genes of pCREB include CREB family members, which consist of cAMP response element modulator (CREM), ICER, and activating transcription factor (ATF). These para- and homologues of CREB also bind CRE elements to

modulate the transcription of particular genes. The CREM gene produces many spliced isoforms that can act as transcriptional repressors or activators. One of these repressor forms is ICER, a group of four proteins made from an internal promoter of the CREM gene. ICER can heterodimerize with CREB and directly block CREB-induced transcription.

Several studies using adult animal models of epilepsy suggest that seizures upregulate CREB or CREM/ICER activity for varying durations. For example, PTZ-induced seizures in adults increase pCREB levels transiently, and electroconvulsive seizures have been found to cause rapid increases of CREM and ICER mRNA expression. Our laboratory, using the pilocarpine model of SE, has found more sustained increases in both pCREB and ICER in the DG of the hippocampus, continuing 1–48 h after SE. This study demonstrated that these increases in pCREB and ICER may mediate the α1 GABAR subunit decreases that occur after SE in the DG, as detailed later. Seizures not only influence CREB phosphorylation, but also influence the association of CREB with CRE sequences on promoters of target genes, a necessary component of CREB transcriptional control. Taken together, these results suggest that increases in the levels or activation state of CREB and its family members, seen after multiple types of seizure induction, may be important mechanisms responsible for the changes in gene expression that occur during epileptogenesis.

Methods

Animal models of epilepsy have been used to study changes in GABARs that occur during the development of spontaneous seizures. In adult rats, the muscarinic agonist pilocarpine induces prolonged seizure activity (SE) and after a latent period of 5–14 days, spontaneous seizures occur in the absence of any provocation. As also occurs in human TLE, GABAR subunit composition is altered in individual DGNs in epileptic rats in this model. We examine tissue from the DG of animals sacrificed during the latent period, and use RT-PCR to examine mRNA levels and Western blotting to examine protein levels of various transcription factors. To better determine if certain transcription factors are regulating GABAR subunit genes, we use chromatin immunoprecipitation (ChIP) to examine if binding of the transcription factor to the subunit gene promoters is increased after SE. To perform ChIP, animals are first perfused with phosphatase inhibitors in saline, then 4% paraformaldehyde, in order to cross-link protein to associated sites on DNA. The DNA–protein complexes are then precipitated with antibodies to the transcription factors of interest: for example, CREB, pCREB, or early growth response factors (EGRs). Once the associated proteins are precipitated with antibodies, the DNA for the α1 CRE or other promoter binding site of interest is detected and quantified with RT-PCR. In our recent studies, we also wanted to detect ICER binding at the α1 CRE site; however, all antibodies to ICER also recognize CREM variants, and so the two transcription factors can only be distinguished by size. We therefore developed a DNA pull-down assay to purify nuclear proteins in DG tissue that specifically recognize the GABRA1:CRE – identified by size using gel electrophoresis. Oligonucleotide duplexes corresponding to GABRA1:CRE and its flanking region are covalently linked to a biotin moiety at their 5′ends, and oligos and their nuclear binding proteins are recovered using magnetic Streptavidin Dynabeads.

Recent Results

Transcriptional Regulation of $GABA_A$ Receptor Subunits

$GABA_A$ receptor α1 subunit gene (GABRA1)

Although many mechanisms of gene regulation remain to be characterized, recent work in our laboratories has established CREB and ICER as critical mediators of GABAR α1 subunit mRNA decreases that occur after SE in the DG. In adult animal models and human TLE, GABAR α1 mRNA levels decrease in DG 24 h to 1 week after SE. Viral delivery of α1 into DG protects rats from development of spontaneous seizures following pilocarpine-induced SE, suggesting that α1 down-regulation is potentially a critical determinant of epileptogenesis. Decreased levels of α1 are accompanied by a reduction in the number of α1-containing synaptic GABARs (as determined by the presence of γ2) and an increase in α4-containing synaptic GABARs. The human α1 promoter contains a functional CRE, making CREB and its family members potential regulators of α1 gene regulation in epilepsy. A study using microarrays to detect changes in multiple mRNAs in response to overexpressed dominant-negative CREB (MCREB) in vivo first suggested that GABRA1 might be regulated by CREB, directly or indirectly. However, it remained unknown whether the CREB system was involved in GABRA1 changes associated with epilepsy. In accordance with previous findings, we found increases in pCREB protein and ICER mRNA and protein in the DG at 1–48 h after SE. Using ChIP and DNA pull-down studies, increased pCREB and ICER binding at the CRE site of GABRA1 were also observed after SE. Further, in vitro studies manipulating CREB and ICER levels showed that overexpression of CREB and ICER together decreases GABRA1 promoter activity in primary hippocampal neurons. In contrast to adults, neonatal rats show increases in α1 levels after SE, and display no changes in pCREB and ICER levels compared with those in controls. These findings suggest that CREB

and ICER are important regulators of seizure-induced GABRA1 changes.

In addition, BDNF has been associated with changes in both α1 and α4 subunit levels associated with epilepsy (discussed later). BDNF treatment of primary hippocampal neurons in culture resulted in similar changes in α subunit levels as observed after SE. After 24 h of BDNF treatment, α1 levels decrease 42% and α4 levels increase 57%. Given the well-documented increases in BDNF that occur after SE, and that CREB has long been associated with BDNF regulation, both of these signaling molecules are likely to be involved in the alteration of GABAR function that occurs in TLE.

$GABA_A$ receptor α4 subunit gene (GABRA4)

GABARs that contain α4 subunits have unique pharmacologic properties such as insensitivity to benzodiazepines and increased sensitivity to zinc blockade. Receptors containing α4 subunits are most often found with the δ rather than γ subunit, in combination with αβ. These α4βδ GABARs are localized to extrasynaptic sites and contribute to tonic inhibition. A minor population of α4βγ2 GABARs are found within DG synapses where they are proposed to affect both the rise time and decay of synaptic currents.

Pharmacological changes in GABAR function in DGCs, including decreased augmentation by type 1 benzodiazepine agonists and increased inhibition by zinc, are observed in isolated DGCs in tissue from both animal models and human TLE. These changes are associated with a marked increase in α4 and decrease in α1 subunit expression. Our studies have shown that the alteration in α4 levels is transcriptionally mediated, and have identified Egr3 as a specific regulator that is responsible for the upregulation of α4 subunit expression. BDNF is believed to be the endogenous signal that induces Egr3 synthesis via a PKC/MAPK-dependent pathway.

These findings are in agreement with the work of Houser and colleagues who observed increases in the levels of α4 and γ2 subunits and decreases in the levels of δ subunits after pilocarpine-induced SE in mice. Taken together, these changes in subunit gene expression may result in a number of functional effects. The loss of δ subunits may lead to a drop in the overall number of extrasynaptic GABARs, decreasing the overall levels of tonic inhibition and lowering the excitatory threshold for affected neurons. In addition, changes in the levels of α4 and α1 subunits suggest that the effect of these changes may extend beyond tonic inhibition and also affect synaptic and/or perisynaptic GABA signaling. Direct evidence for this possibility comes from recent studies in which the decrease in expression of the α1 subunit and increase in expression of the α4 subunit are associated with less α1γ2-containing and more α4γ2-containing $GABA_A$ receptors ($GABA_AR$) in DG after SE. Consistent with these observations, Houser and colleagues reported that there is an increase in the co-localization of α4 and γ2 subunits in DG neurons after SE. The change in receptor subtype from α1βγ2 to α4βγ2 may contribute to epileptogenesis, as α4 containing GABARs have been shown to desensitize rapidly, especially when assembled with β3 subunits. In addition, sensitivity of GABARs to zinc blockade is influenced by the type of α subunit in the receptor complex. GABARs containing the α4 subunit are very sensitive to zinc blockade, as is seen in DGCs of epileptic brain. In epileptic animals, DGCs not only are exposed to a greater amount of zinc as a result of mossy fiber (MF) sprouting accompanying the development of hippocampal sclerosis, but they also undergo a change in GABAR subunit composition that generates a receptor exquisitely sensitive to zinc blockade. Such a change in the sensitivity of GABARs to the zinc released from MF has been hypothesized to contribute to epilepsy development. Whether or not MF sprouting is a necessary prerequisite for TLE, however, is still under intense investigation.

Future Goals

The full range of gene expression changes that are involved in epileptogenesis and the molecular mechanisms that underlie them are just beginning to be characterized. While the transcription factors discussed in this chapter likely compose only a part of the cascade of signaling events that are initiated by seizures, it is clear that BDNF, Egr3, CREB, and ICER play important roles. Recent work characterizing their functions in GABAR α1 and α4 subunit changes in the DG after SE provides an important lead for the future development of molecular therapies. However, the upstream signalers of these pathways, and the exact means through which they confer vulnerability to epilepsy, remain to be further elucidated.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor Plasticity in Epileptogenesis; Molecular Pathology of Genetic Epilepsy Associated with $GABA_A$ Receptor Subunit Gene Mutations; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Molecular Pathology of Genetic Epilepsy Associated with $GABA_A$ Receptor Subunit Gene Mutations

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Introduction

Idiopathic generalized epilepsies (IGEs) affect about 1% of the population worldwide and are among the most common neurological disorders. IGEs are a group of presumably genetic epilepsies that encompass several different clinical phenotypes, including childhood absence epilepsy (CAE), juvenile myoclonic epilepsy (JME), pure febrile seizures (FS), generalized epilepsy with febrile seizures plus (GEFS+), and generalized epilepsy with tonic-clonic seizures. In neuronal networks, either a 'gain of function' of receptor channels mediating excitatory neurotransmission or a 'loss of function' of receptor channels mediating inhibitory neurotransmission could impair the subtle balance of excitation and inhibition in the brain, thus, leading to disinhibition and seizures.

Genetic advances and functional characterizations of the pathophysiological alterations in receptor channel trafficking and function are changing our understanding of IGEs. It is becoming increasingly clear that genetic mutation of transmembrane ion channels is the underlying cause of many forms of human IGEs, both in large pedigrees and in sporadic cases or de novo mutations. The original conceptualization of epilepsy as merely a loss of channel function or an imbalance between excitation and inhibition is probably oversimplified. The imbalance resulting from channel dysfunction is not sufficient to delineate the pathogenesis of all epilepsy syndromes in which the underlying channel abnormalities have been verified. In this article, we will review the molecular pathology of IGEs caused by $GABA_A$ receptor mutations based on the recent studies.

Background: Mutations in $GABA_A$ Receptor Subunit Genes

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Genetic advances and functional characterizations of the pathophysiological alterations in receptor channel trafficking and function are changing our understanding of IGEs. It is becoming increasingly clear that genetic mutation of transmembrane ion channels is the underlying cause of many forms of human IGEs, both in large pedigrees and in sporadic cases or de novo mutations. The original conceptualization of epilepsy as merely a loss of channel function or an imbalance between excitation and inhibition is probably oversimplified. The imbalance resulting from channel dysfunction is not sufficient to delineate the pathogenesis of all epilepsy syndromes in which the underlying channel abnormalities have been verified. In this article, we will review the molecular pathology of IGEs caused by $GABA_A$ receptor mutations based on the recent studies.

Background: Mutations in $GABA_A$ Receptor Subunit Genes

$GABA_A$ receptors are the primary mediators of fast inhibitory synaptic transmission in the central nervous system, and are formed by pentameric assemblies of multiple

subunit subtypes (α1–α6, β1–β3, γ1–γ3, δ, ε, π, θ, and ρ1–ρ3) that form chloride ion channels. The most common $GABA_A$ receptors contain two α subunits, two β subunits, and a γ- or δ subunit. $GABA_A$ receptors mediate both phasic, inhibitory synaptic transmission and tonic, perisynaptic tonic inhibition, and several antiepileptic drugs work through enhancement of $GABA_A$ receptor currents.

Mutations or variants associated with IGEs have been reported in $GABA_A$ receptor α1, β3, γ2, and δ subunits (Figure 1). These mutations include missense mutations (GABRA1(A322D), GABRB3(P11S), GABRB3(S15F), GABRB3(G32R), GABRG2(R43Q), GABRG2(R139G), GABRG2(K289M)), as well as nonsense mutations (GABRG2(Q1X), GABRG2(Q351X)), splice donor site mutations (GABRG2(IVS6 + 2T → G)), or frameshift mutations (GABRA1(975delC, S326fs328X) that generate premature termination codons (PTCs). There are also variants of GABR genes that have been identified as seizure susceptibility genes (GABRD(E177A) and GABRD (R220H)). The mutations and variants are located in both exons and introns of the affected subunit genes, and in signal peptides or mature peptides of the affected subunit proteins. The missense mutations have been shown to impair receptor trafficking of mutant receptors to the cell surface and/or to alter receptor channel kinetics. Impaired trafficking of receptors harboring missense mutations has been shown to be due to posttranslational modifications of subunits, such as impaired folding, impaired assembly, glycosylation arrest, endoplasmic reticulum (ER) retention, or accelerated degradation of the mutant proteins by ER-associated degradation (ERAD). The PTC-generating mutations could trigger degradation of mRNA through nonsense-mediated decay (NMD) due to cellular mRNA quality control, thus eliminating production of the mutant protein.

Mutations in $GABA_A$ Receptor α1 Subunits

$GABA_A$ receptor α1 subunit mutation (A322D)

The α1 subunit mutation (A322D), associated with autosomal dominant JME, introduces a negatively charged aspartate into the middle of the M3 transmembrane helix of the α1 subunit at residue A322. It could be predicted that such a nonconserved mutation in a transmembrane domain would destabilize helix formation and alter α1 subunit folding and pentamer assembly. It has been shown that when coexpressed with wild-type β2 and γ2 subunits, the mutant α1(A322D) subunit reduced both total and surface α1 subunit levels, and had an intermediate effect with 'heterozygous' subunit expression (α1:α1 (A322D – 1:1 cDNA ratio). Peak GABA-evoked currents were significantly reduced in both 'heterozygous' and 'homozygous conditions.' The loss of the mutant protein

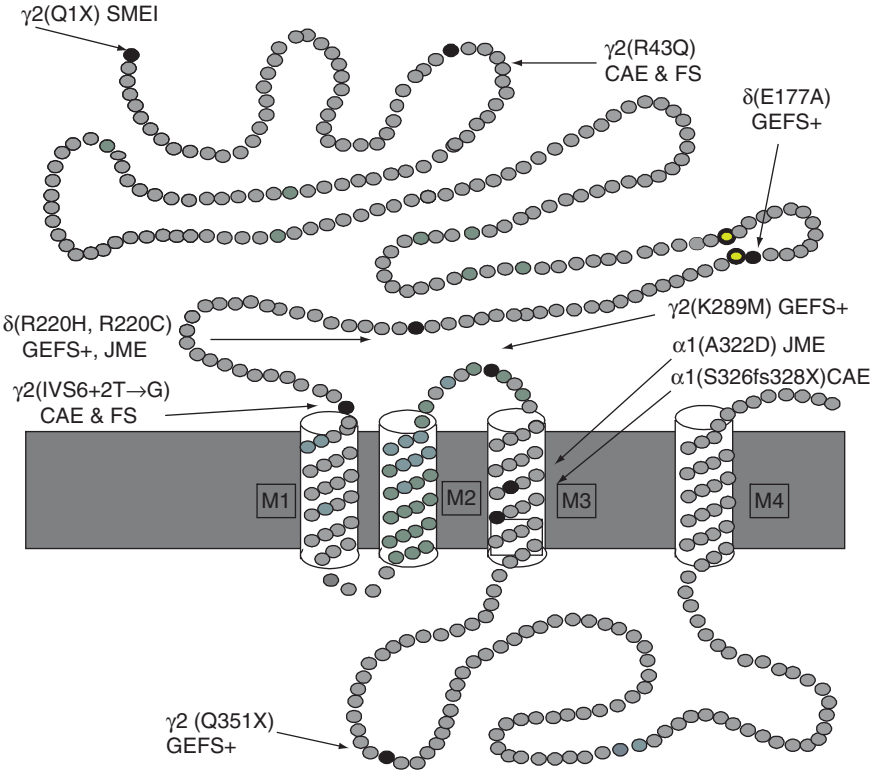


Figure 1 Schematic representation of the $GABA_A$ receptor subunit topology, showing the location of autosomal dominant epilepsy mutations associated with different IGEs.

was due to an ER quality control process, ERAD. A recent study also demonstrated that this mutant protein was subject to rapid degradation through the ubiquitin–proteosome system (Figure 4).

Mutations in GABA$_A$ Receptor β3 Subunits

GABA$_A$ receptor β3 subunit mutations, P11S, S15F, and G32R

Two mutations in β3 signal peptide (exon 1a; P11S, S15F), and a mutation in β3 (exon 2) (G32R) have been associated with CAE. The underlying molecular defect could be abnormal protein expression and/or impaired receptor trafficking, but the mechanism is still under study. Early reports have revealed that heterozygous and homozygous null mutant GABRB3 mice show absence-like features, including the EEG characteristics and pharmacological responses to antiepileptic drugs that are typical for absence seizures. GABRB3 deletion has also been associated with Angelman syndrome in which CAE is present. In addition, a GABRB3 promoter haplotype impairing transcriptional activity has also been associated with CAE. Thus, mutation of the GABRB3 subunit likely contributes to generation of CAE, but this involvement needs to be clarified further.

Missense Mutations in GABA$_A$ Receptor γ2 Subunits

GABA$_A$ receptor γ2 subunit missense mutation, R43Q

The γ2 subunit missense mutation, R43Q, was reported in affected individuals of a large family having both CAE and FS (Figure 1). The functional and cellular consequences of this mutation have been studied extensively from oocyte preparations and mammalian cells to knock-in animals. The data, both in vitro and in vivo, consistently showed reduction of surface γ2 subunit protein with heterozygous expression. We demonstrated that heterozygous expression of mutant α1β2γ2/γ2(R43Q) receptors in HEK 293–T cells resulted in reduced peak current. In vivo in γ2(R43Q) knock-in mice, a small but significant reduction of GABA$_A$ receptors in cortical pyramidal neurons was also observed. Using fusion proteins, in which DNA encoded enhanced yellow fluorescent protein (EYFP) was inserted between amino acids four and five of the mature γ2 subunit (γ2-YFP), we demonstrated (using fluorescence microscopy) that the basis for the reduced surface expression of the mutant protein was ER retention of the mutant protein (Figure 2). Thus, the reduced surface expression of mutant α1β2γ2S (R43Q) receptors was a consequence of receptor ER retention because of impaired protein assembly, folding, and/or trafficking.

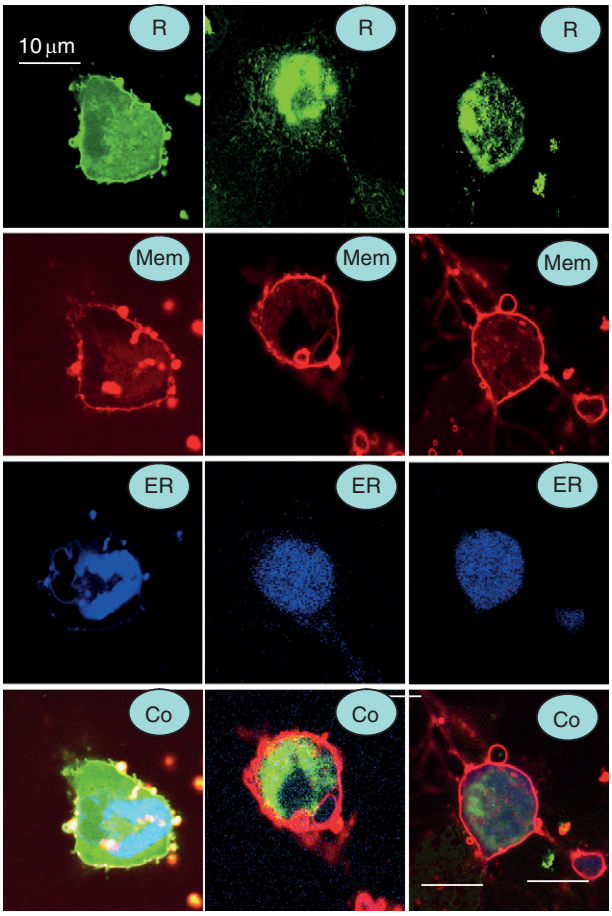


Figure 2 Heterozygous and homozygous α1β2γ2S(R43Q) receptors were retained in the ER. Human α1β2γ2S-YFP wild-type (wt) or α1β2γ2S(R43Q)-YFP heterozygous (het) or homozygous (hom) receptors in COS-7 cells were coexpressed with enhanced CFP-tagged ER marker and labeled with FM4–64 membrane marker. R stands for receptor, Mem for membrane, ER for endoplasmic reticulum and Co for colocalized image. Wt α1β2γ2S-YFP receptors were primarily in the cell membrane. Het α1β2γ2S(R43Q)-YFP receptors were found in both the membrane and intracellular compartments. Hom α1β2γ2S(R43Q)-YFP receptors were primarily found in intracellular compartments with minimal localization on the cell surface. Both het and hom receptors had a fluorescence pattern that was similar in distribution to that of the ER fluorescence pattern. (Modified from Kang J and Macdonald RL (2004) The GABAA receptor gamma2 subunit R43Q mutation linked to childhood absence epilepsy and febrile seizures causes retention of alpha1beta2gamma2S receptors in the endoplasmic reticulum. *The Journal of Neuroscience* 24: 8672–8677).

GABA$_A$ receptor γ2 subunit missense mutation, K289M

A family with an autosomal dominant generalized epilepsy similar to GEFS+ was shown to have a mutation in the GABA$_A$ receptor γ2 subunit, K289M, that is located in the short extracellular loop between transmembrane domains M2 and M3 (M2–M3 loop; Figure 1), a region

implicated in the gating of ligand-gated ion channels. We investigated the effects of this mutation using a rapid application, concentration jump technique (open tip application rise time 1 ms) to apply GABA for brief (2–5 ms) durations and the excised outside-out patch clamp recording technique. We demonstrated that α1β3γ2L(K289M) receptors had faster current deactivation. Single channel currents from homozygous α1β3γ2L (K289M) receptors had mean open times that were one fourth as long as wild-type α1β3γ2L receptor currents, consistent with its more rapid whole cell current deactivation time (Figure 3). Briefly, rapid GABA applications to excised macropatches evoked currents that were similar to inhibitory post synaptic currents (IPSCs). Therefore, reduction of the duration of rapid GABA-evoked current by the γ2L(K289M) subunit mutation suggests that this mutation would result in reduced IPSC duration, thus producing disinhibition that may lead to epilepsy.

$GABA_A$ receptor γ2 subunit missense mutation, R139G

The $GABA_A$ receptor γ2(R139G) subunit mutation was identified in a small FS only pedigree. The R139 residue is conserved among γ2 subunits across species, and basic residues are conserved among other γ subunits. Within the cys-loop family, polar, and charged amino acid residues occur at this position. Residue R139 is located at a highly conserved and likely aqueous accessible part of the amino terminus in a hairpin loop between β sheets 5 and 6. We demonstrated that the mutant α1β3γ2L (R139G) receptors had subtly altered current kinetics and reduced benzodiazepine sensitivity. Future study of the mutant protein maturation and receptor trafficking will further elucidate the molecular defect of this mutation in epilepsy.

Nonsense and Splice Donor Site Mutations in $GABA_A$ Receptor γ2 Subunits

$GABA_A$ receptor γ2 subunit nonsense mutation, Q351X

A γ2 subunit mutation, Q351X, is associated with a family with GEFS+, including two family members with FS and a member with Dravet syndrome. The Q351X mutation is localized in the γ2 subunit intracellular loop between transmembrane domains M3 and M4 (Figure 1) and results in a premature translation-termination codon (PTC) with loss of the downstream 78 amino acids. This mutation was studied by using green fluorescent protein (GFP)-tagged γ2 subunits. The receptor containing this mutation was not expressed on the surface, but was retained in the ER. Consistent with this finding, no GABA-evoked current was recorded from oocytes expressing the mutant receptors. Genetic mutations producing PTCs can result in C-terminally truncated proteins that

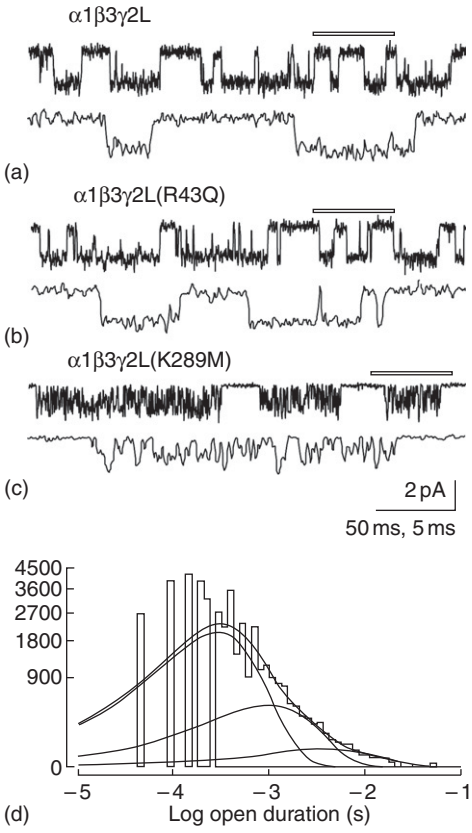


Figure 3 Single channel analysis demonstrated that the mean open time of α1β3γ2L receptor channels was reduced. Single channel recordings obtained in patches held at −75 mV in the presence of 1 mM GABA from wild-type α1β3γ2L receptors (a), α1β3γ2L (R43Q) receptors (b) and α1β3γ2L(K289M) receptors (c). A portion of the top trace in each pair (indicated by the open bar) is expanded below that trace. Calibration bars apply to all three panels. Openings were downward. Similar results were observed from three α1β3γ2L, five α1β3γ2L(R43Q), and seven α1β3γ2L (K289M) patches. (d) Open duration histogram for α1β3γ2L (K289M) single channels. The distribution was best described by the sum of three exponential functions, with each exponential fit shown as a smooth curve. Although three functions were required, the relative contribution of the shortest open state (Leftmost curve) was highest, indicating that the most openings were brief in duration. The time constants were 0.33, 1.17, and 4.54 ms with relative areas 0.81, 0.17, and 0.02 respectively. Data were pooled from seven α1β3γ2L(K289M) patches. Adapted from Bianchi MT, Song L, Zhang H, and Macdonald RL (2002) Two different mechanisms of disinhibition produced by $GABA_A$ receptor mutations linked to epilepsy in humans. *The Journal of Neuroscience* 22: 5321–5327.

can produce dominant negative inhibition on full length proteins, thus potentially harming the cells. Based on the faux 3-UTR model, cellular mRNA quality control mechanisms may also affect γ2(Q351X) subunit expression, but this possibility needs to be clarified. It is also intriguing that there is a great intrafamilial phenotype variation with this mutation; the basis for this variation is unknown.

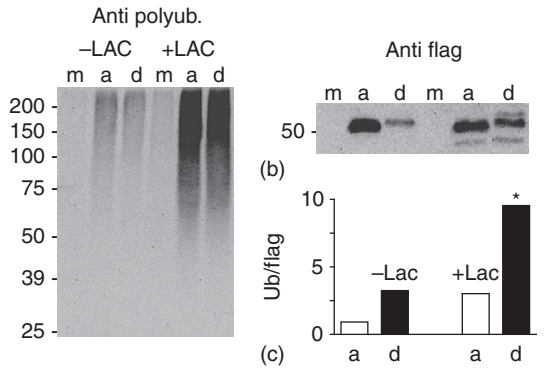


Figure 4 The α1(A322D) subunit was degraded through the ubiquitin–proteosome system.(a) HEK 293T cells were mock transfected (m) or transfected with α1-Flag (a) or α1(A322D)-Flag (d) subunits were incubated in the absence or presence of 10 μm lactacystin ($n \geq 5$) and analyzed by immunoblot that was probed with an antibody directed against polyubiquitin (a) or Flag (b). The relative amounts of polyubiquitinated subunits were plotted (c). The relative amount of polyubiquitinated mutant α1 subunit was greater than the wild-type α1 subunit. Modified from Gallagher MJ, Ding L, Maheshwari A, and Macdonald RL (2007) The $GABA_A$ receptor alpha1 subunit epilepsy mutation A322D inhibits transmembrane helix formation and causes proteasomal degradation. *Proceedings of the National Academy of Sciences* 104(32): 12999–13004.

$GABA_A$ receptor γ2 subunit nonsense mutation, Q1X

A γ2 subunit mutation that introduced a PTC, Q1X, between the signal peptide and mature peptide was identified in a family with severe myoclonic epilepsy of infancy (SMEI). The γ2 subunit mutation, Q1X, would likely trigger NMD, although this has not been demonstrated. However, if the γ2(Q1X) subunit mutation resulted in NMD completely, and if the mutant γ2S subunit does not interfere with transcription of the wild-type γ2S subunit at the mRNA level, no epilepsy would result (as observed in γ2 (+/−) gene deletion heterozygous mice). It is also unclear why in the pedigree, both twins carrying the mutation were diagnosed with SMEI while the father with the de novo mutation was seizure-free. Future studies focusing on gene expression and the completeness of NMD may elucidate the underlying molecular pathology.

$GABA_A$ receptor γ2 subunit splice donor site mutation, IVS6 + 2T → G

The γ2 subunit IVS6 + 2T → G mutation is the first intronic mutation identified in a $GABA_A$ receptor gene. The point mutation in the splice-donor site in the γ2 subunit gene intron 6 (IVS6 + 2T → G) has been identified in a family with CAE and FS (**Figure 1**). The effect of this mutation on $GABA_A$ receptor function is unknown but was predicted to lead to a nonfunctional protein through exon skipping. The predicted exon skipping would result in a new PTC at the fifth and seventh exon junction site. Thus, it is very likely that this prematurely terminated codon may also trigger NMD, thus reducing expression of mutant protein at mRNA level. Therefore, the underlying mechanism for this splice donor site mutation may also be due to haploinsufficiency. In fact, with the NMD-sensitive minigene approach, we demonstrated that the mutant γ2 (IVS6 + 2T → G) minigene only produced a minimal amount of the mutant protein compared with the wild-type γ2 minigene, suggesting that the mutation may activate NMD. Our ongoing research at the transcription level will further elucidate the underlying molecular mechanisms.

$GABA_A$ Receptor δ Subunit Variants (E177A, R220C, R220H)

Monogenic mutations only account for the pathogenesis of a small portion of IGE syndromes. Most of the idiopathic epilepsies are polygenic, requiring additive actions of a set of susceptibility genes. We reported the first $GABA_A$ receptor susceptibility gene, *GABRD*, for IGEs. Two putative missense mutations in *GABRD* were identified: δ(E177A) was detected in a small GEFS+ family, and δ(R220C) was detected in a second small GEFS+ family. Both mutations were heterozygously associated with epilepsy in these pedigrees. In addition, a polymorphic allele, δ(R220H), has been associated with JME patients, but is also found in the general population.

The δ subunit variant, E177A, is adjacent to one of the invariant cysteines that form a disulfide bond, the signature feature of cys-loop receptors. The δ(R220) residue is localized about in the middle between the δ(E177A) variant and the entrance to the first transmembrane domain (M1). The current amplitudes of heterozygous or homozygous receptors harboring either δ(E177A) or δ(R220H) subunits were significantly reduced compared to those of wild-type receptors. The current amplitudes of heterozygous or homozygous α1β2δ(R220C) receptors were not significantly different from those of wild-type receptors, but this finding requires further investigation. Combining both single channel recording and biochemistry data, the basis for the reduced peak current of these δ variant-harboring receptors was determined to be primarily due to the reduced mean channel open time and a small reduction of surface receptor expression.

Methods

Our laboratory uses electrophysiological techniques such as whole cell recording, lifted whole cell recording,

macropatch recording, single channel recording, and slice recording. In addition, we use rapid drug application techniques. With these techniques, we can study the function of any mutant $GABA_A$ receptor composed of various subunit combinations. Toward that end, we have developed a comprehensive $GABA_A$ receptor subunit gene bank.

However, characterization of channel function itself is not adequate to dissect the molecular pathology of these epilepsy mutations. We thus developed numerous other research approaches to study $GABA_A$ receptor subunit mutations at gene, protein, and cellular levels. For each mutation, in addition to electrophysiological study, we run parallel experiments with these additional techniques so as to better understand the molecular pathology of the mutation. At the protein level, these additional approaches include immunoblotting, coimmunoprecipitation, receptor surface biotinylation, immunohistochemistry, and high throughput flow cytometry. We have also generated numerous $GABA_A$ receptor subunit constructs with epitope tags (FLAG, c-myc, HA), including a number of fluorescent protein epitope tags (GFP, YFP, CFP), which allow us to visualize (with confocal microscopy) mutant and wild-type subunit protein trafficking in live cells (**Figure 2**). At the gene level, we have developed real-time PCR (RT-PCR), gene silencing by siRNAs, and BACs (bacterial artificial chromosomes) that contain wild-type human $GABA_A$ receptor subunit genomic sequences. Since expressing BAC constructs in mammalian cells is still problematic, we have initiated studies using transgenic animals that carry the mutations of interest.

Recent Results

Based on our studies, these $GABA_A$ receptor subunit gene mutations could impair neuronal inhibition either through channel gating or receptor trafficking. Our current results suggest that most of the mutations cause reduced receptor subunit biogenesis, including defects in subunit transcription, folding and assembly, or impaired receptor trafficking. These observations are similar to the reports of mutant CFTR in which trafficking-deficient mutations are far more frequent than the mutations with channel gating defects. As shown in **Figure 2**, receptors harboring mutant γ2(R43Q) subunits have a trafficking defect. The YFP-tagged mutant protein was retained inside ER, as indicated by the fact that the membrane/ER fluorescence intensity ratios were significantly lower in both heterozygous and homozygous mutant receptor conditions. Although the mutant $\alpha1\beta3\gamma2$L(R43Q) channel has no gating defect based on our electrophysiology data, the impaired trafficking of the mutant protein suggests that it contributes minimally to synaptic inhibition in a neuronal milieu.

In contrast, receptors harboring mutant γ2(K289M) subunits have a gating defect. As shown in **Figure 3**, mutant $\alpha1\beta3\gamma2$L(K289M) receptor single channel currents had a reduced mean open time. In addition, currents from macropatches excised from cells expressing $\alpha1\beta3\gamma2$L(K289M) receptors displayed accelerated deactivation when evoked with brief, rapid, and saturated GABA concentration. The brief macropatch currents are similar to IPSCs. This reduction of the duration of rapid GABA-evoked current by the γ2L(K289M) subunit mutation suggests that the mutation would result in reduced IPSC durations, thus producing disinhibition that may lead to epilepsy.

What is the cellular fate of those trafficking-impaired mutant proteins that are retained inside ER? Recent studies have demonstrated that the ubiquitin–proteosome degradation of these misfolded and misrouted proteins is a prominent mechanism. Through this mechanism, misfolded proteins are recognized and then dislocated to the cytosol to be ubiquitylated and degraded through a process known as ERAD – ER-associated degradation. A recent study from our laboratory demonstrated that a mutant $GABA_A$ receptor subunit protein could be subject to this ER quality control process. As illustrated in **Figure 4**, the mutant α1(A322D) subunit protein was rapidly degraded by the ubiquitin–proteosome system.

Challenges and Future Directions

In spite of the advances from genetic and functional studies of these epileptic mutations, there are many major challenges remaining in understanding the pathology of these inherited epilepsies. First, it is difficult to obtain a proper and easy-to-carry out experimental model that can adequately reproduce the alterations produced by these mutations in patients. Most of the data to-date are still obtained from studies using heterologous expression in acute transfection systems. The available heterozygous subunit gene deletion mice may reflect the function of a certain gene, but gene knockout may well be different from a loss-of-function mutation. Second, most of the mutations may not be fully penetrant, and there is a great intrafamilial phenotype variation. This variation suggests that there are other modifying genes that influence the development and persistence of the epilepsy, but this possibility needs to be clarified.

Given the complexity of gene transcription and translation, as well as complications involved in receptor trafficking from the ER to cell surface and synapses in neurons, it would not be surprising that mutations in other genes of associated proteins – e.g., chaperones, kinases involved in phosphorylation signaling pathways, etc. – are linked to epilepsy. For example, the non-ion

channel gene mutation in LGI1 has been identified in familial lateral temporal lobe epilepsy, suggesting an additional pathogenetic process in epileptogenesis. It is likely that the concept of ion channelopathy of epilepsy represents only the tip of a gigantic iceberg. Future work directed at the gene expression, protein modification, trafficking, synaptic targeting, and maintenance of cellular homeostasis will eventually present a more accurate picture of epileptogenesis – and hopefully lead to alternative therapeutic approaches.

See also: **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; **Genetics**: *GABRB3* and Childhood Absence Epilepsy; Single Gene Mutations in Inherited and Sporadic Epilepsy.

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Pathophysiology of Status Epilepticus

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Introduction

Broadly, status epilepticus (SE) refers to prolonged seizure activity. The International League Against Epilepsy (ILAE) classification of seizures defines status epilepticus as 'a condition characterized by epileptic seizures that are sufficiently prolonged or repeated at sufficiently brief intervals so as to produce an unvarying and enduring epileptic condition.' This definition does not define the duration of seizures necessary for them to be considered unvarying and enduring. Analyses of seizure duration in epilepsy monitoring units suggest that most complex partial seizures last less than 2 min. Prospective studies of new-onset seizures in children also suggest that if a seizure continues for more than 7 min, it is unlikely to end spontaneously (or soon). These observations suggest that seizures lasting more than 5–7 min are unlikely to end spontaneously, and constitute 'enduring' epileptic condition referred to as SE.

There are at least 126 000–195 000 episodes of status epilepticus associated with 22 000–42 000 deaths each year in the United States. The majority of SE patients have no history of epilepsy. In the 30 days following SE, mortality is approximately 25% – a rate that approaches deaths from gunshot wounds, major head trauma, and myocardial infarction. Much of the mortality associated with SE is a consequence of the underlying cause of seizures. However, prolonged seizures themselves add to the mortality. SE-associated mortality is particularly high in the elderly. In infants and children, SE may impact cognitive development and could potentially increase risk for subsequent development of epilepsy.

Neurobiology of SE is as challenging as its clinical aspects. The primary question is as follows: How do seizures rapidly alter brain function so as to become self-sustaining? SE probably reflects a perversion of normal brain plasticity. The limbic system, and particularly

channel gene mutation in LGI1 has been identified in familial lateral temporal lobe epilepsy, suggesting an additional pathogenetic process in epileptogenesis. It is likely that the concept of ion channelopathy of epilepsy represents only the tip of a gigantic iceberg. Future work directed at the gene expression, protein modification, trafficking, synaptic targeting, and maintenance of cellular homeostasis will eventually present a more accurate picture of epileptogenesis – and hopefully lead to alternative therapeutic approaches.

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Neurobiology of SE is as challenging as its clinical aspects. The primary question is as follows: How do seizures rapidly alter brain function so as to become self-sustaining? SE probably reflects a perversion of normal brain plasticity. The limbic system, and particularly

the hippocampus (which has been the focus of extensive learning and memory research), appears to play a key role in sustaining SE. Experimental animals subjected to SE become permanently epileptic, and have recurrent unprovoked seizures, suggesting long-lasting plasticity as a consequence of a single event. Understanding SE thus offers a direct benefit in advancing the treatment of patients with SE. Such understanding may also provide an indirect but more generally applicable benefit, since mechanisms of brain plasticity involved in/during SE may play a role not only in the pathogenesis of partial epilepsies, but also in mechanisms of learning and memory.

Background

Animal Models and Functional Anatomy

Several animal models are available to study various facets of SE. These models can be divided into two major categories: chemical convulsant-induced and electrogenic models. The progression of behavioral and EEG changes described in humans has also been demonstrated in both the chemical convulsant model and the electrogenic model of SE in experimental animals, thus validating their use in understanding the pathophysiology of SE. Chemical convulsant models of SE have been useful in determining the systemic effects of generalized convulsive SE, elucidating the functional anatomy of SE, and comparing the efficacy of antiepileptic drugs in treatment. In addition, they are convenient experimental tools. However, in studies of SE based on chemical convulsant-induced seizures, there is an inherent difficulty of separating the effects of the convulsant drug itself from the effects of seizures. Electrical stimulation models of SE are helpful in identifying clearly the effect of prolonged and recurrent seizures in a controlled fashion. Electrogenic models are derived from kindling protocols and involve prolonged electrical stimulation of limbic structures. Prolonged electrographic seizures can be induced in hippocampal slices from young animals, using a combination of electrical stimulation and manipulation of extracellular magnesium concentrations.

Anatomical mapping studies using quantitative autoradiography for radiolabeled 2-deoxy glucose and immunohistochemistry for cfos and c Jun have been performed on the electrical stimulation and lithium/pilocarpine models. These studies consistently demonstrated activation of an interconnected circuit of limbic structures, consisting of entrorhinal cortex, dentate gyrus, areas CA1 and CA3, subiculum and parasubiculum, and basolateral amygdala. When seizures progress from limbic to secondarily generalized tonic–clonic events, there is activation of the substantia nigra, somatosensory cortex, head of the striatum, and midline thalamic nuclei.

SE: The GABA Hypothesis

Almost two decades ago, our laboratory proposed that the self-perpetuating nature of SE is due to its impact on GABA-mediated inhibition. We proposed that seizures reduced GABA-mediated inhibition, which then led to more seizures, setting up vicious cycle. We initially investigated (using the paired pulse inhibition method) the hypothesis that GABA-mediated inhibition collapses during SE. The paired pulse technique primarily measures feedback inhibition of CA1 pyramidal neurons. Briefly, the method involves placement of a stimulating electrode in the CA1/CA3 region of one hippocampus and a recording electrode in the homotopic contralateral CA1 region to record extracellular field potentials in response to the stimuli. When two low-intensity pulses were delivered at short interpulse intervals, the inhibition of the response to the second stimulus depends both on the interpulse interval and on stimulus intensity. At higher stimulus intensities, the inhibition is dependent only on the interpulse interval. Paired pulse inhibition in the 20–70-ms interpulse interval range was enhanced by benzodiazepines and diminished by bicuculline.

The relationship of GABA-mediated paired pulse inhibition in the CA1 region of the hippocampus and evolution of SE was studied. The evolution of seizures is closely correlated to the loss of paired pulse inhibition. All three cholinergic-based (i.e., pilocarpine) models of SE have been studied using the paired pulse inhibition technique. While studies using the paired pulse inhibition method have shown the critical role of reduction of GABA-mediated inhibition in the pathogenesis of SE, they did not reveal the mechanisms underlying the disinhibition. Paired pulse inhibition in the CA1 region of the hippocampus could be reduced by diminished release of GABA or diminished responsiveness of postsynaptic $GABA_A$ receptors. These possibilities are discussed later.

While the release of GABA from neurons during SE has not been studied, extracellular amino acid neurotransmitter levels during SE have been measured. In SE induced by kainic acid, there are decreases in glutamate, aspartate, and glutamine concentrations and increases in GABA concentration in the hippocampus. Walton studied amino acid neurotransmitter levels in SE induced by lithium/pilocarpine during various electrographic stages. Both aspartate and glutamate levels decline in the hippocampus, pyriform cortex, motor cortex, and entorhinal cortex during the early phase of SE, and continue to do so in the late phase. GABA levels, in contrast, rise in early SE and continue to do so in the late stages. It is difficult to interpret these observations since increased GABA levels could represent increased synthesis, diminished release, or diminished breakdown of this neurotransmitter. Similar observations pertain to glutamate and aspartate. More

recent electrophysiological studies have further suggested that extracellular GABA levels may be increased.

Methods

Whole-cell patch clamp recordings of $GABA_A$ receptor currents were obtained from hippocampal neurons (CA1 pyramidal neurons and dentate granule cells). In initial experiments, CA1 pyramidal neurons and dentate granule cells were acutely isolated from hippocampal slices after enzymatic digestion. GABA and drugs were applied via pipettes to elicit $GABA_A$ receptor currents. In later experiments, GABA-mediated IPSCs were recorded from dentate granule cells in hippocampal slices by patch clamp technique. Fast glutamatergic currents were blocked by NMDA and AMPA receptor antagonists. Trafficking of $GABA_A$ receptors from neuronal cell membrane into the cytosol was studied using an antibody feeding technique and a biotinylation assay.

Results

$GABA_A$ Receptor Function in the Hippocampus Diminishes During SE

The possibility that the reduction of GABA-mediated inhibition seen in SE is a consequence of changes in CA1 pyramidal neurons has been investigated. Several possible changes in CA1 pyramidal neurons could reduce the efficacy of GABA in producing chloride currents. The number of $GABA_A$ receptors could be reduced, or the efficacy of GABA in opening the ion channel after binding could be reduced. Whole-cell perforated patch recordings of $GABA_A$ receptor currents were made from acutely isolated CA1 pyramidal neurons from rats that exhibited SE or from naive control rats. The 'perforated patch' method was employed and the electrode solution (except for the tip) contained the pore-forming antibiotic, amphotericin. Recordings of $GABA_A$ receptor currents were conducted in whole-cell voltage clamp mode. These experiments showed that marked reductions of $GABA_A$ receptor currents in CA1 pyramidal neurons occurred during SE that produced loss of GABA-mediated inhibition. A third of all CA1 pyramidal neurons isolated from animals undergoing SE showed no response to GABA in contrast to 7% of neurons isolated from naive animals. These experiments also indicated that during SE, $GABA_A$ receptor currents were reduced by reduction of both conductance and the chloride reversal potential.

These findings suggest that the $GABA_A$ receptor is modified during an episode of SE. $GABA_A$ receptors are regulated by numerous positive (barbiturates, benzodiazepines, and neurosteroids) and negative (picrotoxin, bicuculline, and Zn^{2+}) allosteric modulators. We tested whether prolonged seizures of SE altered drug sensitivity of $GABA_A$ receptors. Seizures induced in rats by administration of lithium (Li) followed by pilocarpine were readily terminated by the benzodiazepine diazepam when administered early during the seizures. However, during status epilepticus, there was a substantial reduction of diazepam potency for termination of the seizures. To determine if the loss of sensitivity of the animals to diazepam was due to an alteration of $GABA_A$ receptor functional properties, whole-cell $GABA_A$ receptor currents were recorded from hippocampal dentate granule cells isolated acutely from control rats and from rats undergoing status epilepticus. $GABA_A$ receptor properties were characterized by determining GABA sensitivity, and the sensitivity of $GABA_A$ receptors to regulation by benzodiazepines, barbiturates, and Zn^{2+}. When compared with those from naive littermates, $GABA_A$ receptor currents from rats undergoing status epilepticus were less sensitive to diazepam and Zn^{2+} but retained their sensitivity to GABA and pentobarbital. These studies suggested that the prolonged seizures of status epilepticus rapidly altered the functional properties of hippocampal dentate granule cell $GABA_A$ receptors.

Mechanism of $GABA_A$ Receptor-Mediated Inhibition

The mechanism of reduction of $GABA_A$ receptor-mediated inhibition is being explored in a number of laboratories. $GABA_A$ receptors undergo a cycle of internalization and insertion into the cell membrane via a clathrin-dependent endocytic process. Internalization of the surface $GABA_A$ receptor correlates with a reduced response to GABA, whereas inhibition of internalization results in an increase in the amplitude of synaptic $GABA_A$ receptor currents. An activity-induced acceleration of the internalization of $GABA_A$ receptors from the cell surface could partially explain the reduction in GABA-mediated inhibition observed during the prolonged seizures of SE. The number of receptors at a synapse is an important determinant of synaptic strength; therefore, a reduction in the total number of postsynaptic $GABA_A$ receptors could result in a decline in GABAergic inhibition.

This rapid loss of $GABA_A$ receptor benzodiazepine sensitivity during seizures has been difficult to explain. Recent studies have suggested that benzodiazepine-insensitive receptors are expressed in hippocampal neurons on extrasynaptic membranes. Furthermore, these receptors appear to mediate a novel form of inhibition called tonic inhibition. We tested whether during SE, there is increased internalization of synaptic, diazepam-sensitive $GABA_A$ receptors, which leaves extrasynaptic diazepam-insensitive receptors on the neuronal surface. A combination of immunocytochemical and electrophysiological studies suggested that cultured hippocampal

neurons expressed two types of $GABA_A$ receptors. One type of receptor, containing α1, α2, β2/3, and γ2 receptor subunits, formed clusters that are present at GABAergic synapses and in extrasynaptic membrane. The second type of receptor, containing the α4, β1, and δ subunits, was diffusely distributed in the extrasynaptic membrane. Using an in vitro model of status epilepticus combined with electrophysiological and cellular imaging techniques, we found that prolonged epileptiform bursting results in a reduction in GABA-mediated synaptic inhibition. Furthermore, we found that the constitutive internalization of $GABA_A$ receptors is rapid and is accelerated by increased activity associated with seizures. Reduction of activity reduced the rate of internalization. These findings suggest that the rate of $GABA_A$ receptor internalization is regulated by neuronal activity, and its acceleration contributes to the reduction in inhibitory transmission observed during status epilepticus. More recent studies demonstrate that the rate of internalization of benzodiazepine-insensitive, extrasynaptic $GABA_A$ receptors containing the δ subunit, which mediate tonic inhibition, is much slower than that of synaptic receptors. During acute status epilepticus, tonic inhibition is preserved and the surface expression of the δ subunit containing receptors is unchanged.

NMDA Receptor Function is Enhanced during Limbic Status Epilepticus

Although abnormal GABAergic mechanisms are involved in the pathogenesis of SE, excitatory transmission mediated by glutamate is essential for spread of epileptiform activity. Glutamate activates multiple subtypes of postsynaptic excitatory amino acid receptors during SE, but studies have focused on the *N*-methyl-D-aspartate receptor. The role of non-NMDA receptors in SE is uncertain. Current evidence suggests that NMDA activation sustains SE and may in part cause the characteristic neuropathologic changes in SE. However, NMDA receptor antagonists fail to block initiation of status epilepticus. NMDA receptor antagonist MK 801 administered prior to or shortly after the onset of kainate-induced or lithium/pilocarpine-induced status epilepticus did not control seizures. Although some authors have reported that seizures were less intense after NMDA receptor antagonist administration, others have reported worsening of electrographic seizures by NMDA receptor antagonists.

Recent studies have demonstrated that prolonged status epilepticus can be treated with ketamine, a clinically available NMDA receptor antagonist; these studies indicate that ketamine is more effective in treatment of prolonged status epilepticus compared with early status epilepticus. NMDA receptor function can be inhibited by multiple mechanisms, including competitive block by drugs such as 3-((*R*,*S*)-2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), by open channel block at the phencyclidine (PCP) site by MK-801 or ketamine, and by action at the pH-sensitive site by ifenprodil. NMDA receptor antagonists CPP, MK801, and ifenprodil were compared for their ability to terminate prolonged SE. These studies suggested that noncompetitive NMDA receptor antagonists were superior to competitive and allosteric inhibitors of NMDA receptors in terminating prolonged SE induced by electrical stimulation.

The ability of NMDA receptor antagonists to suppress SE varies from model to model. For example, the NMDA receptor antagonist ketamine terminates diazepam-refractory SE generated by electrical stimulation (see earlier sections), but it is ineffective when used alone in SE induced by cholinergic stimulation. Multiple doses of ketamine, ranging from ineffective to toxic, were examined for their efficacy in terminating diazepam-refractory SE in electrogenic model of status epilepticus. Furthermore, the same range of ketamine doses was used in combination with two doses of diazepam. The principal finding of this study was that a combination of diazepam and ketamine rapidly terminated prolonged cholinergic stimulation-induced SE in a synergistic fashion. Diazepam shifted the ketamine dose response (SE termination) relationship to the left and increased the maximal effect of ketamine.

Conclusions and Directions for Future Research

The current working hypothesis for understanding pathophysiology of status epilepticus is that prolonged seizures diminish $GABA_A$ receptor-mediated inhibition by enhancing the internalization of synaptic $GABA_A$ receptors. Seizures also activate NMDA receptors. The relationship between NMDA receptor activation and diminished inhibition is currently under study. Further studies are needed to characterize the mechanism of NMDA receptor activation. The role of AMPA subtype of glutamate receptors in mediating status epilepticus has not been studied in detail and needs to be explored. Clinical therapeutic trials testing the efficacy of drugs acting on NMDA receptors, in treating refractory status epilepticus, would determine whether treatments developed in laboratories can have clinical application.

See also: **Epidemiology**: Epidemiology: Status Epilepticus; **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; **Hormones and Gender**: Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; **Neurotransmitter Function**: Synaptic Activities and Transmitter Function: Receptor Trafficking.

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Plasticity of GABAergic Systems during Epileptogenesis

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Introduction

Epileptogenesis

An initial insult, be it inherited (i.e., a genetic mutation) or acquired (e.g., brain trauma), is often thought to be causally related to the appearance of temporal lobe epilepsy (TLE). The time interval between the initial insult and the first spontaneous seizure is highly variable (from a couple of hours to decades). It is thus reasonable to propose that modifications in neuronal networks occur during this period. These modifications will eventually favor the emergence of seizures. Such alterations belong to the processes that define epileptogenesis. It is very difficult to study epileptogenesis in humans, since an initial insult, recognized as putting some patients at risk, does not necessarily lead to epilepsy. As a consequence, the results discussed here are derived primarily from animal studies.

Most animal models of TLE are characterized by a chemically or electrically induced initial insult followed by a seizure-free period. Many modifications have been identified just after *status epilepticus* (SE). They include cell death, transcriptional modifications (the expression of hundreds of genes is altered), and posttranslational changes (e.g., modifications of proteins). The impact of these alterations on epileptogenesis remains an open question. It is usually assumed that some of these changes will lead to still further modifications, until an epileptic network is built. In such a dynamical perspective, it seems certain that some of the changes seen during this period are crucial to epileptogenesis (and some may not be). If we can identify the critical (i.e., 'causal') changes, we may be able to interfere with them, and thus delay or stop epileptogenesis. Thus, a related hypothesis states that there is a cascade of deleterious events, and that the earlier we can intervene, the more likely we are to achieve 'antiepileptogenesis.' These hypotheses are very difficult to address experimentally, because of the complex spatio-temporal dimensions of the trauma-induced changes. Alterations occur at several levels – genetic, molecular, cellular, network, and brain region – and they all may evolve over time.

Multiplicity of GABAergic Systems and Functions

Many investigators have focused on the modifications affecting γ-aminobutyric-acid (GABAergic) systems, because GABAergic neurons powerfully control the activity of neurons and neuronal networks. This control has multiple, nonexclusive aspects. When GABA is released by a cell, it will activate $GABA_A$ receptors, leading to a flux of Cl^- and bicarbonate through the associated channels. Because the equilibrium potential for Cl^- is normally close to the cell resting membrane potential, and because the intracellular concentration of Cl^- can be dynamically

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Plasticity of GABAergic Systems during Epileptogenesis

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Introduction

Epileptogenesis

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regulated, the physiological effect of $GABA_A$ receptor activation is context-specific. In adult tissue, activation of perisomatic synapses leads to the hyperpolarization of the membrane. Importantly, a hyperpolarizing action of GABA does not necessarily imply reduced network activity (i.e., inhibition per se), since the coordinated action of perisomatic interneurons can entrain a network into an oscillatory mode. In contrast to perisomatic synapses, the activation of GABAergic synapses on apical dendrites is depolarizing (but not excitatory). Activation of GABAergic synapses on the axon initial segment is depolarizing *and* excitatory. The functional consequences of $GABA_A$ receptor activation are thus dependent upon the cell compartment(s) (initial segment, soma, dendrites) activated, as well as the underlying activity (e.g., oscillations) and (most likely) the type of postsynaptic cell and the specific brain region in question. This complexity is correlated with the heterogeneity of GABAergic neurons in terms of morpho-physiological properties and neurochemical content (tens of GABAergic cell types can be distinguished). For example, most GABAergic neurons in temporal lobe structures have local connectivity, directly controlling local network activity in a cell-type- and brain-state-dependent manner. But there are also GABAergic neurons with long-range connections (spanning different brain regions); the hypothetical role of which is to bind distantly operating networks.

This short presentation focuses on the functional consequences of postsynaptic $GABA_A$ receptor activation. Other important aspects of GABAergic neurotransmission include $GABA_B$ receptors, extrasynaptic receptors (generating a tonic current), and the current shunt effect produced when $GABA_A$ channels are activated. Indeed, one is certainly justified in talking of GABAergic systems – not a single system.

Plasticity of GABAergic Systems

Plasticity within GABAergic systems can occur at all levels of analysis. Modifications may involve gene expression, transcriptional processes, receptors (subunit composition, ability to be translocated to the membrane and to be assembled, anchoring, motility, molecular partners), intracellular medium (Cl^- concentration, phosphorylation processes), extracellular medium (presence of modulators such as benzodiazepines, Zn^{2+}, steroids, etc.), extracellular space, and GABA uptake mechanisms. Upstream, at the presynaptic terminal level, one can distinguish GABA synthesis or recycling, quantal release and content, Ca^{2+} homeostasis, presynaptic terminal excitability, and presynaptic receptors. Further upstream, at the GABAergic neuron level, alterations can be morphological (e.g., axonal sprouting, synaptogenesis or pruning) and functional – i.e., any change affecting the way the GABAergic neuron processes information (e.g., modifications in cell excitability, receptor distribution, and properties). Thus, it is ultimately necessary to take a step backward, and consider not only how the GABAergic cells are embedded in a local network, but also how networks or brain regions *presynaptic* to a given GABAergic network control these GABAergic cells.

Background

Research attempting to assess the state of GABAergic systems during epileptogenesis faces three major difficulties. First, epileptogenesis can only be determined by continuous 24/24-h and 7/7-day video-EEG recordings. Second, the diversity of GABAergic interneurons must be considered. Third, the parameters characterizing the GABAergic circuitry should be adequately chosen. These difficulties make these studies both condition-specific and highly time-consuming.

A large number of studies have been devoted to the characterization of GABAergic systems in the already epileptic animal, in various regions of the temporal pole, and in different experimental models. These studies provide a picture of the state of the system when animals have recurrent seizures. Knowing both the end point of epileptogenesis and the starting point (the state of the system before the initial insult), the strategy for studying epileptogenesis is relatively simple: Determine the temporal profile of the modifications of the chosen parameters. A limitation to this approach lies in the fact that there may not be a real 'end point.' Most studies performed during the 'chronic phase' (i.e., in the epileptic animal) do not take into account the time course of epilepsy progression; further, parameters have been evaluated anywhere between a few days to one year following the first spontaneous seizure. Several experimental models display a progressive sclerosis of the hippocampus, indicating that reorganizations continue to occur once the animal is epileptic. To reduce variability in parameter measurements, an ideal solution would be to evaluate parameters just after the first two spontaneous seizures.

Keeping this limitation in mind, what do we know about the reorganization of GABAergic systems during the chronic (epileptic) period? Morphological studies indicate a specific loss of subpopulations of GABAergic interneurons. Histological and electron microscopy studies performed in the CA1 region demonstrate specific loss of GABAergic synapses on the distal part of the dendritic tree, suggesting the loss of somatostatin-containing O-LM cells, a class of stratum oriens interneurons that project to the lacunosum moleculare (**Figure 1**). The number of GABAergic synapses on the initial segment of pyramidal cells is also decreased, confirming the loss of parvabumin-containing axo-axonic cells. Such reorganization should result in a decreased GABAergic drive, but the functional outcome has been surprisingly difficult to evaluate.

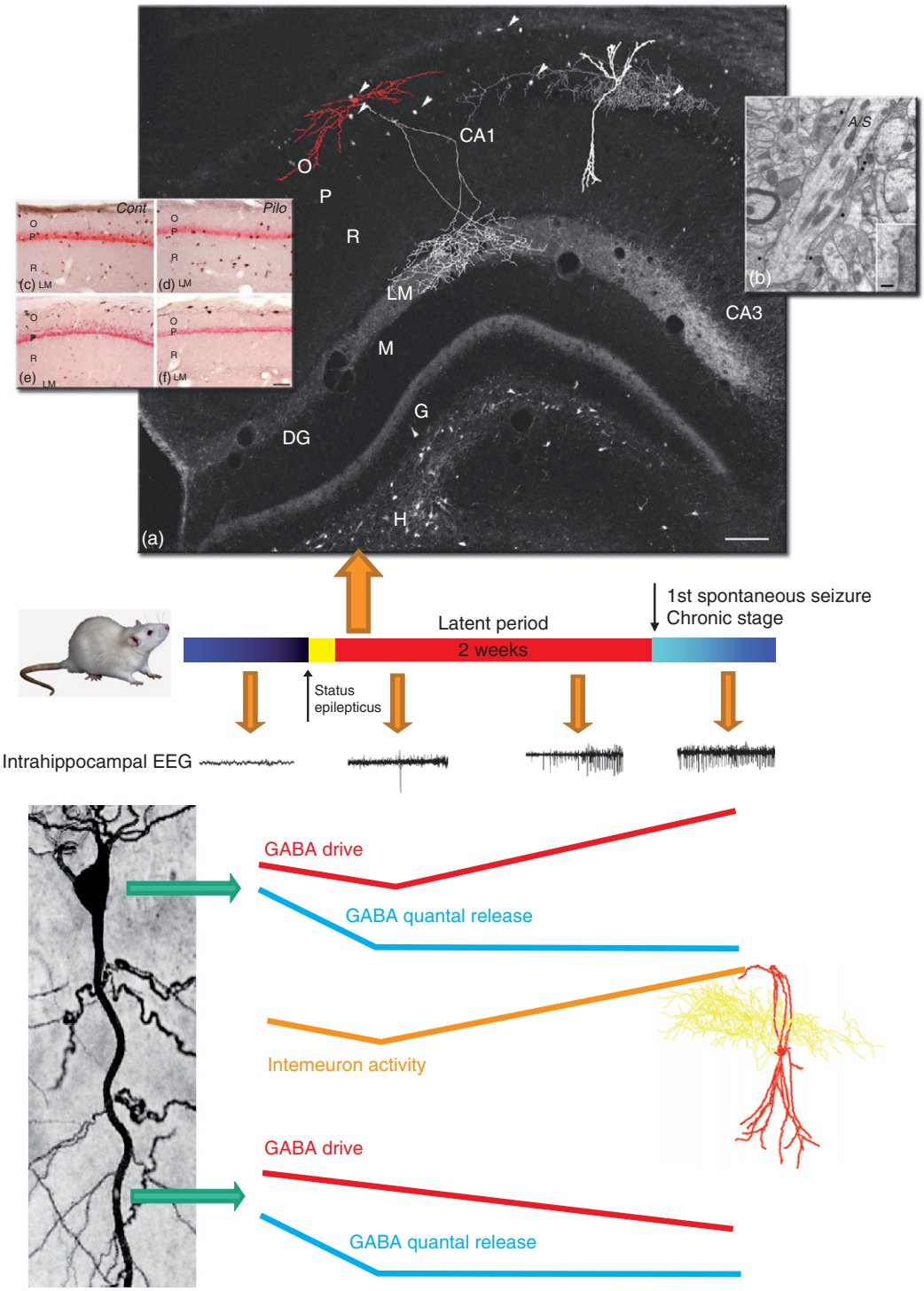


Figure 1 Plasticity of hippocampal CA1 GABAergic pathways in experimental temporal lobe epilepsy. Morphological and physiological parameters are assessed at different times during the latent period and the chronic stage. Morphological analysis (top panel) shows the early loss of O-LM cells and axo-axonic cells (a) 3 days after the pilocarpine-induced *status epilepticus*. (b) Electron microscopy analysis reveals the loss of axo-axonic GABAergic synapses. The loss of GAD-expressing (c, d) and somatostatin-expressing (e, f) interneurons is predominant in CA1 stratum oriens. The middle panel shows the progressive increase in interictal-like activity during epileptogenesis. The bottom panel schematizes the time course of the GABAergic dive and GABA quantal release in the dendritic and somatic compartments in CA1 pyramidal cells at the same time points. Note the transient decrease of the somatic drive and the continuous decrease of the GABAergic drive. Interneuron activity is also modified. For example, after an initial decrease, basket cells become hyperactive.

Physiological studies confirm a decreased GABAergic drive in the distal dendrites of CA1 pyramidal cells, facilitating the transfer of excitatory glutamatergic perforant path inputs coming from the entorhinal cortex. Such a reorganization may qualify as 'proepileptic.' However, the loss of axo-axonic synapses could be considered as 'antiepileptic,' since these GABAergic synapses are excitatory.

The study of the surviving interneurons also provides valuable information about the reorganization of the GABAergic system at the network level. Far from being dormant, the surviving interneurons (including basket cells) are hyperexcitable and hyperactive. An increased glutamatergic drive onto these interneurons contributes to their hyperactivity, showing that glutamatergic networks presynaptic to interneurons are also reorganized. The physiological consequence of basket-cell hyperactivity is an increased GABAergic drive in the soma of CA1 pyramidal cells. However, the functional consequence of this increased drive is difficult to assess, as increased somatic GABAergic drive may mean decreased (increased) firing probability if GABA remains hyperpolarizing (becomes depolarizing), or increased propensity to fire in synchrony. Whether interneuron intrinsic excitability is modified remains to be determined. However, postsynaptic currents in interneurons appear to be modified, suggesting a possible modification in the way they integrate incoming inputs. Such an alteration may modify information processing at the network level, affecting specific functions in a cell-type- and brain-state-dependent manner.

Looking downstream, one needs to consider possible changes in the interneuron output circuitry – e.g., changes in the axonal tree. Whether axonal sprouting occurs for specific interneuron cell types remains debated. There are, however, alterations at the level of presynaptic terminals, including the reduction of GABA quantal release (depending upon the hippocampal region) and a modification of presynaptic receptors.

Finally, major modifications occur at the postsynaptic site. There is a complex reorganization of $GABA_A$ receptors, both spatially (their physical distribution) and functionally (their subunit composition, which determines their biophysical properties and modulation by external and internal compounds). The modifications affecting $GABA_A$ receptors are region-specific, indicating the need for analyses in different networks.

The net functional consequences of these reorganizations are difficult to assess, if only because we still do not have a clear conceptual framework about interneuron function in the normal brain. For example, the functional consequences are likely to be brain-state-dependent and highly variable.

Methods

One strategy used by several investigators – including our laboratory – to determine whether these modifications play a role in epileptogenesis or seizure genesis/propagation is to estimate their time course following the initial insult. Toward that end, we have used pilocarpine-treated rats as an experimental model of TLE. Following an initial SE induced by pilocarpine, animals go through a two-week-long latent (seizure-free) period, until they become 'epileptic' (i.e., have spontaneous recurrent seizures). Continuous 24 h day^{-1}, 7 day $week^{-1}$ video-EEG monitoring is used to relate behavior with electrical abnormalities (ictal episodes and interictal activity). Animals not exhibiting SE following pilocarpine injection, and sham-treated animals, are used for comparison. Animals are sacrificed at different stages during the latent period and during the chronic phase, and directed for morphological and in vitro electrophysiological studies. We have investigated the fate of various classes of interneurons (cell death and synaptic reorganization) using conventional immunohistochemical and electron microscopy techniques, and have tried to correlate morphological alterations with electrophysiological changes. To obtain information about electrophysiological changes, hippocampal CA1 pyramidal cells and different types of interneurons have been recorded in vitro to determine their firing pattern and the properties of their synaptic inputs. In parallel, a computer model able to generate depth EEG-like signals (based on a simplified architecture of the hippocampus) has been used to predict, at the network level, the functional consequences of the physiological modifications identified experimentally. These model-based results are then compared with experimental in vivo recordings.

Recent Results

Using continuous video-EEG recording, we determined that the range between the initial SE and the first spontaneous seizure was 12–18 days. After the first spontaneous seizure, which most of the time was subclinical and limited to the hippocampus, seizures recurred. The state of the GABAergic circuitry was then determined at different time points: 3–5, 5–7, and 7–10 days following the initial SE and during the chronic phase.

The most obvious impact of the initial SE on the CA1 GABAergic circuitry is cell death. According to the classical hypothesis, decreased GABAergic 'inhibition' should be a proepileptic factor. Interneuron cell loss is indeed an early phenomenon that follows SE, but the death of GABAergic cells does not immediately lead to seizure activity. Affected neurons include a subpopulation of somatostatin- and parvalbumin-containing GABAergic interneurons. It is important to stress again that the loss of parvalbumin immunoreactive axo-axonic cells will remove a strong excitatory GABAergic drive onto pyramidal cells. As such, the loss of GABAergic synapses on the initial segment of principal cells may be seen as a 'protective' mechanism during

the early phase of epileptogenesis. The loss of GABAergic synapses on the distal part of the dendritic tree of pyramidal cells (due to the death of somatostatin-containing O-LM interneurons) may have a different functional impact, removing the strong control they exert upon entorhinal cortex glutamatergic inputs.

While the loss of glutamatergic synapses is usually followed by the sprouting of glutamatergic-cell axons and by reactive synaptogenesis, this reaction does not seem to be the case for the GABAergic circuitry. Loss of GABAergic synapses on the initial segment and in the distal part of the dendritic tree is not compensated throughout epileptogenesis and during the chronic phase. Sprouting of GABAergic axons in experimental TLE remains fiercely debated, but direct evidence is still lacking. Sprouting within the GABAergic circuitry has been suggested in tissue from epileptic patients.

What could be the physiological consequence of the loss of GABAergic synapses onto principal cells? Fewer GABAergic synapses should result in decreased functional GABAergic inputs, a hypothesis that can be tested by recording the spontaneous GABA-mediated synaptic activity in pyramidal cells. Since two different cell compartments are involved, we recorded from the soma and the distal dendrites of CA1 pyramidal cells during epileptogenesis. We found that the frequency of spontaneously-occurring GABAergic currents was decreased 3–5 days post-SE, and this decrease persisted throughout the latent period in both the cell compartments. Although the frequency of GABAergic currents remained decreased in the distal dendrites during the chronic phase, it recovered to control (pre-SE) levels in the soma. Although these results correlate with the loss of GABAergic synapses during epileptogenesis, it is important to remember that the frequency of GABAergic inhibitory postsynaptic currents (IPSCs) can also be influenced by many other factors, including the firing behavior of interneurons (which in turn depends upon their endogenous excitability and the synaptic inputs they receive), the probability of GABA release at presynaptic terminals, and postsynaptic $GABA_A$ receptors. We found that the probability of GABA quantal release was decreased by more than 50% at dendritic and somatic GABAergic synapses soon after SE, a phenomenon that persisted during the chronic phase. The loss of GABA quantal release appears to be related to the depletion of the reserve pool of GABA-containing vesicles, not to the loss of GABAergic synapses. Both the loss of synapses and decreased quantal release contribute to decreasing the GABAergic tone during epileptogenesis.

The decrease in IPSC frequency was somewhat compensated by postsynaptic modifications. Consistent with the previously described changes in subunit composition of $GABA_A$ receptors, we found that the kinetics of GABAergic currents were altered during the latent period; slower GABAergic currents compensated for the decreased frequency of GABAergic IPSCs in the soma, but not in dendrites. Interestingly, this modification was transient, as IPSC kinetics returned to pre-SE values during the chronic phase. This late shortening of GABAergic currents resulted in a further decrease of the GABAergic tone in the dendrites, suggesting that there is a gradual erosion of the dendritic GABAergic drive during the course of epilepsy. In the soma, the situation was different as the GABAergic drive was increased during the chronic phase because of the hyperactivity of basket cells, demonstrating that the activity of the surviving GABAergic subnetworks was also modified.

Analysis of the glutamatergic and GABAergic drives received by different classes of interneurons revealed that the GABAergic drive remained unchanged, but that the glutamatergic drive was increased. Such an alteration of the balance between the glutamatergic and GABAergic inputs may contribute to the increased firing rate of interneurons. In keeping with this enhanced activity, the levels of GAD, the GABA synthesis enzyme, were also increased. Glutamate and GABA quantal release, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and $GABA_A$ receptor-mediated current kinetics, and excitability were also modified in a cell-type-dependent fashion; i.e., these measures varied across different interneuron types. It is important to note that several of these modifications have been studied in other TLE models and in other brain regions, and some appear to be model- and region-specific.

A decreased GABAergic drive has been consistently proposed to be causally related to ictogenesis. But decreased GABAergic drive does not appear to constitute a necessary condition for seizure genesis (at least in the CA1 region), for the time window during which the GABAergic drive is maximally decreased is the seizure-free latent period. What, then, are the functional consequences of a decreased GABAergic drive, in particular in the dendrites, during the latent period? Using the computer model described earlier, we found that decreasing the GABAergic drive constitutes a sufficient condition to render the system hyperexcitable, with the occurrence of interictal-like activity. In keeping with this prediction, in vivo recordings revealed the presence of interictal-like activity as soon as 3 days post-SE. As described by others, interictal-like activity increased in frequency during epileptogenesis. Rather than being directly correlated to seizure genesis, a decreased GABAergic drive at the early stages of epileptogenesis may render the system prone to generate interictal-like activity. This activity may, in turn, participate in the construction of a network able to generate seizures.

Future Goals

The results described earlier demonstrate that plasticity of GABAergic systems during epileptogenesis is a very

complex phenomenon. Alterations occur at all levels of analysis, including postsynaptic, presynaptic, cellular, and network levels, in a subnetwork (defined by a given class of interneuron)-dependent manner. To make things more complex, these modifications are time-dependent.

Because there are many different types of interneurons, and because each interneuron type plays a specific role in shaping network dynamics, we have only began to assess the functional consequences of these plastic modifications. Up to now, two broad classes of interneurons have been considered: perisomatic and dendrite-projecting cells. It is now necessary to assess the fate of each cell type. Alterations within a specific GABAergic subnetwork may change specific activity patterns. Future goals include testing the ability of the system to generate physiological activities, including θ and γ oscillations, rhythms that have been associated with many cognitive processes.

Another challenge is to determine whether the interictal-like activity plays a role in epileptogenesis – i.e., whether it is causally related to the construction of an epileptogenic zone. If the occurrence of interictal-like activity is causally related to a decreased GABAergic drive, can we block this pathological activity by boosting GABAergic neurotransmission with conventional antiepileptic drugs as soon as interictal activity appears? Will such a manipulation delay or stop epileptogenesis?

Our results are interpreted within a specific framework that focuses on the inhibitory action of GABA. As pointed out, GABAergic neurotransmission is instrumental for the genesis of oscillatory activities. Whether a system with increased GABAergic drive on the postsynaptic soma is more prone to generate oscillatory activity remains to be tested. A greater ability to oscillate in specific frequency bands may facilitate the emergence of highly synchronized and widespread electrical patterns – such as seizures.

Finally, there is a specific class of interneurons that should be carefully investigated – interneurons with long-range connections. These GABAergic cells have extensive axonal fields spanning different hippocampal and extrahippocampal regions. Some of these long-range connection interneurons contact exclusively other interneurons, while others contact mostly glutamatergic cells. Neurons with long-range connections are believed to play a crucial role in information processing, as they would facilitate the 'binding' of distant brain regions (i.e., coordinate their activities). Hippocampo-septal interneurons (one of these long-range connection cells) survive in epileptic tissue. It will particularly be important to assess the state of these subnetworks, as they may favor synchronization between different brain regions.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor Plasticity in Epileptogenesis; Synchrony Through GABAergic Inhibition; **Transporters**: Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

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Synchrony Through GABAergic Inhibition

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Introduction

Activity in the normal brain is synchronized within a variety of network oscillations, the frequency and spatial scale of which vary with behavior. At one extreme are the ultraslow oscillations (<1 Hz) observed during slow wave sleep, which propagate across the entire neocortical mantle and also recruit neurons in the thalamus and striatum. In contrast, the activity within local circuits can display rhythmic synchronization over much shorter time scales, reaching the high gamma-frequency range (80–120 Hz) and beyond. While neuronal networks can generate rhythmic activity exclusively via mutual excitation, brain rhythms across this physiological spectrum are thought to depend upon GABAergic inhibition to both balance excitation and synchronize neuronal activity.

GABAergic neurons comprise a diverse array of anatomically and physiologically distinct neuronal subtypes, which not only make reciprocal connections with excitatory neurons, but also with each other. It is becoming increasingly clear that different GABAergic loops are recruited during different brain rhythms and contribute to their generation and synchronization. Less is known about the precise mechanisms which determine the pattern of recruitment, or how distributed networks can switch between oscillations at different frequencies. Given the complex and dynamic interplay between excitation and GABAergic inhibition, one can appreciate the difficulties in understanding how perturbations in GABAergic signaling might affect brain rhythms, and conversely, what some pathological changes in neuronal synchronization might imply about the status of GABAergic function.

While epileptic seizures are behaviorally-defined phenomena, a cardinal feature is the presence of atypical oscillations in the electroencephalogram (EEG). These ictal oscillations can be comprised of large-amplitude stereotyped events that are apparent even to the untrained observer, but may also be manifested as more subtle abnormalities in rhythmicity, which are inappropriate for a given brain region and behavioral state. Seizures themselves are clearly hyperexcitable events and are commonly assumed to arise from an underlying failure of inhibition to curb runaway excitation. It is known that blocking GABAergic inhibition produces seizures in animal models, but GABAergic signaling remains viable in epileptic tissue from humans and various chronic seizure models. Indeed, it may sometimes even be increased. GABAergic circuits are recruited during seizure activity and are likely to shape the particular temporospatial patterns of ictal activity observed in different epilepsy syndromes. However, this article will explore the possibility that GABAergic signaling could also play a causative role in the generation of pathological synchronization.

Background

Treatments designed to bolster GABAergic inhibition, or directly suppress neuronal excitability, have proved successful in treating many epileptic syndromes. Moreover, it is very rare for such anticonvulsants to exacerbate seizures. At first glance, this situation appears to rule out a major active role for GABAergic inhibition in ictogenesis. However, it should be noted that drugs acting to inhibit the activity of excitatory neurons will have qualitatively similar effects on GABAergic neurons. Moreover, the transition from interictal to ictal activity involves several stages of temporal development, which may have different mechanisms and be subject to different methods of intervention. In the epileptic brain, the primary underlying deficits in neuronal function give rise to seizures only sporadically. Recent developments in computer analyses of EEG signals, designed to help predict ictal onset, show that changes in brain activity can precede seizures by several minutes, even hours. Immediately prior to the

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Synchrony Through GABAergic Inhibition

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Introduction

Activity in the normal brain is synchronized within a variety of network oscillations, the frequency and spatial scale of which vary with behavior. At one extreme are the ultraslow oscillations (<1 Hz) observed during slow wave sleep, which propagate across the entire neocortical mantle and also recruit neurons in the thalamus and striatum. In contrast, the activity within local circuits can display rhythmic synchronization over much shorter time scales, reaching the high gamma-frequency range (80–120 Hz) and beyond. While neuronal networks can generate rhythmic activity exclusively via mutual excitation, brain rhythms across this physiological spectrum are thought to depend upon GABAergic inhibition to both balance excitation and synchronize neuronal activity.

GABAergic neurons comprise a diverse array of anatomically and physiologically distinct neuronal subtypes, which not only make reciprocal connections with excitatory neurons, but also with each other. It is becoming increasingly clear that different GABAergic loops are recruited during different brain rhythms and contribute to their generation and synchronization. Less is known about the precise mechanisms which determine the pattern of recruitment, or how distributed networks can switch between oscillations at different frequencies. Given the complex and dynamic interplay between excitation and GABAergic inhibition, one can appreciate the difficulties in understanding how perturbations in GABAergic signaling might affect brain rhythms, and conversely, what some pathological changes in neuronal synchronization might imply about the status of GABAergic function.

While epileptic seizures are behaviorally-defined phenomena, a cardinal feature is the presence of atypical oscillations in the electroencephalogram (EEG). These ictal oscillations can be comprised of large-amplitude stereotyped events that are apparent even to the untrained observer, but may also be manifested as more subtle abnormalities in rhythmicity, which are inappropriate for a given brain region and behavioral state. Seizures themselves are clearly hyperexcitable events and are commonly assumed to arise from an underlying failure of inhibition to curb runaway excitation. It is known that blocking GABAergic inhibition produces seizures in animal models, but GABAergic signaling remains viable in epileptic tissue from humans and various chronic seizure models. Indeed, it may sometimes even be increased. GABAergic circuits are recruited during seizure activity and are likely to shape the particular temporospatial patterns of ictal activity observed in different epilepsy syndromes. However, this article will explore the possibility that GABAergic signaling could also play a causative role in the generation of pathological synchronization.

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Treatments designed to bolster GABAergic inhibition, or directly suppress neuronal excitability, have proved successful in treating many epileptic syndromes. Moreover, it is very rare for such anticonvulsants to exacerbate seizures. At first glance, this situation appears to rule out a major active role for GABAergic inhibition in ictogenesis. However, it should be noted that drugs acting to inhibit the activity of excitatory neurons will have qualitatively similar effects on GABAergic neurons. Moreover, the transition from interictal to ictal activity involves several stages of temporal development, which may have different mechanisms and be subject to different methods of intervention. In the epileptic brain, the primary underlying deficits in neuronal function give rise to seizures only sporadically. Recent developments in computer analyses of EEG signals, designed to help predict ictal onset, show that changes in brain activity can precede seizures by several minutes, even hours. Immediately prior to the

final transition to a full-blown behavioral seizure, activity must reach a critical state to 'break out' from the epileptic focus; this state in some cases is seen as a flattening of the EEG and emergence of low-amplitude high-frequency oscillations. Of course, the temporal progression towards seizures is different within and between epilepsy syndromes, but the sequence does not appear to be an inevitable cascade. In some patients with intractable temporal lobe epilepsy, as well as in slice models of this type of seizure, it has proved possible to abort the final transition to ictal (or ictal-like) discharges by disrupting synchronization with low-frequency electrical stimulation. Drugs that reduce the overall levels of excitation might prove broadly effective by blocking different aspects of seizure evolution, and thus imply little about the underlying causes.

Current anticonvulsants that produce gross changes in the balance between inhibition and excitation have severe side effects, and more than 25% of epilepsy cases remain drug-resistant. Whether a therapeutic goal proves to be an enhancement or reduction in certain aspects of GABAergic function, the development of more selective and successful treatments will require a more detailed understanding of the functional diversity of GABAergic signaling at the molecular, cellular, and network levels.

GABA Receptor Heterogeneity

GABA acts via two main types of receptors: the ionotropic $GABA_A$ receptors and the metabotropic $GABA_B$ receptors. $GABA_A$ receptors are composed of pentameric assemblies of seven different classes of subunits (α1–6, β1–3, γ1–3, δ, ε, θ, and π), which form ion channels selective for Cl^- and HCO_3^- ions. Not all the possible combinatorial permutations exist in the brain and the rules governing $GABA_A$ receptor assembly and function have been reviewed extensively elsewhere. Suffice it to say that most receptors in mature neurons probably contain either a γ or δ subunit, and that the precise composition determines not only the biophysical properties of $GABA_A$ receptors, but also their cellular localization and pharmacology. For example, δ subunit-containing $GABA_A$ receptors have the highest affinity for GABA, display slow desensitization, and appear to be excluded from synaptic sites. In contrast, $\alpha1\beta2\gamma2$ $GABA_A$ receptors, which are the most abundant in the brain, display a much lower affinity and rapid desensitization, and are localized at synapses.

Benzodiazepines, which are positive allosteric modulators of $GABA_A$ receptors, display powerful anticonvulsant properties. Benzodiazepine-sensitivity is also determined by subunit composition, with binding occurring at the interface between the γ subunit and the α1, α2, α3, or α5 subunits. Switching these α subunits for α4 or α6, or substituting the γ subunit for a δ subunit, eliminates benzodiazepine-sensitivity.

Metabotropic $GABA_B$ receptors display much less diversity. Receptors are composed of either the $GABA_{B1a}$ or $GABA_{B1b}$ subunit isoform, in combination with a $GABA_{B2}$ subunit, to form two types of heteromeric receptors. $GABA_B$ receptors are located both pre- and postsynaptically, where their major respective roles appear to be in regulating transmitter release via the inhibition of Ca^{2+} channels and mediating slow inhibition through the activation of inwardly-rectifying K^+ channels. The subtype of the $GABA_B$ receptor may determine its subcellular localization within a given cell type, but both isoforms can be either pre- or postsynaptic and they cannot yet be distinguished pharmacologically.

Tonic & Phasic GABAergic Inhibition

The activation of synaptic $GABA_A$ receptors, in response to a transient exposure to GABA released from the presynaptic terminals, produces postsynaptic currents with fast rise and decay kinetics, termed phasic inhibition. However, GABA can also escape the synaptic cleft and pool in the extracellular space to activate peri- and extrasynaptic $GABA_A$ receptors and thus generate an 'inhibitory tone.' The ability to detect and transduce such volume transmission requires nonsynaptic $GABA_A$ receptors with a high affinity and low rate of desensitization, and in many brain regions, these criteria appear to be met by δ or α5 subunit-containing $GABA_A$ receptors. While many studies have demonstrated the importance of phasic inhibition for synchronizing neuronal activity, the possible roles of tonic inhibition in modulating network function are yet to be fully explored.

Hyperpolarizing and Depolarizing $GABA_A$ Receptor-Mediated Responses

The response to GABA receptor activation can be hyperpolarizing or depolarizing, depending on the difference between the membrane potential and the reversal potential across the activated ion channel. Unless an experimenter artificially clamps the membrane potential, activation of postsynaptic $GABA_B$ receptors, coupled to K^+ channels, will always lead to hyperpolarization. However, the relationship between the reversal potential across the $GABA_A$ receptor and the neuronal membrane potential varies throughout development, between neurons, and during the course of disease.

In immature neurons, the expression of the NKCC1 chloride transporter produces a net accumulation of intracellular Cl^-, yielding a $GABA_A$ receptor reversal potential positive to the resting membrane potential, and thus enabling GABA to be depolarizing. During the course of development, the expression of the KCC2

chloride transporter becomes upregulated, leading to active extrusion of intracellular Cl^-. Accordingly, in the adult brain, $GABA_A$ receptor-mediated signaling is predominantly hyper-polarizing. However, the expression of KCC2 does not become developmentally-upregulated in at least some classes of GABAergic interneurons and it is likely that the precise nature of GABAergic signaling varies throughout the mature brain.

Recently, there have been several studies suggesting that $GABA_A$ receptors on axonal compartments, including the axon initial segment and presynaptic terminal, may mediate depolarizing responses at resting membrane potentials. This idea harks back to classical electrophysiological studies showing that GABA could produce primary afferent depolarization in the peripheral nervous system. However, as with such studies, it is not yet clear how Cl^- homeostasis is independently controlled in different neuronal compartments, or whether the predominant effect during ongoing activity would be stimulation of release or presynaptic inhibition through inactivation of voltage-dependent conductances.

Rebound Excitation

Even when GABAergic events are hyperpolarizing, they can paradoxically excite neurons by interacting with intrinsic voltage-dependent conductances, thus producing a rebound excitation. An example of this effect is shown in Figure 1, in which GABAergic inhibition across a population of neurons leads to the activation of an intrinsic inward current with a slow time constant (I_h) and subsequently to synchronized rebound firing. GABAergic hyperpolarization could produce similar effects via the deactivation of outward currents, such as I_M, or by the deinactivation of inward currents, such as Na^+ and Ca^{2+} currents.

Diversity of GABAergic Interneurons

Interneurons can be classified into subtypes based on their axonal arborization, with the simplest distinction being those interneurons that selectively target the perisomatic region of excitatory neurons, those that target the dendritic tree of excitatory cells, and those that selectively target other interneurons. Finer distinctions within these broad classes can be made on the basis of intrinsic physiological and synaptic properties, as well as the expression of neuropeptides, calcium-binding proteins, and neurotransmitter receptors. There is increasing evidence that these subtypes of GABAerigc neuron are recruited differentially during different brain rhythms, both within and between distinct brain regions. Moreover, each subtype is thought to modulate different aspects of cellular and network function. We still have a long way to go to link the molecular, cellular, and network aspects of

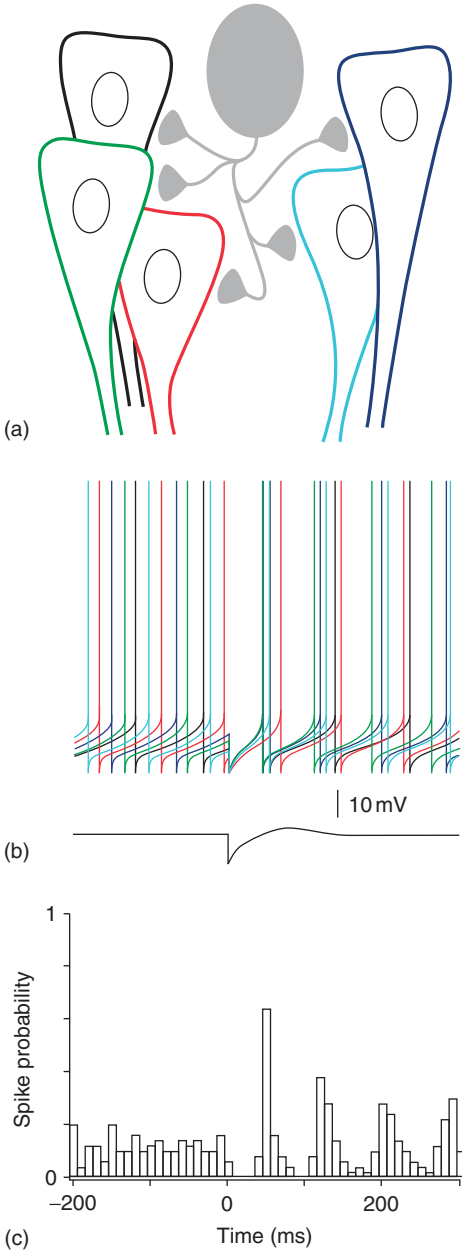


Figure 1 Synchronization via inhibition and rebound excitation. This figure is based on work by Peter Somogyi's laboratory, showing that a single perisomatic-targeting GABAergic interneuron can actively synchronize the spontaneous spiking activity of multiple pyramidal neurons, not only by vetoing spike generation during the period of synaptic inhibition, but also by generating rebound excitation. (a) A single perisomatic-targeting interneuron (grey) makes divergent projections onto a population of pyramidal neurons. (b) A spike in the interneuron produces a $GABA_A$ receptor-mediated inhibitory potential in the pyramidal neurons, which is followed by rebound excitation due to the activation of I_H (lower subthreshold trace). If the pyramidal cells are depolarized to a level that gives spontaneous asynchronous spiking, the postinhibitory rebound induces synchronization over several oscillatory cycles (upper traces). (c) Spike probability histogram centered on the timing of the GABAergic input, and averaged over eight trials, to demonstrate the increase in population synchronization.

GABAergic signaling, but the ability to modulate these GABAergic circuits independently would provide a powerful pharmacological armory for probing neuronal function and also for combatting brain disorders.

Methods

Our laboratory is focused on exploring the multiple facets of GABAergic inhibition using model rodent systems. The various projects take advantage of transgenic mice and chronic disease models to determine both the normal function GABAergic signaling and how these systems change in response to perturbations and contribute to pathogenesis. The analysis of the biophysical and pharmacological properties of different modes of GABAergic signaling is performed routinely using patch-clamp recording from acutely prepared brain slices. How such GABAergic inhibition shapes network activity is explored further in slice models that display intrinsic rhythmic activity and computational models, and with EEG recordings from awake behaving animals.

Recent Results – Evidence for a Causative Role for GABAergic Signaling in Seizure Evolution

While exploring the changes in synaptic transmission and cellular excitability associated with seizures, in both resected tissue samples from human patients and in animal models of epilepsy, it is difficult to distinguish between those changes that predispose the network to seizures and those that represent a homeostatic response to pathological activity. This problem is compounded in patients that have been on extended drug treatments, which are also likely to cause adaptive changes in the neuronal circuitry. Nevertheless, there are a few cases where a role for GABAergic signaling in driving hyperexcitability is emerging.

Interictal Spikes in Mesial Temporal Lobe Epilepsy

The neuronal circuits that are disturbed in epilepsy may give rise to abnormal EEG patterns during the interictal period. These interictal events can be used to localize the seizure focus and aid the diagnosis of the epilepsy syndrome. In focal epilepsies, these EEG abnormalities often include interictal spikes lasting 50–200 ms. Intracranial recordings from patients with mesial temporal lobe epilepsy have shown that interictal spikes do not correlate with massive burst discharges across the neuronal population, but rather suggest that these events are predominantly inhibitory. Indeed, slices of the subiculum resected from these patients continue to display interictal spikes in vitro, where they have been associated with high-frequency firing in GABAergic interneurons and inhibition in the majority of excitatory pyramidal neurons. The small spatially-distributed proportion of pyramidal neurons that do display interictal bursting was found to exhibit decreased KCC2 expression and depolarizing GABAergic responses. The interictal spikes in these slices can be blocked by antagonists of either glutamatergic or GABAerigc receptors, suggesting that interictal spikes are generated by positive feedback loops between interneurons and a selective population of pyramidal cells – an interaction that results in surround inhibition in the bulk of the network.

There is continued debate as to whether interictal spikes represent a pathological state of a network that might be involved in ictogenesis, or whether these events are in fact protective against seizures. Interictal spikes are, by definition, inversely correlated with seizures (i.e., they occur when there are no seizures), but the area of generation of interictal spikes does not always overlap with the determined epileptic focus (i.e., the site of seizure generation). Further, the frequency of interictal spiking has been shown to decrease prior to seizure onset. Greater understanding of the network interactions during the temporal evolution of seizure onset will be required to resolve this relationship between interictal and ictal phenomena.

Changes in Excitability Induced by Febrile Seizures

Complex febrile seizures in children are associated with an increased risk for the development of epilepsy. Experimentally-induced hyperthermia also induces seizures in rat pups and leads to a subsequent reduction in seizure threshold. Interestingly, recordings from hippocampal slices prepared form these animals reveal an enhancement of GABAergic inhibition, without any significant changes in excitatory transmission. A clue as to how such increased inhibition could increase network excitability came from the finding that this change in GABAergic transmission is accompanied by a positive shift in the activation curve of I_h, and thus an enhancement of post-inhibitory rebound firing (see **Figure 1**). Hyperthermia-induced seizures may also lead to other changes in the activity-dependent modulation of GABAergic transmission, but these studies have fostered the idea that increased inhibition might be proictogenic in cortical circuits by promoting both synchrony and hyperexcitability via rebound excitation.

GABAergic Circuits Involved in Thalamocortical Spike-Wave Seizures

Absence epilepsy is characterized by generalized non-convulsive seizures, which are observed in the EEG as periods of repetitive and bilaterally-synchronous ~3 Hz

spike-wave discharges. Understanding the mechanisms underlying absence epilepsy has been aided by several genetic and pharmacological animal models, which display qualitatively similar spike-wave seizures. While there is still debate over the location of seizure focus, there is a general agreement that the stereotypical pattern in the ictal EEG depends on synchronizing mechanisms in the thalamus. A minimal circuit for these paroxysmal oscillations appears to be the reciprocal connections between the inhibitory neurons of the thalamic reticular nucleus (nRT) and the excitatory thalamic relay neurons. In this circuit, excessive synchronized inhibition from the nRT deinactivates T-type Ca^{2+} channels into thalamic relay neurons, leading to rebound bursts and re-entrant excitation. This scenario is consistent with the fact that ethosuximide, which is a clinically-effective treatment for absence seizures, can act to reduce T-type Ca^{2+} currents in thalamic relay cells.

Support for the role of GABAergic inhibition in driving spike-wave seizures comes from the observation that the systemic administration of $GABA_A$ or $GABA_B$ receptor agonists can lead to spike-wave seizures in animal models. Perhaps unexpectedly, benzodiazepines, which augment $GABA_A$ receptor-mediated transmission, have also proved effective in treating both spike-wave seizures in animals and absence seizures in humans. The key to this paradox may lie in the cell type-specific distribution of $GABA_A$ receptor subunits. Many neurons in the nRT appear to be rich in benzodiazepine-sensitive γ-containing $GABA_A$ receptors, while a large number of thalamic relay nuclei contain δ and $\alpha 4$ subunits, which convey insensitivity to benzodiazepines. Therefore, while increased inhibition onto thalamic relay neurons may precipitate absence seizures, benzodiazepines may nevertheless be therapeutic by selectively increasing inhibition within the nRT and thus disrupting pathological synchronization in the thalamic circuitry.

Increased Inhibition in a Mouse Model of Autosomal Dominant Nocturnal Frontal Lobe Epilepsy

Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is characterized by frontal lobe motor seizures that arise most frequently during slow wave sleep. Three loci associated with ADNFLE have been mapped to mutant genes coding for the $\alpha 4$ (CHRNA4), $\alpha 2$ (CHRNA2), and $\beta 2$ (CHRNB2) subunits of the nicotinic acetylcholine receptor (nAChR), while candidates genes for alternate loci remain to be identified. Studies exploring the effects of ADNFLE nAChR mutations in heterologous in vitro expression systems have produced inconsistent results, suggesting a variety of changes in receptor properties that lead to both increases and decreases in nAChR function. These differences may reflect the details of expression and the particular experimental paradigms used. More compelling insights come from a set of mouse strains engineered to carry either the ADNFLE $Chrna4^{S252F}$ or the $Chrna4^{+L264}$ mutant alleles; both the homozygous and heterozygous mice carrying these mutations display spontaneous recurrent seizures. Recordings from pyramidal neurons in the frontal cortex of these ADNFLE mutant mice in vitro demonstrate a large increase in nicotine-evoked inhibition, without significant changes in nicotine-evoked excitation. Furthermore, the animals spontaneous seizures can be inhibited by subconvulsive doses of picrotoxin, a use-dependent antagonist of fast GABAergic transmission. It has yet to be shown how this gain of inhibition could lead to paroxysmal activity, but again it has been suggested that synchronization through post-inhibitory rebound bursting may play a role.

Further Goals

One of the future challenges in this area of research will be to understand how a certain change in inhibition, measured at the level of a single cell, translates into the aberrant activity of a local network. Individual neurons may 'titrate' their inhibition by very small amounts, but these small cellular changes can translate into massive pathological alterations of excitability at the network level. Moreover, to understand how inhibition will affect the local network, we will have to study its modulation at the level of each individual network component. More genetic, molecular biological, and optical approaches are needed to alter inhibition or activity in specific neuronal components of the network, thus providing the needed functional insight into generation of epileptic activity. Considering the importance of inhibition in pathological synchrony, it will be well worth the challenge to design new drugs that are effective only against pathological inhibitory activity.

See also: **Absence Seizures**: Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamocortical Synchronization and Absence Epilepsy; Thalamic Neurons and Networks Related to Absence Epilepsy; **GABA**: $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations.

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Epilepsy Genes that do not Encode Ion Channels
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Sleep-Related Epilepsies and Mutations in Acetylcholine Receptors

ARX Gene Mutations and X-linked Infantile Spasms

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Introduction

The *ARX* gene is a transcription factor, which belongs to a family of paired-class homeobox genes that play a pivotal role in the embryogenesis of all animals, including the development of the central nervous system. *ARX* is a relatively small gene of 12.5 kb, with an open reading frame (ORF) that spans 1686 bp and has an unusually high GC content (72.5%), which complicates polymerase chain reaction (PCR) and sequencing. In the central nervous system, *ARX* is expressed in the cerebral cortex, amygdala, corpus callosum, caudate nucleus and hippocampus, areas implicated in the pathophysiology of seizures, neuropsychiatric disorders, involuntary movements, and learning and memory formation. Studies in humans have demonstrated remarkable pleiotrophy, as several apparently unrelated disease phenotypes have been associated with mutations of this gene. Thus, *ARX* is emerging as one of the most important disease-causing genes on the X chromosome and ought to be considered for routine screening.

One of the disorders in which *ARX* has been implicated is infantile spasms (ISs). ISs are a unique form of seizure disorder, the occurrence of which is limited almost

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entirely to infants during the first year of life, and that is refractory to conventional anticonvulsant drugs. ISs are usually associated with developmental retardation or deterioration and a characteristic electroencephalographic (EEG) pattern (hypsarrhythmia) that together configure a syndrome that is also known as West syndrome (WS). The seizure type is defined as an epileptic spasm; it is seen predominantly in infants, although spasms may also occur in childhood or even in adult patients. ISs have multiple causes, and their pathophysiological mechanism is unknown. Infants with developmental brain abnormalities and those who are small for gestational age are more apt to develop spasms. Familial cases are probably the expression of several genetic disorders, some of which are well characterized, including X-linked lissencephaly and band heterotopia, or X-linked mental retardation and ISs due to mutations in the *ARX* gene.

Background

The first three-generation family with an X-linked recessive form of infantile spasms (ISSXs) was described in 1977. Support for an X-linked form of ISs was also provided by reports of monozygotic male twins and male siblings with this condition. Linkage analysis performed in two additional families with ISSX suggested localization to Xp, in a region distal to DXS1068, with a maximum lod score of 2.36. The mapping was confirmed to Xp22.1-p21.3 and the interval containing the candidate gene was refined to 7.0 cM by studying a western Canadian family with X-linked ISs. *ARX* was considered as a candidate gene underlying ISSX primarily on the basis of its expression pattern in fetal, infant, and adult brain.

In 2002, *ARX* was screened in five ISSX families, and mutations were found in four of them. An additional stretch of seven tandem GCG repeats (c.333_334ins$(GCG)_{10+7}$) within the first normal stretch of 10 GCG triplet repeats in exon 2 (resulting in 16–23 polyalanine repeat expansion) was found in two of the five screened families. A third family had a duplication of 24 bp within the second stretch of GCG triplet repeats in exon 2 (428-451dup (24 bp)), causing 12–20 polyalanine repeat expansion. The fourth family was found to have a deletion of 1517 bp, removing 816 bp of intron 4 and 701 bp of exon 5 (IVS4-816_EX5701del). This deletion was predicted to result in an alternative carboxyl terminus of the ARX protein. In the last family screened, no mutations were found.

In addition to patients with ISSX/WS, *ARX* mutations were identified in patients with Partington syndrome, X-linked lissencephanly with ambiguous genitalia, a rare form of X-linked myoclonic epilepsy with generalized spasticity and intellectual disability, and nonsyndromic X-linked mental retardation. The majority of ISSX mutations identified to date (summarized in **Table 1**) are located in exon 2, which is also the largest coding exon, representing 52% of the *ARX* ORF. In particular, 5 of 6 families described to date with ISSX/WS had a polyalanine expansion in exon 2, harboring the 24-bp duplication 428-451dup (which is considered an *ARX* mutation hot spot), or the 23 polyalanine $(GCG)_{10+7}$ repeat expansion. In addition, a 24-bp duplication (441-464dup) has also been found in a patient with cryptogenic WS.

The role of the polyalanine tracts in the ARX protein, and in other proteins in general, is poorly understood. On the basis of their overrepresentation in transcription factors and in homeobox genes in particular, it has been suggested that they play a role in transcription regulation. Among the four polyalanine tracts present in *ARX* gene, two of them – the polyalanine tracts 1 and 2, (both located in exon 2) – have been directly affected by *ARX* mutations. Little is known about the molecular pathology associated with polyalanine expansions, and in order to explain how these mutations in the *ARX* gene cause disease, a series of polyalanine expansions in *Arx* have been generated. Expression studies in COS cell cultures and mouse brain slices showed that these constructs result in nuclear protein aggregation, filamentous nuclear inclusions, and increased cell death. Furthermore, it has been suggested that different *ARX* polyalanine expansion mutations may aggregate differently: while the $(GCG)_{10+7}$ repeat expansion seems to lead to protein aggregates mostly in the cytoplasm, the 428-451(24 bp) duplication seems to favor precipitation of aggregates in the nucleus. Both mutations were also found to be associated with the repression of the transcription of reporter genes.

Methods

The genetic mapping of the ISSX/WS locus was obtained through classic two point and multipoint linkage analysis, using sets of microsatellite markers on chromosome X. PCR amplification and DNA sequencing of the candidate genes present in the region (selected for their expression profile and/or function) allowed investigators to identify *ARX* as the mutated gene. The wide spectrum of phenotypes associated with *ARX* mutations is a reflection of the complexity associated with the ARX protein structure and function. A detailed analysis of the clinical findings of the patient, and an accurate analysis of the family pedigree, are fundamental in order to consider *ARX* genetic testing – in particular, for less severe *ARX* mutations such as trinucleotide expansions in exon 2 or missense mutations outside the homeobox. In fact, these latter mutations cause syndromes without malformations, including ISs, mental retardation with or without seizures, intellectual disability, and myoclonic epilepsy. EEG analysis is also very important, leading to the identification of typical electroclinical features, such as hypsarrhythmia. For the

Table 1 Reported families and sporadic cases with X-linked infantile spasms, West syndrome, and related disorders

Family	*Year*	*Country*	*Phenotype*	*Affected males*	*Mutation*	*Exon*	*Protein alteration*
1	2002	Canada	ISSX/WS	8	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
2	2002	Belgium	ISSX/WS	2	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
3	2002	Norway	ISSX/WS	7	428-451dup(24 bp)	E2	A12 > A20 polyA expansion
4	2002	Australia	PRTS/WS	11	428-451dup(24 bp)	E2	A12 > A20 polyA expansion
5	2002	Belgium	PRTS/WS	2	428-451dup(24 bp)	E2	A12 > A20 polyA expansion
6	2002	Norway	ISSX/WS	2	IVS4-816_EX5-701del	I4-E5	R483FS/ truncation
7	2003	Japan	WS	1	441-464dup(24 bp)	E2	A16 > A20 polyA expansion
8	2005	Switzerland	WS	3	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
9	2007	Japan	EIEE/WS	1	298_330dupGCGGCA (GCG)9	E2	A16 > A27 polyA expansion
10	2007	Japan	EIEE/WS	1	298_330dupGCGGCA (GCG)9	E2	A16 > A27 polyA expansion
11	2007	Italy	EDE	2	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
12	2007	England	EDE	1	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
13	2007	USA	EDE	1	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion
14	2007	Japan	EDE	2	c.333_334ins(GCG)10+7	E2	A16 > A23 polyA expansion

ISs, X-linked infantile spasms; WS, West syndrome; PRTS, Partington syndrome; EIEE, early infantile epileptic encephalopathy with suppression-burst pattern; EDE, epileptic-dyskinetic encephalophaty.

purpose of genetic testing, genomic DNA is usually extracted from blood samples according to standard procedures; then all coding regions of the gene and the flanking intronic regions are amplified by PCR. PCR products are then analyzed using denaturing high performance liquid chromatography (DHPLC), a fast and reliable technique for screening DNA that can detect variations within minutes, with close to 100% sensitivity and specificity (including single-base substitutions as well as small deletions and insertions in DNA fragments). All the PCR fragments that show an abnormal DHPLC profile are then directly sequenced on an automated capillary sequencer.

Recent Results

Previous reports have described remarkable pleiotrophy among the phenotypes associated with mutations of *ARX*, including several syndromes with or without malformations of the brain. Most patients with ISSX/WS harbor expansions of the two GCG (polyalanine encoding) repeat regions in exon 2 of the *ARX* gene. It is, however, well documented that among these patients there can be considerable variability in clinical presentation, making the phenotypic classification difficult.

Recently, our group broadened the phenotypic spectrum of *ARX* mutations. To investigate the role of *ARX* mutations in cryptogenic ISSX, we performed mutation analysis in 115 boys, including two pairs of brothers, with ISSX and developmental delay. The $(GCG)_{10+7}$ polyalanine expansion was found in six boys from four families (Table 1).

Males carrying the expansion exhibited an epileptic-dyskinetic encephalophaty (EDE), with cognitive impairment, early-onset ISs, and severe generalized dystonia – reflecting the most severe nonmalformation phenotype described in patients with any *ARX* mutation.

A novel observation in this group of severely affected patients is the magnetic resonance imaging (MRI) finding of multiple small foci of abnormal cavitation, possibly reflecting progressive multifocal loss of tissue, in basal ganglia. The basal ganglia have long been implicated in the pathogenesis of dystonia and other movement disorders. Several additional lines of evidence support the hypothesis that abnormalities in basal ganglia might also play a role in the

pathogenesis of ISs. The basal ganglia are, in fact, rich in GABAergic interneurons, the developmental fate of which is known to be influenced by *ARX*. In addition, lesions in subcortical structures, including the basal ganglia, have been implicated in the generation of ISs.

Recently, a novel 33-bp duplication (298_330dup GCGGCA$(GCG)_9$) has been identified in two unrelated patients showing early infantile epileptic encephalopathy with suppression-burst pattern (EIEE, **Table 1**). This epilepsy syndrome is considered as an early variant of ISs; both EIEE and IS patients developed WS.

While early reports in multiplex families suggested that duplication of the second polyalanine tract was the most common mutation of the *ARX* gene, most recent studies (including our own) suggest that expansion of the first polyalanine tract may be more common in sporadic patients. Furthermore, the severity of the phenotypes described in these reports, and the observation that small in-frame c.333_334(GCG) insertions (leading to one to three amino acid expansions of the first polyalanine tract) cause only mild to moderate nonsyndromic mental retardation suggest that expansion of polyalanine tracts is harmful to neurons in a size-dependent manner.

Future Goals

Although the function of polyalanine tracts remains unknown, repeats of several alanine residues are frequent in transcription factors and other proteins with nuclear localization. Recently, the human ARX protein was shown to be a potent transcriptional repressor and, although the domain encompassing polyalanine tract 1 and 2 of *ARX* gene seems not to have a direct role in the repression activity, it has been demonstrated that the expansion of either polyalanine tract 1 or 2 enhances the transcriptional repression activity in a size-dependent manner. This effect could occur by a number of different mechanisms. For example, aggregation of the mutant protein due to polyalanine expansion could result in a loss of transcriptional activity or it could disrupt normal cellular processes due to the aggregates themselves. More systematic studies are needed to better elucidate how polyalanine expansions affect *ARX* function. Toward this goal, the development of mouse 'knock-in' lines harboring the expansion found in children with ISs will provide an essential genetic tool to assess how these mutations affect *ARX* transcriptional repression activity, and will help to clarify the pathogenetic mechanisms leading to the disease. Hopefully, the development of animal models carrying polyalanine expansions will also provide insights regarding the mechanisms by which *ARX* regulates the cell fate (in particular, the fate of GABAergic interneurons) in the developing brain. Valuable insights to address these points will be also obtained from the characterization of the transcriptional activity of the *ARX* gene in patients with polyalanine expansion mutations. Finally, since no neuropathologic studies have been performed so far in boys with polyalanine expansion in *ARX*, such studies should be carried out.

See also: **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood.

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Cytoskeletal Genes and Idiopathic Epilepsies

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Introduction

The neuronal cytoskeleton consists of cellular scaffolding or skeleton, contained within the cytoplasm of the perikarion as well as within adjacent neurites (dendrites and axons). In all cells, the cytoskeleton is a dynamic structure that maintains cell shape, enables cellular motion (using structures such as flagella, cilia, and lamellipodia) and cellular division. In nerve cells, it plays important roles in axonal transport (e.g., the movement of vesicles and organelles). The neuronal cytoskeleton is essentially composed of three types of fibrous structures namely, in decreasing order of their diameters, microtubules (15 nm in diameter), neurofilaments (the intermediate filaments of the neurons; 8–12 nm), and microfilaments (7 nm). The constituent proteins include, respectively, polymers of α- and β-tubulin, neurofilamins (NF-L, NF-M and NF-H), and actin.

Some epileptic syndromes are associated with mutations in genes encoding proteins implicated in the dynamic properties of this neuronal cytoskeleton. This article reviews some of these disorders. As will be illustrated, these initial genetic defects often lead to the production of important brain malformations, due to defaults in either nerve cell division or migration occurring during brain development. Brain development is a very complex process that can be divided into three phases: cell proliferation, neuronal migration, and cortical organization. Dynamic rearrangements of cytoskeleton are clearly implicated in all of these steps. Different microtubule-associated proteins (MAPs) and proteins localized at the centrosome – such as doublecortin (DCX), Map1b, Tau, Lis1, dynein, NudE – are implicated in the regulation of microtubule dynamics and have been shown to be essential for brain development. The epilepsies associated with the genetically based disruption of these cytoskeletal elements are often classified as secondary, lesional, or focal – or even 'acquired' epileptic diseases. The epileptogenic mechanisms of such syndromes are therefore also considered to be 'secondary,' with neuronal hyperexcitability due to an abnormal cortical environment – similar to abnormalities associated with other 'lesional' disorders such as tumors or posttraumatic lesions.

However, in some cases such gene mutations may give rise to a nonlesional epilepsy syndrome. In the latter part of this article, we present our strategy for investigating the biological properties of an unknown gene whose mutations are segregated with juvenile myoclonic epilepsies (JMEs), a clear nonlesional, inherited syndrome. While in most cases, such nonlesional epileptic syndromes have been associated with channelopathies, we present evidence to support our conculsion that in some idiopathic generalized epilepsies (IGEs), the mutated gene is not directly coding an ionic channel protein, but rather a protein associated with the cellular cytoskeleton. Specifically, we focus on a gene encoding a protein called EFHC1 (or Myoclonin 1) that has been found to be mutated in some pedigrees suffering from JME.

On the basis of our work on this genetic disorder, we propose that some IGEs are a consequence of electrical disorders due to subtle microdysgenetic lesions within the cortical mantle.

Background – Epileptic Syndromes Associated with Mutated Cytoskeleton Genes

Some epileptic conditions are now identified as phenotypes co-segregating with defaults in genes encoding proteins of the cytoskeleton and/or vesicle membrane trafficking-interacting proteins. These genes are summarized on Table 1.

Periventricular Nodular Heterotopia

Periventricular nodular heterotopia (PNH) is characterized by aggregates or masses of grey matter along the lateral ventricular wall. This disorder is due to an arrest in the migrational progress of neuroblasts from the periventricular zone to the cortical layers (occurring between the seventh and sixteenth gestational weeks) or a failure in programmed cell death of some neuroblasts within the periventricular germinal matrix. The associated epileptic features are variable: seizures may be generalized or focal, suggesting mesial or neocortical temporal and parieto-occipital onset, with the age of onset usually in the mid teens. PNH has an X-linked dominant mode of inheritance. It displays high rates of embryonic hemizygous male lethality, often exhibiting in females a cell-autonomous mosaic phenotype due to random X-inactivation. Neurons expressing the mutant X chromosome fail to migrate, while those having the normal X chromosome migrate normally.

This condition is, in most cases, associated with mutations of the *filamin 1* gene (*FLNA*) that maps to Xq28. The gene is composed of 48 exons, spans a 26-kb genomic region and codes for the F-actin-binding cytoplasmic cross-linking phosphoprotein Filamin A (FLNA).

Table 1 Some epileptic conditions associated with defaults in genes encoding cytoskeleton and/or vesicle and membrane trafficking interacting proteins

Cytoskeleton and vesicle trafficking interacting proteins	*Genes*	*Loci*	*Properties*	*Types of disorders*		*Inheritance*	*Epileptic features*
				Cellular level	*Tissue level*		
Filamin A, alpha	FLNA	Xq28	Actin binding protein 280 Vascular function	Radial migration	Bilateral PNH	X-linked	Epilepsy very frequent (90%) mild to intractable
Doublecortin	DCX	Xq22.3	Microtubulesassociated protein (MAP)	Radial migration	SBH	X-linked	Epilepsy very frequent Onset before 6 months Intractable
Non catalytic subunit of PAF acétylhydrolase isoform Ib	PAFAH1B1 or LIS1	17p13.3	Nuclear migration along microtubules via dynein motor function	Radial migration	Type 1 Lissencephaly	AD	Epilepsy frequent Intractable
ADP ribosylation factor for guanine nucleotide exchange factor 2 (Brefeldin A inhibited)	ARFGEF2	20q13.13	Intracellular vesicular trafficking	Proliferation; radial and tangential migration	PVH and microcephaly	AR	Early onset seizures Infantile spasms

PAF, platelet activaton factor; ADP, adenosine diphosphate; PNH, periventricular nodular hetrotopia; SBH, subcortical band heterotopia or double cortex; PVH, periventricular heterotopia; AD, autosomal dominant; AR, autosomal recessive.

This protein binds other membrane-associated proteins such as integrins, presenilin1 and glycoprotein Iba, and promotes orthogonal branching of actin filaments. Filamin A is thought to act early during development, and could be part of the molecular machinery leading to the 'switch' that permits a neuron to become competent for subsequent migration. FLNA functions also by structuring actin networks at the cell periphery. In PNH, instead of acting at the leading edge of the migrating neuron, FLNA maintains a static focal contact between a stationary neuron and a neighboring radial glial cell.

Subcortical Band Hetrotopia or Double Cortex and Type 1 Lissencephaly

Subcortical band hetrotopia (SBH) and type 1 lissencephaly (LIS) are both cortical malformations secondary to abnormal migration of neurons during early brain development. Lissencephaly consists in an absence (agyria) or various degrees of decrease (pachygyria) of cortical gyration, leading to the development of an abnormally smooth cerebral surface. In lissencephaly, neurons migrate partially toward their proper cortical destination, but subsequent formation of gyri and sulci fails to occur. Subcortical band heterotopia (SBH) is a related disorder in which there are bilateral bands of grey matter found interposed within the white matter between the cortex and the lateral ventricles ('double cortex').

Mutated genes have been found to cosegregate in families with either classical LIS or SBH. One gene responsible for the autosomal form of LIS is *LIS1*, located on chromosome 17p13.3. A second gene, *doublecortin* (*DCX*), is X-linked. Interestingly, mutations in both genes can result in either LIS or SBH; however, the majority of cases of classical LIS are due to deletions or mutations of *LIS1*, whereas the majority of cases of SBH are due to mutations of *DCX*. *LIS1* mutations result in lissencephaly more severe in posterior brain regions, whereas *DCX* mutations result in LIS more severe in anterior brain regions. Epileptic features vary according the site of the malformation. Patients with missense mutations have less severe defects; they may present pachygyria (and only rarely SBH) and much milder neurological and cognitive impairment. Truncating mutations, in contrast, cause severe lissencephaly.

LIS1 was initially identified as the noncatalytic subunit of platelet-activating factor acetylhydrolase Ib (PAF-AH), an enzyme that degrades the bioactive lipid PAF. Experimentally, treatment with a PAF agonist caused a drastic reduction of the migration of cerebellar granule cells in vitro. LIS1 protein function in PAF-AH could, therefore, be related to its essential role in migration. Moreover, LIS1 protein has been shown to colocalize with microtubules and to promote their stabilization. An ortholog of LIS1 in *Aspergillus nidulans*, nudF, mediates nuclear translocation, probably by interacting with microtubules. Thus, LIS1 protein might exert its effects on migration through microtubules.

DCX mutations classically produce a double cortex phenotype in which a second band of cortical neurons appears within the white matter below the normal cortical layers. This gene encodes isoforms of a highly hydrophilic 40-kDa protein containing several potential phosphorylation sites. DCX proteins are homologous to other CNS protein containing a Ca^{2+}/calmodulin kinase domain, suggesting that the DCX may belong to a novel class of intracellular proteins involved in neuronal migration through Ca^{2+}-dependent signaling. In fact, DCX is a microtubule-associated and stabilizer protein, expressed almost exclusively in immature neurons. DCX stabilizes microtubules and inhibits neurite outgrowth during nerve growth factor-induced differentiation. Neuronal precursors start expressing DCX early after exiting the cell cycle, and continue to express DCX for 2–3 weeks as the cells mature into neurons. Downregulation of DCX begins after 2 weeks, and occurs at the same time that these cells begin to express a marker for mature neurons. Due to the nearly exclusive expression of DCX in developing neurons, this protein has been used increasingly as a marker for neurogenesis.

The interaction of both DCX and LIS1 with microtubules may explain the striking similarities between the lissencephalic phenotypes produced by mutations in these two genes.

Methods – The Genotype–Phenotype Correlations in Epilepsy: A Real Way of the Cross

In recent decades, thanks to advances in molecular biology techniques coupled with those in medical diagnosis, many gene mutations cosegregated with inherited neurological diseases have been identified. However, before declaring that any isolated mutation is responsible for the morbid phenotype, a pathophysiological mechanism must also be proposed and demonstrated. Even with the current capabilities for rapid genotyping, elucidation of the genotype–phenotype relationship may require many years of research. This difficulty is particularly true if the properties of the mutated gene have yet to be established. Examples of such obvious difficulties include the *huntingtin* gene in Huntington's disease, *parkin* for Parkinson disease, and *preselinin* in Alzheimer disease. Much work has been carried out to characterize these new proteins, but full explanation of how the gene is related to the relevant morbid process is often still unclear.

In the field of epilepsies, diseases of brain cortical excitability, the story is slightly different. Many of the initial gene mutations detected within families with inherited epilepsy corresponded to proteins known to be involved in the control of neuronal excitability

(i.e., nicotinic and $GABA_A$ receptors). Thus, many of these seizure disorders have been classified as 'channelopathies.' However, despite these important discoveries, studies of these gene mutations have sometimes failed to produce a complete explanation of the clinical picture.

Recently, a generalized idiopathic epileptic syndrome, JME, has been associated, in some families, with mutations in a gene encoding a protein with unknown properties. How should one go about establishing the correlation between the genotype and phenotype in this case? In our laboratory, such research consists of two phases.

The first phase starts by a systematic screening that include the following steps:

1. Understanding/characterizing the phenotype.
2. Establishing the complete sequence of the gene and the protein, and comparing them to sequences in available databases to identify possible regions with known properties.
3. Determining the distribution of the transcript in the adult organism and during development using Northern blot and/or in situ hybridization methods.
4. Generating and validating a poly- or monoclonal antibody against a sequence of the protein in order to estimate its level of translation and its distribution within the organism (and in particular in the brain), both in adulthood and during development. This goal can be achieved using Western blot and/or immunohistochemistry methods.
5. Determining the subcellular distribution of the protein in vitro in cell lines using immunocytochemistry and/or overexpression of protein coupled to fluorescent marker.
6. Identifying the protein partners using yeast two hybrid, pull down and/or immunoprecipitation methods.

Investigations during the *second phase* depend upon the results obtained during the first phase. Within the context of a gene related to an epilepsy phenotype, the studies will obviously focus on brain tissue, with functional electrophysiological experiments used within relevant experimental models in which plausible pathophysiological mechanisms can be tested. Production of a knock-in mouse model, reproducing the human genotype of interest (together with at least a part of the human phenotype) is the 'gold standard' for models studies focused on the role of the target gene in the development of the particular epileptic disease.

Results – An Example of Genotype–Phenotype Work: JME and EFHC1 or 'Myoclonin1'

First Phase

Characterizing the phenotype

JME is the most common form of IGEs and the primary cause of hereditary grand mal seizures. Most reports calculate that JME accounts for 10–30% of all epilepsies. JME is a familial disorder inherited with a complex mode of transmission.

JME phenotypes can be distinguished as two major and two minor subsyndromes. Classic JME (72%) is characterized by the adolescent (13–15 years) onset of myoclonic jerks on awakening, tonic–clonic seizures appearing two years later, with no absence seizures. The second major subsyndrome is characterized by childhood absence evolving to JME (18%). Pyknoleptic absence, with 3 Hz spike and wave complexes and 4–6 Hz spike and polyspike-waves complexes then persist into adolescence, when myoclonias and convulsive seizures appear. The two minor forms consist of adolescent myoclonias, and 'grand mal' seizures with absences that start during adolescence (or even after 18 years), some rare cases being associated with astatic drop attack. Electroencephalographs consistently show diffuse 3–6 Hz multispike wave complexes between seizures and diffuse rapid spikes during actual myoclonias.

To date, 15 chromosomal loci have been associated with JME by linkage analysis. Three genes (below) are known to be involved in different pedigrees of monogenic JME. The first two genes are obviously implicated in the neuronal excitability, whereas the last one is a gene of unknown function.

GABRA1 (EJM5 – 5q34)

This gene encodes the α_1 subunit of the $GABA_A$ receptor. It exhibits an A322D mutation in one French and one large French–Canadian JME family. This mutation results in a lesser amplitude GABA current in vitro, and also reduces the α_1 subunit trafficking at the synaptic level.

CLCN2 (EJM6 – 3q26)

This gene encodes a voltage-gated chloride channel. Mutations (M200fsX231, 74–117del and G715E) have been shown to reduce in vitro the transmembrane chloride gradient, thus potentially reducing GABA inhibition.

EFHC1/Myoclonin (EJM1a – 6p12)

This gene encodes a protein of unknown function. Mutations in EFHC1 seem to be the most common in JME patients. Molecular analysis reveals missense, nonsense, frameshift-deletion and deletion mutations found in six separate cohorts. Mutations appear more commonly in Hispanic JME population from California, Mexico, and Honduras, but also appear in Caucassian families from Italy and Austria.

What can we learn from the gene structure and the encoded protein?

The *EFHC1* gene (EF-hand domain containing 1) is located on chromosome 6p12.3, has a size of 72 kb and encodes a 640 amino acid protein of 73 kDa. This protein

contains three DM10 domains of unknown function and a single EF hand motif. This last sequence is part of protein structure often implicated in calcium homeostasis. In human, alternative splicing at exon 4 generates a splice variant encoding a truncated protein of 278 amino acids, with 240 common to the long transcript.

Distribution of the transcript in various tissues

The distribution of EFHC1 mRNA in various tissues has been verified by quantitative RT-PCR, northern blot and in situ hybridization. EFHC1 transcript is present in all studied organs of adult mouse. It is expressed at high levels in testis, lung, muscle, and thymus. Lower transcription levels were observed in the cerebral cortex, uterus, ovary, stomach, intestine, kidney, liver, and heart.

We also studied the EFHC1 transcript expression during mouse brain development. The expression was highest at E14, and then progressively decreased until 10 days after birth, at which time it stabilized at the adult level. In brain, EFHC1 mRNA is therefore essentially expressed at early stages of development, when nerve cells are dividing and organizing the cortical mantle cytoarchytecture.

The distribution of the transcript is also available on the Allen Brain Atlas web, indicating the presence of the transcript in various neuronal layers, in particular, in hippocampus (dentate gyrus) and cerebellum, as well as in ependymal structures and choroids plexus. In human, Northern blot and EST counting has demonstrated that the transcript is expressed in many tissues (including testis, pharynx, pituitary gland, lymph nodes, bone marrow, kidney, brain, heart, and pancreas at high levels, and many more at lower levels).

Distribution of the protein in various tissues

Using validated antibodies, we started to revisit (previous results were presented with nonvalidated antibodies) the distribution of the protein in the adult mouse brain and during development. Expression of EFHC1 in the adult brain was shown in neurons in the hippocampus CA1, cerebellum, and cerebral cortex. The staining appeared to be localized in the nucleus and in the cytoplasm. Immunostainings using a different antibody also showed the presence of EFHC1 in neuronal cells in the hippocampus and the cortex, with stronger signal in pyramidal neurons. In hippocampal neuronal cells in culture, the same cellular localization was observed; the neuronal processes were also weakly stained. A significant staining was also observed on the border of the lateral ventricles lining with ciliated ependymal cells. These data are in agreement with results from in situ hybridization performed on mouse brain (see the on-line Allen Brain Atlas).

Immunohistochemistry in developing mouse brain shows protein expression at E16, with strong staining at the border of the cerebral ventricles as well as in the choroid plexus. Significant immunostaining was also observed in neocortex, piriform cortex, and striatum. At the subcellular level, EFHC1 antibody recognized principally a cytoplasmic material, quite radially organized. Therefore, not only the quantitative expression but also the subcellular localization of EFHC1 appears to be modulated during development. Western blot has confirmed that the protein expression is also higher during development compared to adult. All together, these findings strongly suggest that EFHC1 may contribute to the development of the nervous system.

Intracellular localization

To study the subcellular distribution of EFHC1, we overexpressed EFHC1 coupled to EGFP, a fluorescent marker, in different cells lines (HEK293, COS7, HeLa, NIH3T3, and neuroblastoma). In interphase cells, the fusion protein was present throughout the cytoplasm and in the nucleus, but was absent from the nucleoli. One, or occasionally two, intensively fluorescent spots were observed close to the nuclei. However, the most prominent characteristic was observed during mitosis, as EGFP–EFHC1 showed a strong colocalization with the mitotic spindle poles and the midbody (the microtubule-based intercellular bridge at cytokinesis). These results suggest the involvement of EFHC1 in cell division, and in particular, in the organization of the mitotic spindle and the midbody.

The localization of the above-mentioned strongly fluorescent spots close to the nucleus, and the presence of two spots in some cells, suggested an association of the protein with the centrosome. To substantiate this hypothesis, we performed colocalizations by using a fluorescent anti-γ-tubulin antibody, a well-established centrosomal marker. We indeed observed a strong colocalization of γ-tubulin with the EGFP–EFHC1 spots in interphase cells and at spindle poles during mitosis. Thus we suggest that EGFP–EFHC1 could be a centrosomal component.

We also compared the distribution of the EGFP-tagged protein with the endogenous EFHC1 using a specific antibody. By indirect immunofluorescence we observed the presence of EFHC1 in the cytoplasm and nuclei during interphase in different cell lines. In interphase cells, intense spots close to the nuclei were detected. These spots were identified as centrosomes using an anti-γ-tubulin costaining. During mitosis, the distribution of the endogenous EFHC1 was the same as the overexpressed protein. The association with the mitotic spindle and the midbody (**Figure 1**) was observed in all above-mentioned cell lines.

EFHC1 contains three DM10 domains of unknown function. To investigate the role of DM10 domains in its association with the mitotic spindle, HEK293 cells were transfected with various truncated forms of EGFP-tagged EFHC1 and processed for immunofluorescent staining

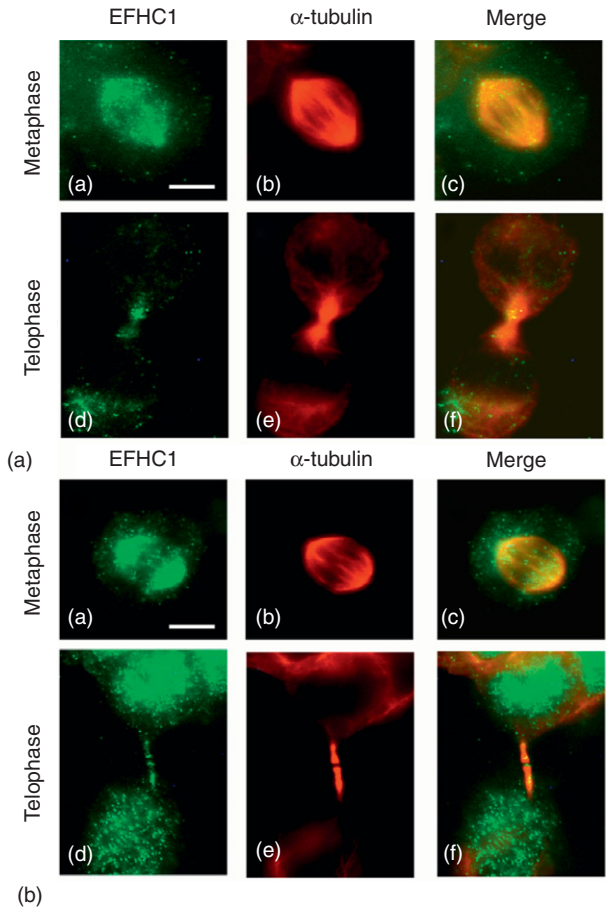


Figure 1 Association of EFHC1 (Myoclonin1) with the mitotic spindle in HEK293 and neuroblastoma cells. HEK293 (A) and N2A (B) cells were immunostained using anti-EFHC1 antibody (a, d, in green) and anti-α-tubulin antibody (b, e, in red) during metaphase (a–c) and telophase (d–f). The corresponding merged images are shown on right panels (c, f). Epifluorescence images. Scale bar: 10 μm. Note the characteristic staining of the midbody in (B-f).

using an anti-α-tubulin antibody. Different EFHC1 chimeras demonstrated their distinct capacities for localization to the mitotic apparatus. All constructs that contained the N-terminal portion of the protein localized to the mitotic spindle and midbody, whereas C-terminal constructs did not. The N-terminal part of the protein, containing only the 92 first amino acids of EFHC1, still associated with the mitotic apparatus; however, the truncated form containing the DM10 domains and EF-hand did not. We therefore conclude that the N-terminal portion is required to address EFHC1 to the mitotic spindle and the midbody.

Protein–protein interaction

To date, interaction between EFHC1 and the C-terminus of R-type voltage-dependent Ca^{2+} channel ($Ca_v2.3$) has been suggested by immunoprecipitation studies. Another potential interacting partner, TEX11 (a testis-expressed sequence of unknown function), has also been proposed after yeast two hybrid experiments. In our hands, preliminary results indicate a specific EFHC1 interaction with nucleoporin 54, creatine kinase B, C18orfl, SUI1, ProSApiP1 and three unknown sequences. However for all of them, additional experiments (such as pull down and immunoprecipitation assays) are needed to confirm these putative interactions.

Second Phase

Taking all the above information together, the role of EFHC1 is still unclear. However, based on these data, we have hypothesized that EFHC1 can either play a role in intracellular calcium signaling and/or in cell division during neuronal development.

Is EFHC1 a calcium sensor?

C-terminal EF-hands are found in neuronal calcium sensors of the calmodulin family, Ca^{2+} ATPase, Ca^{2+} activated K^+ and Ca^{2+} channels, and various novel calcium binding proteins such as calsenilin, DREAM, and KchIP3. By modulating calcium concentrations, the EF-hand superfamily regulates neurotransmitter release, excitability and synaptic plasticity. Comparison of EFHC1 with calcium binding proteins from mammals, *Drosophilia*, *Caenorhabditis elegans*, and yeast revealed significant evolutionary conservation for key residues in the EF-hand motif. A recent study demonstrated that the EF-hand domain of EFHC1 bind to one divalent ion (Ca^{2+} or Mg^{2+}) under reduced condition. Further reports indicated that the overexpression of EFHC1 in hippocampal neurons in vitro induced apoptotic cell-death, which was significantly reduced by any of the mutations. This proapoptotic effect of EFHC1 was assigned to its enhancement of calcium influx through the R-type voltage-dependent Ca^{2+} channel ($Ca_v2.3$). EFHC1 mutations partly reversed this effect. The investigators carrying out those studies suggested that an incomplete reversal of EFHC1-induced calcium influx through $Ca_v2.3$ may be responsible for a precarious state of calcium homeostasis in JME patients. A higher density of neurons with precarious calcium homeostasis could, in theory, enhance a hyperexcitable state. In a sense, this hypothesis is similar to hypotheses suggested for other epileptic syndromes in which channelopathies or ion exchange modifications have been proposed as central causes of the clinical pictures.

Is EFHC1 implicated in the organization of the cytoskeleton?

More interestingly, in 2005, Ikeda et al. found that EFHC1 is a homolog of *Chlamydomonas* axonemal protein Rib72 and is expressed in tissues that have motile cilia and flagella. Since it is known that motile cilia of ependymal

cells are necessary for the correct migration of neuroblasts in the adult mouse brain, the mutations of EFHC1 could produce ependymal ciliary motility defects leading to abnormal GABAergic neuron cytoarchitecture in the neocortex, resulting in epileptogenesis. This hypothesis is supported by recent work demonstrating the presence of EFHC1 in ependymal cells.

As shown above, we have demonstrated that EFHC1 associates with the centrosome and the mitotic spindle through its N-terminal region. These results strongly suggest a role for EFHC1 in the regulation of the cellular cytoskeleton. Consequently, our current efforts have focused on the following questions:

Can EFHC1 plays a role during cell division?
Does EFHC1 have particular interactions with some proteins of the cytoskeleton?
If EFHC1 can participate in cell division, how can it affect CNS function?

Since neuronal precursors are dividing in CNS during the early stage of brain development, what could be the influence of the inactivation of the EFHC1 gene on neuronal proliferation and migration?

We recently started to answer this latter question using the in utero electroporation technique combined with RNA interference (RNAi). In brief, this approach involves an intraventricular injection, in utero, of a DNA plasmid solution, subsequently submitted to a brief electrostatic field. The plasmid is incorporated within the dividing cells along the ventricular wall of an appropriately developing hemisphere, so that the migration patterns can be further observed either in vivo or in vitro (using brain slices), under different conditions. With this technique, we have confirming the subcellular localization of EFHC1, suggesting that EFHC1 could have similar functions in vivo to those previously demonstrated in vitro. Next, we will analyze the effect of EFHC1 loss of function on radial migration. Embryos will be subjected to electroporation with plasmids encoding control shRNA or EFHC1 shRNA along with plasmid encoding EGFP to mark transfected cells. The position of $EGFP^+$ cells in different neocortical subregions will be analyzed four days after electroporation. Our unpublished results look very promising.

Directions for Future Research – Working Hypothesis: IGEs as a Disorder of Neuronal Cell Division or Migration?

Based on all the above-mentioned data, we hypothesize that IGE, as JME, could be due, in part, to subtle neuronal migration disorders. If EFHC1 controls cell division and neuronal migration, a gene mutation causing functional protein abnormalities could lead to subtle migration defects. During the later phases of development, subsequent reshaping of synaptic circuits could lead to progressive epileptogenic characters. Rare and still controversial indications in the literature support this working hypothesis. For example, autopsy studies of a patient with JME have shown cortical and subcortical 'dysplastic' neurons and other subtle abnormalities of intracortical architecture called 'microdysgenesis.' Quantitative MRI studies have demonstrated an increase in cortical grey matter in some JME patients. Such defects could be caused by abnormalities during specific stages of cortical development.

Therefore, the main goal for future research is to determine if JME is indeed a developmental disorder. To answer this question, we will modulate EFHC1 expression during brain development by in utero electroporation combined with RNAi, and study the influence of EFHC1 inactivation on neuronal migration. If any neuronal migration defect is found, we will let the animals carrying such a lesion survive and study (in adulthood) the consequence of these defects on neuroanatomical and electrophysiological properties of the cerebral cortex. We hypothesize that a subtle abnormal migration defect could produce an epileptogenic lesion under these conditions. If this hypothesis is correct, then categorization of all IGEs as classical channelopathies must be revised.

See also: **Genetics**: *ARX* Gene Mutations and X-Linked Infantile Spasms; Genetic Contributions to Brain Structure and Epileptogenesis; Genetics of Lafora and Juvenile Myoclonic Epilepsies.

Further Reading

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Epilepsy Genes that Do Not Encode Ion Channels

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Introduction – Non-ion Channel Genes in Human Epilepsy

Epilepsy can be viewed as arising from the inability of the brain to control the balance of excitatory and inhibitory neurotransmission. Since ionic content determines the electrical potential of a cell, proteins that allow the flow of ions across the plasma membrane are the primary determinants of excitability state. Naturally, mutations in the genes that encode these channels are logical candidates for causing disruptions in brain excitability – such as seen in familial idiopathic epilepsies. It is therefore no surprise that two-thirds of the few known genes for idiopathic epilepsy encode ion channels. Still, several known human epilepsy genes do not encode ion channels. Furthermore, since most idiopathic familial epilepsy is genetically complex, the 20 or so known human epilepsy genes are likely to be just a fraction of those that are at play in nature.

It is tempting to consider, as a group, epilepsy genes that do not encode ion channels. As shown in **Table 1**, whether proven or proposed as candidates based on association studies, this group consists of a very small number of genes – too small, in fact, to draw systematic conclusions. However, with ion channels so important for epilepsy, one might at least expect that some of these genes may be directly involved in modifying channel activity. Indeed, this may be the case for *EHFC1*, which encodes a Ca^{2+}-sensing protein that may be involved in regulating the activity of R-type voltage-dependent calcium channels; EFHC1 mutations can cause juvenile myoclonic epilepsy, but the role of calcium channel misregulation in disease is not yet understood. More generally, one can imagine how a candidate gene such as *BRD2*, which is likely involved in gene transcription, may be an upstream regulator of certain ion channel genes. But as noted, we cannot yet draw any general conclusions.

Background – Insight from Epileptic Mice

At the present time, a richer source of information about candidate epilepsy genes is the larger number that renders seizure disorders when mutated in laboratory mice. We can use this group to begin to systematically assess the pathways that can lead to epilepsy. The Mouse Genome Informatics group at The Jackson Laboratory has assembled the world's largest mammalian genetics database, annotating information from all published articles according to mammalian phenotype ontology, and reflecting a community effort to provide standard terms for annotating mammalian phenotypic data.

Given 'seizure' as a search term, the database yields 467 allelic variants, from some 335 independent genes that, when mutated, yield a seizure-related phenotype of one kind or another. When considering relevance to epilepsy, it seems best to limit this set to genes that are associated with spontaneous (or naturally induced – e.g., by routine handling) seizures and for which the experimental evidence is the clearest. Therefore, after excluding mutants that only showed altered seizure threshold (but not spontaneous seizure), after excluding those spontaneous seizures noted in mice over 10 months of age (because some mixed strain backgrounds can exhibit age-related spontaneous seizures due to polygenic effects), and after eliminating several ion channels (but not metabotropic receptors or electroneutral ion transporters), 82 genes remained (**Table 2**). Although only some papers describing these mutant mice have included electroencephalographic evidence of seizure discharge, it is likely that most of these examples are mice that exhibit bona fide tonic–clonic and generalized seizures; the behavioral descriptions usually include characteristic features such as Straub tail, ventral or dorsal neck flexion, jaw clonus, forelimb clonus, loss of balance, and excessive salivation and/or defecation, followed by a quiescent period.

At a glance, the 82 genes appear to represent a rather wide set of cellular processes, ranging from cytostructural (e.g., myosin), to neurotransmitter receptor ($GABA_B$), to synaptic vesicle functions (e.g., synaptojanin-1), to cellular signaling (sonic hedgehog). One advantage of database-friendly annotation is that computational tools can be exploited to gain a systematic overview of how functional classes relate to each other and to the group that defines them (i.e., seizure disorder). Using a prototype visualization tool called VLAD (VisuaL Annotation Display), we can observe how these 82 molecules cluster according to gene ontology (GO) functional annotation (**Figure 1**; *NB*:For the analysis, we also added the few non-ion channel human epilepsy genes listed earlier). The first impression supports the anecdotal observation that a wide variety of functions are encoded by genes associated with seizure disorder in mice. The GO category with the largest number of seizure genes is involved in cellular component organization/biogenesis. Naturally, the composition of this group is very mixed – containing synaptic vesicle component proteins (*Syn1*, *Syn2*, *Snap25*),

Table 1 Non-ion channel genes known or suspected in human idiopathic epilepsy

Epilepsy type	*Gene*	*Encoded protein*	*Biol. Function*	*Chr*
Autosomal dominant epilepsy with partial auditory features	*LGI1*	Leucine-rich repeat LGI family, member 1	Unknown (secreted glycoprotein)	10q24
Febrile seizures	*GPR98 (MASS1)*	G protein-coupled receptor 98	Unknown (membrane protein)	5q14
Febrile seizures	*IMPA1*	Inositol (myo)-1/4 monophosphatase 1	Inositol metabolism	8q21.13-q21.3
Idiopathic generalized epilepsy	*ME2*	Malic enzyme 2, NAD(+)-dependent, mitochondrial	Malate metabolism	18q21
Juvenile Myoclonic Epilepsy	*EFHC1*	EF-hand domain (C-terminal) containing 1	Ca^{2+} binding; regulates VDCC	6p12-p11
Juvenile myoclonic epilepsy	*BRD2*	Bromodomain-containing 2	Transcriptional regulation?	6p21.3

metabolic enzymes (*Sod2*, *Hexa*), as well as genes encoding adaptor complex proteins (*Ap3b2*, *Ap3m2*). However, VLAD also shows the relative significance of each functional category, as a function of the number of seizure genes relative to the number of genes that exist altogether in a GO category. The darker circles in **Figure 1** highlight the more statistically significant groups. The two most significant groups are cell–cell signaling and nervous system development – the latter including transcriptional regulators *Bmi1*, *Emx1*, *Neurod2*, *Otx1*, and *Pten* as well as other cell-signaling molecules. One can imagine several ways in which these genes are crucial for preventing seizures; for example, they may regulate the maturation of cellular networks and synapses, or regulate the expression of ion channel gene transcription directly.

As large as this list may seem, 82 is certainly an underestimate of non-ion channel genes, which, when mutated, can cause epilepsy – especially given the ascertainment bias inherent in the dataset. That is, most alleles are man-made gene knockout mutations, resulting in little or no normal protein. Only a few mutant alleles encode missense or gain of function mutations. In nature, one finds a good mix of both reduced function and altered function. Still, even for the list of 82 genes, an entire encyclopedia would be required to discuss the potential roles of these molecules in epilepsy. As a recent example, we will focus on a new non-ion channel seizure gene, *Brunol4*, which causes a complex seizure disorder in mice.

Methods

Genetic approaches to understanding gene function are often classified in one of two categories: 'forward genetics' and 'reverse genetics.' Forward genetics means simply that the molecular identity of the gene is not known ahead of time, and that 'bottom–up' genetic or genomic methods are used to find it. The canonical 'forward' approach is called *positional cloning*, whereby the causative gene is identified by virtue of its position on a chromosome. Classically, this process consists of 'walking' along the chromosome (from a genetic marker on one side of the chromosomal interval associated with a disease to the other side), characterizing and evaluating molecular variants (e.g., by gene expression or structure analysis) along the way until one is found to be a strong possibility (i.e., a gene likely to be the cause of the disease). This possibility is then independently tested (e.g., by creating and testing a new allele, or performing a rescue experiment, or both). A variant of *positional cloning* is *positional candidate* – a slightly less systematic approach whereby the chromosomal interval or region of interest is known, and researchers make educated guesses about the causative gene – based on the position or nature of a nearby gene – and then evaluate molecular variants. Some transgenic approaches may be considered to be positional candidate – for example, when a transgene inactivates an endogenous gene (as in the case of *Brunol4*, described later); the endogenous gene is then retrieved based on its proximity to the transgene and is evaluated in a similar manner.

'Reverse genetics' is the opposite of forward genetics, in that the gene is already hypothesized to be a candidate for a particular phenotype (e.g., based on other studies) and then is purposefully mutated (e.g., in a 'knockout' or 'knockin' mouse) to test the hypothesis. Most targeted mutation studies in mice to date represent a reverse type of approach.

Recent Results

Complex Seizure Phenotype in *Brunol4* Mutant Mice

In 2007, a new mouse model was described with features of a genetically complex seizure disorder. The most obvious abnormality in heterozygous mutants was recurrent, tonic–clonic, and generalized seizures, which could be precipitated by routine animal care (e.g., moving mice into a clean pen), with onset during middle age (e.g., after 2–3 months). Because the behavioral manifestion of the tonic–clonic component can be quite wild, the mutant

Table 2 Non-ion channel genes associated with spontaneous or handling-associated seizures in mutant mice

Gene	*Encoded protein*	*Gene*	*Encoded protein*
Acp2	Acid phosphatase 2, lysosomal	*Htra2*	HtrA serine peptidase 2
Adam22	A disintegrin and metallopeptidase domain 22	*Jrk*	Jerky
Aldh5a1	Aldhehyde dehydrogenase family 5, subfamily A1	*Myo5a*	Myosin Va
Amph	Amphiphysin	*Neurod2*	Neurogenic differentiation 2
Ank3	Ankyrin 3, epithelial	*Npy*	Neuropeptide Y
Ap3b2	Adaptor-related protein complex 3, beta 2 subunit	*Nr4a3*	Nuclear receptor subfamily 4, group A, member 3
Ap3m2	Adaptor-related protein complex 3, mu 2 subunit	*Numb*	Numb gene homolog (Drosophila)
Asc1	Anterior suture cataract 1	*Numbl*	Numb-like
Atcay	Ataxia, cerebellar, Cayman-type homolog (human)	*Otx1*	Orthodenticle homolog 1 (Drosophila)
Atxn7	Ataxin 7	*Pcmt1*	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase 1
Bax	Bcl2-associated X protein	*Pitpna*	Phosphatidylinositol transfer protein, alpha
Bckdha	Branched chain ketoacid dehydrogenase E1, alpha polypeptide	*Plaur*	Plasminogen activator, urokinase receptor
Bmi1	Bmi1 polycomb ring finger oncogene	*Plcb1*	Phospholipase C, beta 1
Brunol4	Bruno-like 4, RNA binding protein	*Plp1*	Proteolipid protein (myelin) 1
Bsn	Bassoon	*Pmp22*	Peripheral myelin protein
Cdk5r1	Cyclin-dependent kinase 5, regulatory subunit (p35) 1	*Ppt1*	Palmitoyl-protein thioesterase 1
Cnp	2′,3′-Cyclic nucleotide 3′ phosphodiesterase	*Psap*	Prosaposin
Cplx1	Complexin 1	*Pten*	Phosphatase and tensin homolog
Cstb	Cystatin B	*Pura*	Purine-rich element binding protein A
Ctnnb1	Catenin (cadherin associated protein), beta 1	*Qk*	Quaking
Ctsd	Cathepsin D	*Scg5*	Secretogranin V
Dbnl	Drebrin-like	*Serpine2*	Serine (or cysteine) peptidase inhibitor, clade E, member 2
Dbp	D site albumin promoter binding protein	*Sgce*	Sarcoglycan, epsilon
Drd1a	Dopamine receptor D1A	*Shh*	Sonic hedgehog
Emx1	Empty spiracles homolog 1 (Drosophila)	*Slc12a5*	Solute carrier family 12, member 5
Epm2a	Epilepsy, progressive myoclonic epilepsy, type 2 gene alpha	*Slc13a1*	Solute carrier family 13 (sodium/sulfate symporters), member 1
Fgf14	Fibroblast growth factor 14	*Slc1a2*	Solute carrier family 1 (glial high-affinity glutamate transporter), member 2
Fyn	Fyn proto-oncogene	*Slc25a12*	Solute carrier family 25 (mitochondrial carrier, Aralar), member 12
Gabbr1	γ-Aminobutyric acid (GABA-B) receptor, 1	*Slc2a1*	Solute carrier family 2 (facilitated glucose transporter), member 1
Gabbr2	γ-Aminobutyric acid (GABA) B receptor 2	*Slc9a1*	Solute carrier family 9 (sodium/hydrogen exchanger), member 1
Gad2	Glutamic acid decarboxylase 2	*Snap25*	Synaptosomal-associated protein 25
Gjb1	Gap junction protein, beta 1	*Sod2*	Superoxide dismutase 2, mitochondrial
Gjc2	Gap junction protein, gamma 2	*Sox1*	SRY-box containing gene 1
Gnao1	Guanine nucleotide binding protein, alpha O	*Syn1*	Synapsin I
Gng3	Guanine nucleotide binding protein (G protein), gamma 3	*Syn2*	Synapsin II
Grm7	Glutamate receptor, metabotropic 7	*Synj1*	Synaptojanin 1
Helt	Hey-like transcription factor (zebrafish)	*Tal2*	T-cell acute lymphocytic leukemia 2
Hexa	Hexosaminidase A	*Tef*	Thyrotroph embryonic factor
Hexb	Hexosaminidase B	*Ube3a*	Ubiquitin protein ligase E3A
Hlf	Hepatic leukemia factor	*Usp18*	Ubiquitin-specific peptidase 18
Htr2c	5-Hydroxytryptamine (serotonin) receptor 2C		

allele was dubbed 'frequent-flyer' (allele symbol *Ff*). Heterozygotes also have a reduced seizure threshold, are slightly hyperactive, and gain more weight later in life compared with wild-type littermates. Interestingly, homozygous mutants (two copies of the transgene) have a much more severe phenotype, including lethality of some mice within the first week of life; those mice that survive show convulsive seizures by 3–4 weeks of age. Moreover, homozygotes that are not on a C57BL/6J strain background display spike-wave discharges (SWD), the hallmark of

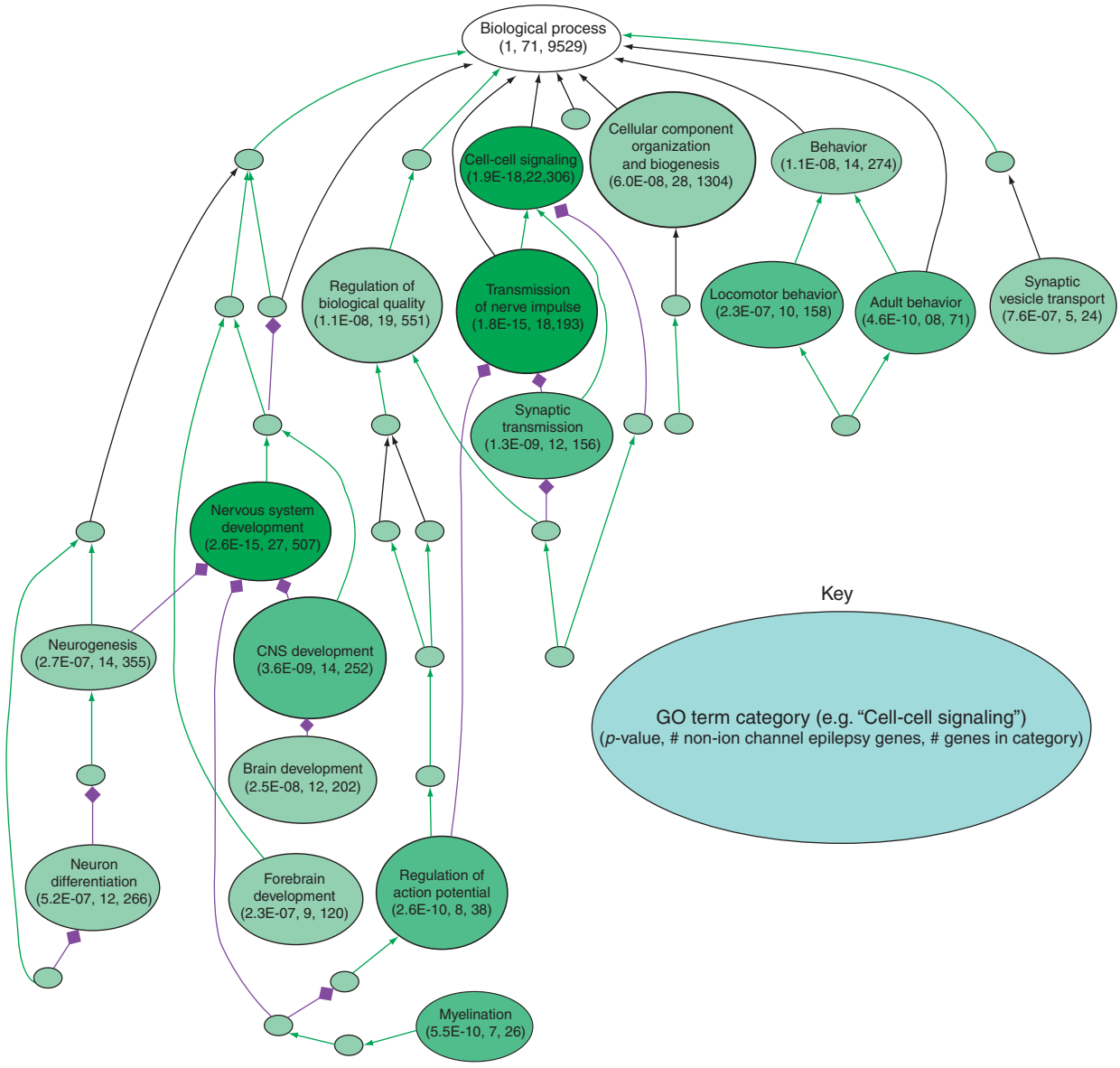


Figure 1 A classification of non-ion channel epilepsy genes using gene-ontology (GO) functional annotation. The prototype clustering tool, VLAD (see text), was used to categorize 82 mouse genes associated with convulsive seizures, and the human non-ion channel epilepsy genes from **Table 1**, according to their GO functional annotation. Individual genes can be members of different categories. The ovals show the categories according to statistical significance (p value obtained from the number of epilepsy genes compared with the total number of genes in a particular GO category). Darker green indicates more significant.

absence epilepsy (**Figure 2**). Gross neuroanatomical abnormalities, such as cell death or hypoplasia, were not found in either heterozygous or homozygous mutant mice, although assessment of specific neuronal populations was not done.

Because the phenotype arose in a strain carrying a non-functional transgenic insertion, and since seizures and the transgene cosegregated with each other, the abnormalities were most likely due to insertional inactivation of an endogenous gene. Genomic sequencing of the transgene insertion site revealed that the inactivated gene encodes the RNA binding protein, BRUNOL4 (Bruno-like 4), and that the effect of transgene insertion was to reduce the amount of steady-state Brunol4 mRNA to a very low level. Subsequent analysis of gene-targeted *Brunol4* null mutants, with the same phenotype, confirmed the gene identity.

Bruno-like RNA binding proteins have been shown, mostly from molecular biology studies, to be involved in multiple aspects of RNA processing such as pre-mRNA splicing, mRNA editing, and RNA stability and translation. There are six Bruno family members in humans and mice, with orthologs in nematode and fruit fly. *Brunol4* is expressed broadly during midgestation, but with maturity the expression becomes limited to the brain and is

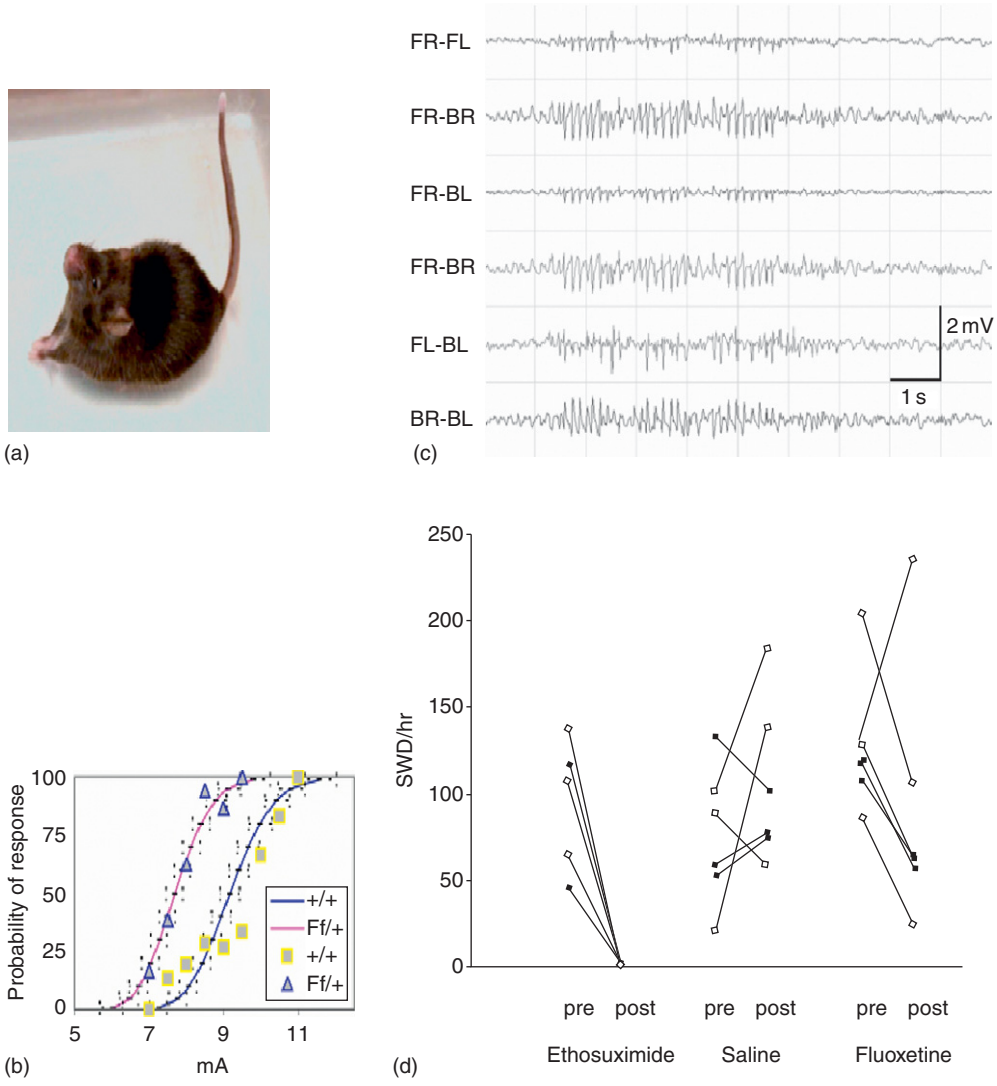


Figure 2 Seizure phenotypes of frequent-flyer mutant mice. (a) The posture of a 3-month-old *Brunol4*$^{Ff/+}$ heterozygote mouse at the beginning of a seizure episode. Note the contraction of the muscles of the neck, ears, and forelimbs and the vertical position of the tail. (b) Reduced ECT of *Brunol4*$^{Ff/+}$ mice. Squares (▫) and triangles (△) indicate individual data points used to construct CC curves in +/+ and *Brunol4*$^{Ff/+}$ mice, respectively. (c) EEG recording from a *Brunol4*$^{Ff/Ff}$ homozygous mouse. Shown are the six differential recordings from four subcranial electrodes, one in each quadrant corresponding to front-right (FR), front-left (FL), back-right (BR), and back-left (BL). These traces correspond to one of the longer spike-wave discharge (SWD) episodes observed. (d) Treatment of SWD in *Brunol4*$^{Ff/Ff}$ homozygous F2 hybrid mice from crosses to FVB (white box) or 129S1 (black). Recording sessions began at least 1 h prior to ethosuximide, saline, or fluoxetine (Prozac) treatment, and the rate of SWD (minimum criteria: ≥0.5-s duration, amplitude ≥2× baseline) was determined and plotted ('pre'). Animals were then injected with drug, monitored for an additional hour, and SWD incidence was recorded (post). Lines between data points correspond to each animal pre- and posttreatment. Only the ethosuximide and fluoxetine results were significantly different comparing treatments (ETX, $p = 0.009$; fluoxetine, $p = 0.019$; saline, $p = 0.804$ matched pairs test). Modified from Yang Y, Mahaffey CL, *et al*. (2007) Complex seizure disorder caused by Brunol4 deficiency in mice. *PLoS Genetics* 3(7): e124.

mostly enriched in the hippocampus – in particular, in the CA3 region. In vitro, BRUNOL4 itself has been shown to bind to U/G-rich intronic sequence and promotes the inclusion of the adjacent exons, at least when measured in cell lines with constructs designed to measure alternative splicing. However, in vivo – i.e., in the whole organism and particularly in the brain – its function is completely unknown.

The discovery that a single gene mutation in *Brunol4* has a striking neurological phenotype presents an opportunity to learn more about the true function of *Brunol4*. But it also raises a question: How can a complex seizure disorder result from a single gene defect in an RNA binding protein? The current hypothesis is that *Brunol4* somehow quantitatively regulates the levels of several different mRNA target genes simultaneously – and in mutants, it is the *combination* of

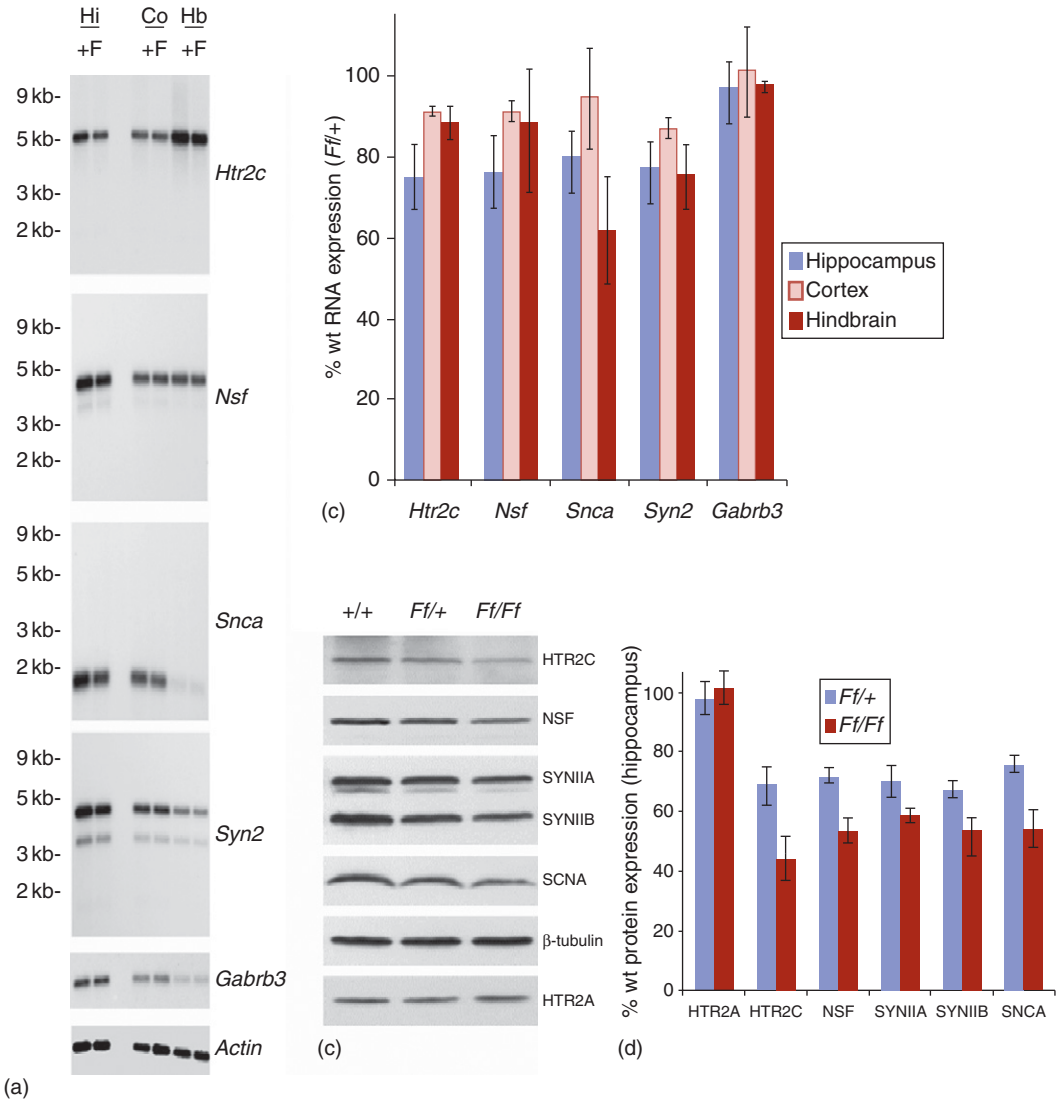


Figure 3 Reduced expression of four candidate genes in *Brunol4*$^{Ff/+}$ brains. (a) Representative images from three independent RNA blots. Sequential hybridization using *Htr2c*, *Nsf*, *Snca*, *Syn2* (experimental), *Gabrb3*, and β-actin (control) probes was carried out on a blot containing poly-A RNA extracted from dissected brain regions. CO, cerebral cortex; Hb, hindbrain; Hi, hippocampus. F, *Brunol4*$^{Ff/+}$; +, +/+. The position of size markers was shown on the left. (b) Histogram summarizing results from northern blots, expressed as % of wild type after normalization to β-actin. (c) Reduced expression at the protein level – western blot for HTR2A, HTR2C, NSF, synapsin II, α-synuclein, and β-tubulin. HTR2A, a molecule with distribution and property similar to HTR2C, was used as a control in addition to β-tubulin. Representative images from experiments with three independent adult mice on a mixed background are shown. (d) Histogram summarizing results from panel (c), in *Brunol4*$^{Ff/+}$ and *Brunol4*$^{Ff/Ff}$ mutants on a mixed background, expressed as % of wild type after normalization to β-tubulin, summarizing data from three independent animals. Error bars throughout are 1 standard deviation. Reproduced from Yang Y, Mahaffey CL, *et al*. (2007) Complex seizure disorder caused by Brunol4 deficiency in mice. *PLoS Genetics* 3(7): e124.

these effects that leads to the seizure disorder. Because the consequence of *Brunol4* deficiency might lead to a decrease in levels of steady-state mRNA(s), genome-wide microarray analysis was performed on brain RNA from homozygous mutants and compared with those of wild-type littermate control mice in order to look for candidate targets. Considering that tens of thousands of transcripts are represented on an array, a surprisingly small number (~75) had significantly lower levels in mutant as compared with control. Four epilepsy-related candidates stood out from this group: *Htr2c* (encoding a serotonin receptor, for which the null mutation has tonic–clonic convulsions, hyperactivity and middle-age weight gain), *Syn2* (encoding synapsin 2, which, when combined with synapsin 1 deficiency, lowers seizure threshold), *Nsf* and *Snca* (two known synaptic proteins also enriched in hippocampus, although heretofore not specifically linked to seizure disorders). The decrease of the mRNAs and protein for these genes was confirmed in mutants, being reduced some 25–40% compared with that in controls (**Figure 3**).

Genes for two of these candidate target mRNAs were already associated with epilepsy in mice. Further, *Htr2c* null mutants are also hyperactive and slightly obese as adults. Still, the relatively modest decrease in *Htr2c* transcripts in frequent-flyer mice with their severe seizure phenotype, in contrast with its complete absence in the knockout, suggests that its quantitative reduction is not sufficient (if it is indeed involved in the seizure phenotype). Moreover, *Htr2c* knockout mice did not have absence seizures, whereas the frequent-flyer homozygotes did. Interestingly, fluoxetine (Prozac®), the 5-HT reuptake inhibitor, partially reduced the incidence of SWD in frequent-flyer mutants, suggesting that the *Htr2c* deficiency was somehow involved in the etiology of SWD in frequent-flyer mice. To gain more direct evidence for the assertion that other genes were also involved in the frequent-flyer seizure syndrome, double-mutant mice (*Htr2c* null, *Brunol4*$^{Ff/+}$) were constructed and their seizure threshold was compared with that of single mutants. This procedure revealed that double mutants had a significantly lower threshold than either single mutant (**Figure 4**) – confirming that additional downstream players (i.e., not just *Htr2c*) in frequent-flyer heterozygous mice affect the seizure threshold. Although this initial study did not resolve whether *Syn2*, *Nsf*, *Snca*, or other potential BRUNOL4 targets were the coconspirators, it is consistent with the likelihood that frequent-flyer mice have a seizure disorder that mimics genetically complex seizure disorders in human idiopathic epilepsies.

Brunol4^{Ff} mutants illustrate, in a striking way, how genes other than those encoding ion channels can cause a complex form of epilepsy. Although many of the 75 downregulated mRNAs seen in animals with this mutation were of unknown function (and have not yet been tested), some of them may also contribute to the phenotype – and none encode ion channels. Still, if *Htr2c* and the other genes indeed contribute to the seizure phenotype associated with *Brunol4*^{Ff} mutant, one can hypothesize that the genetic effect is due to subtle modulation of synapse maturation or function.

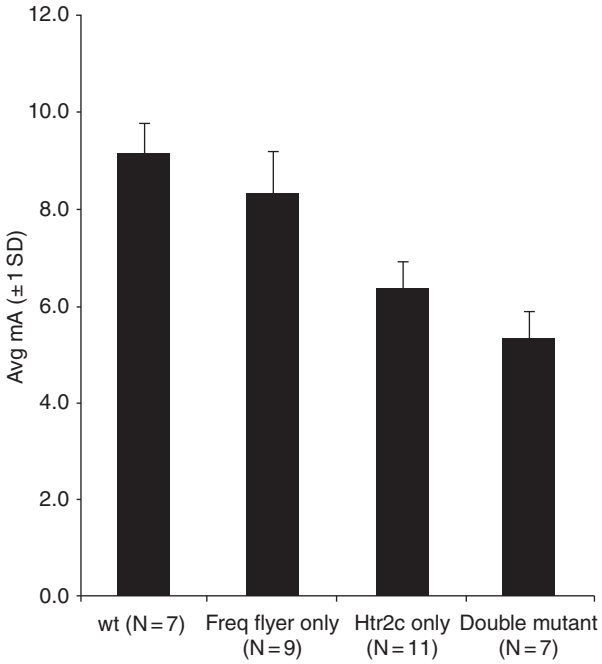


Figure 4 Genetic interaction between *Brunol4* and *Htr2c* for electroconvulsive threshold. Shown is a histogram of average threshold (in mA) to a minimal clonic or maximal tonic hindlimb extension seizure, for wild-type (+/+, +/+), single mutant (*Brunol4*$^{Ff/+}$, +/+ or B6 – *Htr2c*^{tm1Jul}/Y), or double-mutant (*Brunol4*^{Ff}/+; *Htr2c*^{tm1Jul}/Y) littermates from crosses between B6-*Htr2c*^{tm1Jul}/Y and B6-*Brunol4*^{Ff}/+ mice. Reproduced from Yang Y, Mahaffey CL, *et al*. (2007) Complex seizure disorder caused by Brunol4 deficiency in mice. *PLoS Genetics* 3(7): e124.

Directions for Future Research

It is somewhat difficult to chart a specific way forward for understanding the role of non-ion channel genes in idiopathic seizure disorders, short of first identifying all the genetic factors underlying human epilepsy – a daunting, if not impossible task even in the 'postgenome' era. While it seems essential to gain a better understanding of the function of natural variants of ion channels in human epilepsy, the mouse research literature alone clearly suggests that many different types of genes – beyond ion channels – have a role too, including transporters, transcription and RNA processing factors, synaptic proteins, components of metabolism, and many other types of molecules. Furthermore, players at the 'upstream' end of pathway hierarchies, such as *Brunol4*, imply how even just one such gene can have complex consequences on multiple genes that more directly influence excitability. Over the next few years, the genetics research community will undoubtedly be systematically mutating a variety of genes (e.g., in the Knockout Mouse Project, KOMP). These studies will foster and better standardize the assessment of seizure phenotypes in new mutants, so that we can come to a more thorough understanding of what kinds of molecular changes can lead to seizures. In this way, when candidate genes are eventually nominated for more complex human epilepsies, the community will have a better grasp of which ones merit further pursuit in human genetic studies and in studies of potential targets for therapy.

See also: **Genetics**: *ARX* Gene Mutations and X-Linked Infantile Spasms; Cytoskeletal Genes and Idiopathic Epilepsies; Genetic Contributions to Brain Structure and Epileptogenesis; Genetics of Lafora and Juvenile Myoclonic Epilepsies; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future.

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GABRB3 and Childhood Absence Epilepsy

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Introduction

γ-Aminobutyric acid is the major inhibitory neurotransmitter in the brain, and the ligand-gated ion channel $GABA_A$ receptors (GABARs) mediate the bulk of rapid inhibitory transmission. Any dysfunction of GABA causes seizures; conversely, several anticonvulsant medications act by enhancing GABA-mediated inhibition. Human epilepsies have an overall prevalence of about 1% across various countries. Amongst these epilepsies are absence or petit mal epilepsies which account for 3–12% of all epilepsies. Mutations in GABAR gamma2 (γ2), alpha1 (α1), and beta3 (β3) subunit genes have now been identified in human childhood absence epilepsy (CAE) sub-syndromes, namely (a) febrile seizures and CAE, (b) juvenile myoclonic epilepsy with CAE, and (c) remitting CAE, respectively. Mouse models of these mutated GABAR subunit genes are consistent with their role in producing inherited human absence epilepsies. This article focuses on the pathogenic role of GABAR beta3 subunit (*GABRB3*) in remitting CAE.

Background

$GABA_A$ Receptors

GABARs are heteropentameric membrane chloride channel proteins composed from a superfamily of 19 related subunit genes. These genes are constituted into a family of about 25 receptor subtypes which exhibit different regional, age-dependent, and subcellular localization. Plastic changes in subunit composition are observed with experience, disease, and chronic drug administration. Each receptor protein molecule contains two copies of an α subunit, two copies of a β subunit, and one other subunit. The γ2 subunit-containing GABARs are primarily synaptically localized and sensitive to benzodiazepines; extrasynaptic, primarily δ subunit-containing GABARs also control neuronal and network excitability (through tonic currents), and are important targets for drugs like anesthetics, ethanol, and neurosteroids. Genetically engineered mice have been valuable in deducing GABAR functions.

GABAR Mutations in Human Idiopathic Generalized Epilepsy (IGE) and Angelman Syndrome (AS)

Mutations in GABAR have been observed in multiple families affected with CAE syndromes. The GABAR subunit α1 has been found to be mutated in several positions within their protein sequences in autosomal dominant juvenile myoclonic epilepsy with CAE (JME/CAE) and in the syndrome of CAE only. Mutations have also been found in the γ2 subunit in the syndromes of febrile seizures with CAE, and generalized epilepsy with febrile seizures (GEFS+) and CAE. Some evidence also implicates the GABAR subunits δ and β3 in CAE. Current studies have investigated the reasons why a heterozygous mutation in these GABAR subunits can alter inhibitory function and produce petit mal seizures. Evidence suggests that in most cases there is reduced GABAR protein expression, reduced physiological inhibition, and hyperexcitability. The explanation for the dominant genetics remains unknown, but can include altered physiological properties of the GABAR channels, reduced trafficking of receptor proteins to the cell surface, and dominant-negative reduction of normal heteropentamer assembly.

An interesting variant on *GABRB3* pathology involves the neurodevelopmental genetic disease Angelman syndrome (AS). AS is associated with an imprinted maternal-inherited mutation in chromosome 15q involving the gene *UBE3a*. The majority of patients have a large deletion that includes, along with *UBE3a*, the GABAR subunit gene cluster α5/β3/γ3; these patients are thus heterozygous for these three subunits, including *GABRB3*. Compared to

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UBE3a mutations alone, this deletion seems to contribute to a more severe syndrome of mental retardation and epilepsy, typified by atypical absence and myoclonic seizures generalizing to grand mal, as well as a characteristic epileptiform EEG. We ascribe these abnormalities to the reduced expression of GABAR β3. Genes in this region of 15q also show reduced expression with potential functional consequences in autism spectrum disorder.

Methods

Mouse Knockout

Gene targeting in embryonic stem cells was employed to produce a mouse harboring a deletion mutation in the *gabrb3* gene, in a mixed 129/C57 genetic background. These animals were null (knockouts) for the GABAR β3 subunit mRNA and protein in the homozygous −/− animals.

Human Genetics

Probands with remitting CAE (with frequent, 20–200 per day, seizures), and family members, were screened for *GABRB3* mutations using DNA heteroduplex analysis with denaturing high performance liquid chromatography and gene sequencing. Microsatellite markers in the vicinity of *GABRB3* were assessed in all the family members to determine allelic linkage and segregation with the disease phenotype.

Molecular Cell Biology and Physiology

Human *GABRB3* cDNAs mutated in the three residues observed in human CAE patients were expressed in heterologous cells for examination of protein expression, in vitro transcription and translation, with analysis of the glycosylated product, and for functional expression of Cl^- channels using electrophysiological recording.

Results

GABRB3 Mouse Ko

Mice lacking the GABAR β3 subunit have a complex and pathological phenotype that includes epilepsy in the homozygous (−/−) animals. There appears to be little or no compensatory increase in other GABAR β subunits in the knockouts. The seizures have been characterized by EEG and show spike and wave discharges, accompanied by arrested locomotion similar to an absence seizure, beginning at about 2 months of age. The seizures become progressively more frequent with age, not remitting in adult, and the mice exhibit myoclonic jerks during the abnormal EEG. The seizures often are accompanied by a circling behavior in the cage, sometimes becoming frantic with 'whirling dervish' behavior, and sometimes generalizing to tonic-clonic generalized seizures. The EEG includes some absence-like discharges that continue so long that they can be considered nonconvulsive status epilepticus. The seizures, like human absence, respond favorably to ethosuximide and are worsened by carbamazepine; they respond poorly to other clinical antiepileptic medications.

The homozygous β3 knockout mice, but not heterozygotes, clearly show epileptic seizures that resemble human absence and the epilepsy of AS. These animals also show motor incoordination, disturbed rest/activity cycles, and cognitive deficits, possibly also a model of the developmental human genetic disease AS (of which autism is overlapping).

The *gabr3* mouse gene product is expressed widely in brain, especially early in life. It forms heteropentamers with varying α subunits, γ2 or δ, and is enriched in subtypes lacking α1 (which normally partners with β2). These heteropentamers are abundant in GABAergic interneurons, which are therefore low in functional β3. The β3 subunit polypeptide is expressed in the embryonic thalamus and decays to low levels in the adult, except in the thalamic reticular nucleus. Rhythmic activity of inhibitory synapses in the thalamic reticular nucleus and thalamic relay neurons produces synchronous thalamocortical firing typical of sleep spindles; loss of inhibition in one of the two regions, but not both (lack of β3 in the knockout in reticular nucleus but not in the relay cells), increases synchronicity and thus gives rise to the absence seizure activity.

Human Remitting CAE

CAE (petit mal) with remission at adolescence (remitting CAE) is a common idiopathic generalized epilepsy typified by frequent brief loss of consciousness with bilateral 3–4 Hz spike-and-wave without motor convulsions. Family studies have proved inherited distribution of the disorder, consistent with a major gene interacting with additional genetic and environmental factors. Association with the *GABRB3* gene was reported for Austrian CAE patients, and in California we found linkage of genetic polymorphisms near *GABRB3* with remitting CAE. Analysis of the DNA from remitting CAE patients and family members revealed mutations in 4 of 48 families from Mexico and Honduras. These mutations included one heterozygous missense mutation P11S (exon 1A, position 11 in immature polypeptide) that segregated with 4 CAE patients in a two-generation Mexican family, and the same mutation in a singleton from Mexico. We also found a heterozygous missense mutation S15F (exon 1A, position 15 in immature polypeptide) in a singleton from Honduras, and a heterozygous missense mutation G32R (exon 2, position 10 in the mature polypeptide) in 2 CAE diagnoses and 2 abnormal EEG cases in a single family from Honduras. All mutations were absent in 630 controls.

The three versions of mutated *GABRB3* were studied by expression in heterologous cells using immunoblotting, and shown to exhibit no significant difference from wild type. But all exhibited hyperglycosylation in in vitro transcription and translation, and reduced GABAR inhibitory current amplitude, measured by patch clamp recording. All the three mutated gene products appeared to undergo abnormal hyperglycosylation and possibly impaired leader peptide cleavage, leading to reduced assembly and/or trafficking to the cell surface and reduced physiological inhibition consistent with seizure-susceptibility.

Future Directions

How Does GABA β3 Mutation Cause Seizures?

GABAR-mediated inhibition cannot be significantly impaired without dangerous consequences in hyperexcitability, namely seizures. The β3 subunit is a component of about 40% of the adult GABAR and an even higher percentage of GABAR in embryonic and neonatal brain. It is a constituent of the only subtype(s) of GABAR present in certain neurons in critical circuits needed for myriad behaviors. Its function is apparently especially important in the nucleus reticularis of thalamus, and perhaps in hippocampal CA1. Further, the β3 subunit is part of the only GABAR present in a number of regions neonatally and deficiency results in permanently altered wiring and dire consequences such as deficits in cognition, sensorimotor integration, motor coordination, sleep patterns, and seizures.

Preliminary characterization of the mutations in *GABRB3* suggests loss of function via a variety of protein assembly, trafficking, and functional deficits resulting in reduced brain inhibition, even in the heterozygotes (dominance).

The role of GABRB3 in *remitting* absence epilepsy is particularly intriguing. This age-dependent change in seizure expression is probably related to the important developmental role of this gene product. The β3 subunit-containing GABARs are prevalent in the embryonic and neonatal brain. Interestingly, the β3 protein is expressed in neonatal thalamic relay neurons, but not in adult, and the remission of CAE at adolescence may reflect this changing expression pattern, suggesting that the β3 is no longer needed for function in the adult in some circuits that employ this GABAR subtype earlier in life.

How General is the Pathophysiology of GABAR Mutations in Human IGE?

Current information suggests that mutations in GABAR are one of a few molecular genetic mechanisms to explain idiopathic epilepsies. Even more epilepsies are due to channelopathies in general. However, mutations in four different GABAR subunits, α1, γ2, δ, and β3, have now been found in four sub-syndromes of human epilepsies – all of which have in common the presence of CAEs; at least three of these mutations – in γ2, α1, and β3 – segregate in family members affected with CAEs. These subunits are so prevalent that it is difficult to pinpoint the effect of a mutation/deficiency to a specific brain region/circuit, but evidence in both man and mouse suggests a likely thalamocortical involvement, and especially the GABAR in the reticular nucleus of thalamus.

Is Any Therapy (Other Than Gene Delivery) Suggested by the Genetic Lesion?

GABAR functional enhancement is already a strategy for anticonvulsant drug development. The implication of GABAR genes in human IGEs strengthens the rationale to spend more effort on efficacious GABAR-directed medications, and in solving related problems such as tolerance to the anticonvulsant therapeutics after chronic treatment. Development of GABAR subtype-selective drugs that would modulate the appropriate GABAR and circuits while minimizing side-effects (such as sedation, inhibition of learning, intoxication, dependence, and withdrawal) could be particularly valuable. In particular, a beta3-selective drug might be useful and recent advances in GABAR pharmacology suggest that such drugs exist and/or could be developed.

In conclusion, a major and common type of human epilepsy, namely CAE, involves pathogenic mutations in GABAR and should be amenable to anti-seizure medications aimed at these molecular lesions. Further knowledge about the ontogenetic and epigenetic modulation of GABAR molecular lesions could lead to curative strategies to treat absence epilepsies.

See also: **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; **Genetics**: Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy.

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Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs

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Introduction

Family and twin studies demonstrate that genetic factors are important contributors to the etiology of the majority of human epilepsy phenotypes. Some epilepsy phenotypes are known to be caused by mutations in a single gene, and these disorders are transmitted through families in predictable patterns based on classic Mendelian genetics. Such disorders are very rare, however, with less than 1% of all epilepsy patients acquiring their illness because of a single mutation in a gene of major effect. Current research suggests that the vast majority of patients with common forms of epilepsy acquire their disease because of genetic variation in multiple genes of small effect and that these collectively increase the risk for expressing recurrent seizures. This genetic risk then interacts with environmental factors (i.e., diet, sleep deprivation, alcohol consumption, etc.) to elicit the onset of disease in some individuals. Thus, common forms of epilepsy are considered to be complex trait diseases that are caused by interaction between DNA sequence variations in multiple genes, combined with exposure to environmental factors.

Identification of genetic variants that influence epilepsy susceptibility would be of exceptional value in the field and would open new avenues of research both in terms of diagnostics and treatment. Variants associated with specific epilepsy phenotypes could be used immediately as molecular markers for more accurate diagnosis. More importantly, genetic variations associated with epilepsy would help elucidate those molecular mechanisms that cause disease. An understanding of mechanisms that contribute to the etiology of disease would help identify novel neural pathways and lead to new pharmacological or mechanical interventions devised to improve medical treatment.

Pharmacogenetics is a relatively new field dedicated to identifying genetic variation that can predict response to pharmacologic agents. Currently, antiepileptic drugs (AEDs) and other medications are prescribed based on physician experience alone. In the future, genetic testing will identify DNA sequence variation that can accurately predict drug resistance or risk for an adverse side effect. Since 30% or more of all epilepsy patients are either resistant to, or suffer intolerable side effects from current AEDs, the identification of DNA sequence variation that accurately predicts resistance or adverse effects would revolutionize treatment. Prescriptions could be based on scientific tests with acceptable clinical validity and accuracy, and would eliminate much of the 'trial and error' process currently employed.

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Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs

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Introduction

Family and twin studies demonstrate that genetic factors are important contributors to the etiology of the majority of human epilepsy phenotypes. Some epilepsy phenotypes are known to be caused by mutations in a single gene, and these disorders are transmitted through families in predictable patterns based on classic Mendelian genetics. Such disorders are very rare, however, with less than 1% of all epilepsy patients acquiring their illness because of a single mutation in a gene of major effect. Current research suggests that the vast majority of patients with common forms of epilepsy acquire their disease because of genetic variation in multiple genes of small effect and that these collectively increase the risk for expressing recurrent seizures. This genetic risk then interacts with environmental factors (i.e., diet, sleep deprivation, alcohol consumption, etc.) to elicit the onset of disease in some individuals. Thus, common forms of epilepsy are considered to be complex trait diseases that are caused by interaction between DNA sequence variations in multiple genes, combined with exposure to environmental factors.

Identification of genetic variants that influence epilepsy susceptibility would be of exceptional value in the field and would open new avenues of research both in terms of diagnostics and treatment. Variants associated with specific epilepsy phenotypes could be used immediately as molecular markers for more accurate diagnosis. More importantly, genetic variations associated with epilepsy would help elucidate those molecular mechanisms that cause disease. An understanding of mechanisms that contribute to the etiology of disease would help identify novel neural pathways and lead to new pharmacological or mechanical interventions devised to improve medical treatment.

Pharmacogenetics is a relatively new field dedicated to identifying genetic variation that can predict response to pharmacologic agents. Currently, antiepileptic drugs (AEDs) and other medications are prescribed based on physician experience alone. In the future, genetic testing will identify DNA sequence variation that can accurately predict drug resistance or risk for an adverse side effect. Since 30% or more of all epilepsy patients are either resistant to, or suffer intolerable side effects from current AEDs, the identification of DNA sequence variation that accurately predicts resistance or adverse effects would revolutionize treatment. Prescriptions could be based on scientific tests with acceptable clinical validity and accuracy, and would eliminate much of the 'trial and error' process currently employed.

Background

A combination of linkage and association studies is currently being used to identify the multiple gene variations that underlie complex trait phenotypes, including risk of disease and response to medication.

Genetic Linkage

Genetic linkage studies have been performed for many decades and a seminal paper in this field was published in 1996, describing the advantages and disadvantages of these techniques (see Risch and Merikangas in Further Reading). Genetic linkage analysis is a technique used to identify disease causing genetic 'mutations' by examination of the genomes of related individuals, both affected and unaffected subjects. Linkage techniques can be applied to humans (typically, multiple generations of a single family) and animals. Linkage analysis in inbred strains of mice has been used to identify genes related to epilepsy.

The term 'mutation' is used for a genetic variation that has been proved to cause a disease. Linkage analysis is most effective when it is used to localize mutations that underlie a specific phenotype inherited in a predictable Mendelian fashion. The linkage approach is a global one that examines many DNA markers across the entire genome of all the subjects collected. Typical DNA markers used for linkage analysis include single nucleotide polymorphisms (SNPs) and variable nucleotide repeat elements (VNRE, or microsatellite repeats). Linkage analysis identifies chromosomal regions that harbor mutations by quantifying the form and frequency of specific DNA markers, assigning genotypes, and then calculating differences between those genotypes found in affected family members and those in nonaffected members. DNA markers are tested to determine if they cosegregate with the disease phenotype in affected individuals compared to unaffected; if so, it is concluded that those markers are physically 'linked' to the disease-causing mutation. In the best scenario, the marker tested is the actual disease-causing mutation. However, this best case scenario is generally not the case.

Linkage is a descriptive term to indicate that the causative mutation and DNA marker are inherited together, likely because the two are physically in close proximity on the same chromosome. Linkage equilibrium is a term used to imply that all DNA markers have an equal chance of being segregated during meiotic recombination. We know this 'equality' is not the case, however, because recombination rates between various DNA markers can be experimentally calculated and the rate of recombination has been shown to be dependent on the physical distance that separates the DNA markers of interest on the chromosome. That is, the farther apart two DNA markers are on a chromosome, the more likely it is that they will be separated from each other during meiosis. The term linkage disequilibrium (LD) is used to describe the phenomenon whereby two markers tend to be inherited together because of their close proximity on the chromosome.

To date, all of the confirmed epilepsy-causing gene mutations have been discovered using linkage analysis in large multigeneration families, where specific forms of seizures were found to be inherited in predicable Mendelian patterns. The majority of the identified gene mutations result in alteration of structure and function of proteins related to ion channels and maintenance of ion homeostasis across excitable membranes. These results have led to the conclusion that many types of epilepsy are 'ion channelopathy' diseases. However, there are several examples of epilepsy-causing DNA mutations in nonion channel genes, including mutations in genes related to development and metabolism.

Genetic Association

By contrast, genetic association analysis is performed on DNA samples from either unrelated or related individuals. One common paradigm for this type of analysis is the use of a case control study design. Unrelated patients are grouped into a 'disease' cohort (cases) and the genotypes of specific DNA markers are quantified and compared between these cases and healthy unrelated control subjects. This study design has the advantage that collecting unrelated cases and controls is logistically easier than collecting multiple members of specific pedigrees. However, one potential drawback is that subtle genetic differences between unrelated individuals may lead to an undetected population stratification that could confound results of an association study. This stratification can result, for example, from undetected differences in ancestry in cases and controls. DNA allele frequency varies greatly based on ancestral background, and most studies depend on self reporting of ethnicity. Many of these self reports may be limited in their reliability, and thus undetected differences in allele frequency based on ancestry could lead to false positive results. Numerous researchers have, however, argued that the threat of population stratification as a confounding variable in case control genetic association studies is very minor if a factor at all. The topic remains controversial.

Another type of association study uses relatives of an affected individual as a control. Most often, unaffected parents are used as controls to test for transmission of specific DNA sequences to their affected progeny. This type of association study is know as the Transmission Disequilibrium Test (TDT) and was first introduced by Spielman and colleagues as an alternative to the case control association design (see Further Reading section). The TDT is immune to population stratification confounds because it uses related individuals as controls.

In addition, the TDT can establish linkage in the presence of association, and was originally devised to address situations where a population association was found in the absence of evidence for linkage from traditional family-based study designs. Thus, the TDT can establish linkage if a positive association exists.

Methodology

One excellent source for information on genetic influences on disease traits is the Online Mendelian Inheritance in Man (OMIM) website (http://www.ncbi.nlm.nih.gov/sites/entrez?db = OMIM) maintained by the National Center for Biotechnology Information (NCBI) at the National Library of Medicine (www.ncbi.nlm.nih.gov). The NCBI site contains numerous databases related to human and animal genome and genetic resources used in biomedical research, including DNA, RNA, and protein sequences, information about specific DNA markers and their distribution in the genome, and links to many other genomic and proteomic resources on the internet.

For an association study, DNA markers are typically chosen in high priority candidate genes. High priority candidate genes are those for which there are existing linkage data or other biological evidence to suggest that dysfunction of the gene (and its encoded protein product) could play a role in the etiology of the phenotype under study. For example, genes and proteins related to GABA and glutamate neurotransmitter systems have long been considered excellent candidate genes that may act as epilepsy susceptibility factors based on their role in the biology of neurotransmission of inhibitory and excitatory signaling, respectively. More recently, ion channel genes have been implicated as epilepsy susceptibility factors and have been considered to be high priority candidate genes.

One unresolved question in modeling and designing association studies is whether DNA variations contributing to illness are very rare or very common. Most researchers study markers that are present in at least 5% or more of the population (common variants). The assumption is that rare variations will not be found in large proportions of patients, and therefore these variations would not be useful in generalizing treatment and diagnoses options to the majority of patients. If, however, complex disease traits are caused by rare mutations, then the current methodologies in place will be generally ineffective in identifying these factors.

The statistical interpretation of simple association studies is generally very straight-forward. Most researchers use standard contingency testing such as χ^2 or *t*-tests to compare the form and frequency of DNA variation between cases and controls. In the TDT, a χ^2 statistic is also used to compare the calculated transmission of the suspected disease-linked marker to the alternative form of the marker hypothesized to be unlinked to the disease. Typically, SNPs are used for the TDT; and since they are bi-allelic, only two possible forms of the marker exist to be tested. If a SNP allele is unlinked to disease, then its transmission from a heterozygous parent to an affected child should occur at a transmission rate of 50%, the random probability of transmission for a bi-allelic marker. However, a SNP allele associated and linked with a disease will be transmitted at a rate significantly different from the random distribution of 50%.

The first association studies used limited numbers of DNA markers to study any given candidate gene ranging from one marker (single SNP) to a half dozen or so. The use of multiple markers complicates the statistical analysis in that correction for multiple testing must be considered and factored into any final calculation for measures of significance. In addition, the use of multiple markers introduces the concept of 'haplotypes.' A haplotype refers to multiple linked markers that are physically located on the same sister chromatid of the chromosome pair, and tend to be inherited together based on strong LD. Haplotypes can be easily determined by DNA sequencing especially if DNA from both the patient and their parents is available for study. Since each parent contributes one chromosome of each pair to the progeny, genotyping the parents' DNA provides information on which marker is derived from which parent. If parental DNA is not available, there are computer-based algorithms that can estimate haplotypes in groups of unrelated individuals based on population genotypes. In some cases, haplotype analysis can be more powerful than single marker analysis in identifying the location of a disease-causing mutation or location of a DNA variation that acts as a susceptibility or resistance factor for the phenotype under study. The Ott laboratory at The Rockefeller Institute maintains an extensive database of statistical genetic programs, and is an outstanding resource for those working in (or interested in learning about detailed analyses used for) linkage and association studies (http://linkage.rockefeller.edu/group/).

Technology has progressed to the point where over 500 000 individual SNP markers can be genotyped from a DNA sample from a single individual in a matter of hours. This technology has led to genome-wide association studies (GWAS), where 500 000 or more DNA markers are genotyped and results quantified in hundreds to thousands of cases, and compared to equal (or more) numbers of controls. This type of study is not immune to potential confounding variables. Indeed, performing over 500 000 tests in thousands of individuals introduces a difficult problem in choosing appropriate methods for correction for multiple testing. Quantifying the false discovery rate, to determine potential numbers of false positive or negative results, can also be quite problematic. These studies offer much promise but require intensive

statistical genetic support to 'clean' the large data sets that are generated.

Currently there are no published reports of GWA studies in epilepsy; however, unpublished results were recently obtained by this author in a North American cohort. Similar work is being performed by groups in Europe, Asia, and elsewhere; utility of the GWAS technique will become apparent when analyses of these data sets are published.

Results

In general, although genetic association studies in epilepsy have been performed for over a decade, there has been no gene variation(s) discovered that has a major effect on increasing risk of any of the common forms of epilepsy. Recently, a strong association was discovered that can predict the adverse reaction known as Stevens–Johnson syndrome in some patients of Asian ancestry in response to carbamazepine, a commonly prescribed AED. This association, however, is highly restricted to Han Chinese and Taiwanese patients, and was not found in Japanese or Caucasian patients. Further studies are required before this association is proved valuable for its possible predictive value.

The single most important issue to resolve for genetic association studies in all complex trait disease studies is that of independent replication. Few genetic polymorphisms that have been reported to show association with a complex trait in the initial study have been replicated by an independent group with a separate but similar cohort. One success story is the strong association between the APOE4 allele and common sporadic cases of late onset Alzheimer's disease, initially described by Corder and colleagues (see Further Reading section) and replicated many times since. However, associations of similar strength have not been found for epilepsies, response to AEDs, or most other complex traits.

In the past decade, numerous association studies have been published in an attempt to correlate genetic variation to increased risk of acquiring epilepsy. For example, Sander and colleagues have collected a large and well characterized cohort of patients with common forms of idiopathic generalized epilepsy, and conducted a large number of genetic association studies. This cohort facilitates the ability to investigate novel hypotheses as well as to quickly attempt to replicate the findings of other laboratories. Although some modest associations have been identified while examining novel candidate genes, none of the positive findings has been independently replicated. In addition, attempts to replicate prior claims of positive association by other groups have, in most all cases, led to negative results.

Currently, only one initial positive report of association between a gene variation and epilepsy has been successfully replicated in a separate population. We found a variation in the KCNJ10 gene (that encodes an inwardly rectifying potassium ion channel subunit) to be associated with common forms of human epilepsy, and this result was subsequently replicated in a separate population (Lenzen *et al.*, 2005). The function of this gene in extracellular potassium buffering makes it an ideal epilepsy candidate gene, and offers opportunities to develop pharmacological interventions that target this type of potassium channel to treat common forms of epilepsy. Further work is needed to clarify the role of DNA variation in this region and its possible relation to increasing risk of epilepsy.

The lack of ability to replicate initial positive data has generally led to the conclusion that initial results represent a false positive finding. However, in many cases, the attempts to replicate a study used different methodologies and/or populations. Standards for publication of association studies have been raised substantially in recent years, as the number of investigators performing such work has increased. Many journals have introduced very stringent guidelines to accept such manuscripts, and it is unclear yet if these guidelines will have the desired effect, to reduce the number of false positive findings that are published. However, if the guidelines are too stringent, then many gene variations that *do* associate with illness, but have only a minor contribution, will likely be dismissed – even though they may represent false negatives. Ott has published a suggested set of guidelines that many journals and authors are adapting (see Further Readings) and which address many of the common problems and pitfalls encountered in this field.

Summary and Directions for Future Research

Changes and improvements in the technology to genotype, sequence, and store large numbers of DNA variations is moving at a rapid pace. In addition, our understanding of the structure and function of the genome continues to increase, and research has documented that even DNA variation in nongene coding regions that do not alter primary protein sequence can affect transcription and translation of encoded protein products. These data demonstrate how all DNA variations, including deletions and duplications, must be considered during the course of association studies. Furthermore, research is showing that alteration of DNA and/or its histone protein core, by methylation and other processes, can lead to 'epigenetic' modification of the genome. Such epigenetic factors can be affected by environmental factors and, in turn, can influence the structure and function of DNA that is inherited by progeny without changing the primary DNA sequence. Epigenetic regulation of DNA function would

likely not be discovered by techniques that exploit DNA sequence variation alone.

There are serious challenges facing the field of genetic association in complex traits. Many caveats exist in the design and analysis of association studies. Some of these include heterogeneity of phenotype in cases, population stratification in cases and controls, small numbers of subjects for study, different methods to calculate statistical significance, different methods of correction for multiple testing, different models for gene dose effect, variations in penetrance and modes of transmission, haploype structure and function, control sample collection and use, ethnic variations in DNA structure, epigenetic and environmental factors, and other issues. Many investigators now recognize that collection of tens of thousands of case and control samples may be required to identify many of the contributing genetic factors that exist and influence complex traits. The biggest challenge in the future may well be the development of statistical genetic techniques that can correct all the confounding variables inherent in these studies as they are currently performed.

In conclusion, the search for genetic variation that is linked or associated with complex trait disease will continue far into the future, and perhaps represents one of the best ways to identify new avenues of research into the underlying pathology of the illness under study. The eventual correlation between DNA variation and response to medication (efficacy and tolerability) will revolutionize the way in which drugs are prescribed. Improvements and modifications are continually being made in the design and execution of genetic association studies, and the excitement surrounding their potential impact on disease and pharmacogenetics remains very high.

Acknowledgements

This material is the result in part from support of facilities and resources from the Coatesville VA Medical Center, Coatesville PA. The contents of this article represent the views of the author and do not represent official views of the Department of Veteran Affairs or any branch of the US Government.

See also: **Genetics**: Clinical Genetics of Epilepsy; Genetics of Lafora and Juvenile Myoclonic Epilepsies; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future; Large-Scale Mapping and Epilepsy Gene Discovery; Sixty Years in the Making: A Multifactorial Mouse Model of Seizure Susceptibility.

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Genetic Contributions to Brain Structure and Epileptogenesis

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Introduction

The number of mutant genes responsible for brain malformations is rapidly expanding and their associated syndromes often include seizure disorders as part of the mutation-related clinical phenotype. It was once generally accepted that increased cortical excitability accompanies displacement or disorganization of neuronal somata. However, recent advances in understanding the function of molecules encoded by these genes (mutations of which give rise to brain malformations) has significantly altered our view of their roles in epilepsy and indeed in normal behavior. In fact, subtle mutations in brain malformation genes may not produce severe malformations detectable on MR imaging. Nevertheless, loss- or gain-of-function changes in these genes can alter neuronal excitability and so may underlie some idiopathic seizure disorders that are unrelated to a known injury or toxic cause. Thus, the search for genes causing brain malformations in mouse and man is providing an unexpectedly rich source of insight into epilepsy. Some of these recent advances will be discussed concerning research into the function of genes associated with two major types of brain malformation, lissencephaly (smooth brain), and microcephaly (small brain).

Methods

There are several approaches to the identification of the genetic defects underlying brain malformation. They all hinge on the successful characterization of the salient features of a particular malformation – whether in patients or in animal models. In the clinical arena, this detailed phenotyping allows investigators to group like patients to enable physical mapping and cloning of the causative gene. It also makes it possible to compare the structural outcome of genetic manipulations in animal models with the counterpart in human brain, thereby permitting one to take a candidate gene approach to disease gene discovery. Once the responsible gene is identified, comparison of specific mutations with the developmental outcome (establishing genotype–phenotype relationships) provides insight into the important functional domains of the gene product.

Identification of a brain malformation gene in patients can be approached via several routes, which will be dictated by circumstances of the inheritance of the disorder. When malformations appear in families as isolated occurrences, sporadic dominant mutation is a likely mechanism. Cytogenetic investigations of chromosome spreads, including fluorescence in situ hybridization (FISH), may reveal large gene deletions, duplications, and translocations such as were found in Miller Deiker Syndrome (MDS) involving chromosome 17. More recently, smaller mutations and rearrangements have been detected by comparative genome hybridization (CGH) using bacterial artificial chromosome (BAC) probes. Finally, genome arrays are becoming the standard for detection of copy number variants (CNVs) in gene representation that may underlie many sporadic occurrences of brain malformation.

When brain malformation is seen to follow simple Mendelian inheritance – indicating autosomal dominant, autosomal recessive, or X-linked transmission patterns – linkage analysis will often provide the critical interval that must contain the mutant gene. This approach is based on the use of polymorphic microsatellite or single nucleotide polymorphisms (SNPs) that cover the genome at a density that allows the detection of a small portion of the genome that is always inherited with the disease phenotype. Such analyses may be carried out in a single large family, or more often in these conditions, several small families may be grouped (multiplexed) to compare their combined inheritance patterns.

Once a locus is established, the critical interval of the gene containing that mutation is physically studied, by DNA sequencing. Regions to be sequenced may be prioritized based on the nature of the genes known to be present within the critical interval. Patient sequences are compared with normal controls to establish the likely significance of a particular variation. Sometimes a mutation would be predicted to create a premature stop, a nonsense change (i.e., frame shift) that abrogates the protein sequence downstream of the mutation, or a missense mutation that would strikingly alter the size and charge of an amino acid in the coding region. Other more subtle changes could occur in the promoter or enhancer regulatory regions, or in the sequences that direct the splicing of RNA transcribed from the gene. These types of relationships must be investigated in order to decipher the meaning of genetic variation in a locus associated with a genetic disorder.

Brain malformation genes may also be identified through candidate genes, identified either by positional cloning of a naturally occurring mutation associated with brain malformation in an animal model or through recognition of a malformation produced by gene knockout,

most often in mouse models. Examples of such mutations include those found in the reeler mouse, whose human counterpart on chromosome 7q22 was identified because of the marked cerebellar hypoplasia in both the reeler mouse and in patients. Another example of the advantageous use of mouse–human phenotype comparisons was the realization that knockout of the ARX (X-linked aristaless) gene in the mouse displayed remarkable similarity to children with the XLAG syndrome (X-linked lissencephaly with ambiguous genitalia).

Once a gene defect associated with brain malformation is in hand, initial clues to function can be gleaned from comparison with clinical phenotypes. A gene, the mutation of which leads to lissencephaly, is likely to include neuronal migration and other cell motility modalities among its functions. A mutation leading to microcephaly would be expected to impact neural cell proliferation and/or cell survival. Point mutations associated with the phenotype may provide critical clues to the most important functional domains within the encoded protein. Approaches to probing gene function then include manipulation of gene expression levels by RNA interference and overexpression by transfection into cells in culture, or even into tissues in utero (gene transfer made using viral particles or electroporation). Finally, the investigation of the systems-level impact of a gene mutation will be carried out in genetically engineered mouse or rat models.

Background and Results

Lissencephaly Genes and the Neuronal Cytoskeleton

Derived from the two Greek words *Lissos* (smooth) and *encephalos* (brain), lissencephaly is characterized by loss of cortical convolutions (either total absence or widening of gyri called pachygyria) and thickening of the cortical gray matter due to impaired neuronal migration. In classical lissencephaly, the cortical mantle measures a centimeter or more, compared with the usual 4 mm or less, and the normal six layers are replaced with a poorly organized four-layer cortex that includes a cell sparse zone appreciated on MRI. Most of the classical lissencephaly encountered in the clinic can be accounted for by two genes, *LIS1* and doublecortin (*DCX*). More recently, mutations in an alpha tubulin subunit, TUBA1–3, have been found to produce a range of lissencephaly phenotypes in mouse and man.

Lis1

This gene was named as the first lissencephaly gene to be identified from patients with MDS or isolated lissencephaly (ILS). *Lis1* was recognized as encoding a 45 kDa soluble protein with structural similarity to G-protein β-subunits, in which a seven-bladed propeller structure is made from 7-WD40 repeat units. Although it resembles a G-protein subunit, it was soon realized that *Lis1* is a noncatalytic 1b1 subunit of a ubiquitous mammalian protein called platelet-activating acetylhydrolase (PAFAH) and that *Lis1* is evolutionally conserved, bearing striking homology with NUDF (nuclear displacement F) in *Aspergillus*. The NUD family of proteins was functionally defined as causing defects in nuclear migration in fungus, which requires NUD interactions with cytoplasmic dynein in a dynein protein motor complex.

Numerous studies have now linked *LIS1* and function of the dynein protein motor complex. In this role, *Lis1* has been shown to affect neuronal migration, which has often been attributed to its central role in nuclear translocation and coupling of the nucleus to the centrosome. The impact of *Lis1* on dynein also has profound influence on neural progenitor proliferation through their interaction with the kinetochore and mitotic spindles.

The association of *Lis1* with the dynein protein motor complex has become so strong that several investigators have even equated manipulation of *Lis1* or dynein as synonymous with alteration in the other. However, our studies have revealed an additional role for *Lis1* in the regulation of small GTPases of the Rho family to impact the actin-based cytoskeleton. The Rho-GTPases are potent signaling molecules that regulate cytoskeletal dynamics involved in processes that include microtubule growth, pausing (brief halting of growth), and catastrophe (disintegration of microtubules into tubulin monomers), establishment of cell polarity and centrosomal localization, and regulation of actin polymerization as well as capture of microtubule ends to the cortical actin meshwork at the inner plasma membrane Such effects on cytoskeletal dynamics may alter growthcone motility, synaptogenesis, and neuronal excitability.

Given these involvements of *Lis1*, there are several potential ways in which *Lis1*-deficient neurons may generate abnormal discharges and seizures, which have little or nothing to do with the placement of these neurons. The first way in which *Lis1* may impact neuronal physiology is that cells lacking, or expressing defective, *Lis1* may have impaired membrane and protein trafficking through effects on cytoplasmic dynein. Although an impact of *Lis1* on these events has yet to be directly shown, cytoplasmic dynein complex proteins (including dynactin) have been implicated in aspects of endocytosis and in differential protein trafficking contributions to cell polarity. Indeed, evidence points to the ability of *Lis1* to regulate the ATPase activity of dynein, thereby influencing intracellular protein transport.

A second way in which *Lis1* might impact cortical excitability is through aberrant neuronal connectivity stemming not from the misplacement of neuronal cell bodies but from effects on axon outgrowth and actin remodeling events. Electrophysiological evidence supports

synaptic transmission abnormalities in the CA1 region of *Lis1* haploinsufficient mice, and abnormalities of hippocampal pyramidal and granule neuron dendritic arbors have been observed in *Lis1* +/− mice. The impact of *Lis1* on calcium dependent signaling through NMDA-receptor has been shown to occur through modulation of the Rho-family GTPases. Moreover, NMDA receptor and Rho GTPase-dependent mechanisms are known to have a significant regulatory influence on activity-dependent neurite development. The role of *Lis1*, and other neuronal migration genes, in these processes is just beginning to be explored.

Yet a third mechanism likely contributing to epileptogenesis in *Lis1* deficiency is a function of the differential severity of migration defects among neuronal subsets. The long tangential migration of cerebral cortical interneurons from their origins in ventral forebrain was previously shown to result in selective deficits in GABAergic interneurons in *LIS1* deficient human brain. This neuron subtype-specific deficit would presumably reduce not only direct inhibitory inputs to excitatory neurons but would also reduce the GABA content or 'GABA tone' of the extracellular milieu.

DCX

Doublecortin was named for the popular designation of the X-linked syndrome phenotype in affected females, double cortex (aka subcortical band heterotopia, SBH). This band of misplaced neurons collects beneath the relatively normal appearing cortical gray matter as the result of X-inactivation in females. Boys inherit only one X chromosome and so all their neurons use the same *DCX* mutant gene if present. Since female cells possess two X chromosomes (each with its own *DCX* allele or version of the gene), one of them must be inactivated early in embryogensis to avoid excess gene dosage. Selection of which of the two chromosomes gets to be inactivated is a random event. In girls, neurons that inactivate the X chromosome that bears the mutant *DCX* allele use the normal *DCX* gene, and so migrate normally; those neurons that inactivate the X chromosome bearing the normal *DCX* allele must use the mutant copy of the gene and so are impaired in their migration. Thus, the brain of a girl with one *DCX* mutant gene will be 'mosaic' – some neurons will be normal and others will use the mutant form of the protein, creating the displaced band. *DCX* encodes a 40 kDa soluble microtubule-associated protein (MAP) that defines a novel functional class of proteins with microtubule binding domains (*DCX*-like or DCL domains). Within this family is a kinase (doublecortin-like kinase, DCLK) that also interacts with microtubules and phosphorylates *DCX*, among other proteins, including those contributing to the mitotic spindle apparatus. Although *DCX* is expressed primarily in postmitotic neurons, *DCLK* is transcribed in both dividing progenitors and postmitotic neurons. Unlike *Lis1*, which is ubiquitously expressed in most if not all cells, *DCX* is highly selective for neurons of the central and peripheral nervous system. Indeed, *DCX* expression has become a widely accepted marker for young neurons as they emerge from the cell division cycle.

The major mode of *DCX* action is thought to involve regulation of the stability and bundling of microtubules. When there is a mutation, the resulting motility defects include altered nuclear translocation, abnormal branching of the primary neurite, and altered axon extension. *DCX* is a phosphoprotein that is regulated by kinases (including cyclin-dependent kinase 5 (CDK5), Dclk, protein kinase A (PKA), and MARK/Par1) and is dephosphorylated by phosphatases (including spinophillin). This regulation enables *DCX* to finely modulate the motility of neuronal growth cones at the transition point between the microtubule shaft of the axon and the growth cone.

As a classical MAP, *DCX* can impact neuronal function in several ways. When hypophosphorylated, it will bind primarily to microtubules and may influence microtubule dynamics. Hyperphosphorylation of *DCX* shifts the protein from the microtubule to the actin cytoskeletal compartment. *DCX* is also capable of binding to *Lis1*, and may modulate the cytoskeletal effects of that migration protein.

Yeast two hybrid screens have identified the interaction of *DCX* with the mu subunit of clatherin-coated adapter proteins, suggesting a role for *DCX* in protein sorting or vesicle trafficking. This role is supported by observations made in double mutant mice in which both *DCX* and *DCLK* have been inactivated. Such mice display defects in axon outgrowth and in transport of vesicular proteins. A critical demonstration of the important role of *DCX* in neuronal function has been made by the recognition that certain *DCX* mutations in patients can cause mild mental retardation and epilepsy, even though no cortical malformation can be detected (on high resolution MRI) in these patients. Thus, *DCX* mutations need not result in significant neuronal migration abnormalities to impact cortical function in substantive ways.

Other lissencephaly genes and epilepsy

Other forms of lissencephaly include varying degrees of pachygyria because of loss of extracellular signaling molecules. One such example is a mutation that affects reelin (RLN), which gives rise to a form of lissencephaly with severe cerebellar hypoplasia in which the cerebellum of affected patients is nearly absent. *RLN* encodes a large extracellular matrix protein thought to provide guidance, or start/stop cues, to migrating neurons. Mutations in one of the RLN receptor proteins, VLDLR, produce a very similar picture as seen with mutations of the RLN gene – moderately thickened cortex, pachygyria, and significant cerebellar hypoplasia. Genetic interaction evidenced by the cross breeding of mutant mice indicate that RLN signaling is positioned upstream of *Lis1* function. Interestingly,

seizures are not a prominent feature of the clinical presentation of RLN mutations, perhaps because much of the basic neuronal physiology remains intact.

In contrast, lissencephaly because of mutation in the *ARX* gene is associated with severe forms of epilepsy. A transcription factor in the aristiless gene family, *ARX* loss of function leads to profound deficits of GABAergic inhibitory interneurons. These deficits are associated with defects, evident on MRI, in the basal ganglia and even thalamus. During embryogenesis, it is the lateral ganglionic eminence (LGE) that gives rise to caudate/putamen, while the medial ganglionic eminence (MGE) is a major source of interneurons that make tangential migrations to reach the cerebral cortex. The caudal ganglionic eminence (CGE) also contributes interneurons to the cortex and is the embryonic source of much of the thalamus. *ARX* is expressed (used to make ARX protein) in these LGE, MGE, and CGE regions during brain formation, leading to basal ganglia and thalamic abnormalities when *ARX* is mutated. Knockout mice that are deficient in *ARX* reveal marked deficits in interneurons, along with other severe malformation features. Children with *ARX* mutations are prone to infantile spasms, a seizure type that is especially difficult to control. Because of the marked abnormalities in these deep diencephalic structures, *ARX* disorder supports the notion that basal ganglia and especially thalamic structures (including thalamocortical connections) may play a significant role in the pathogenesis of infantile spasms.

A recent addition to the list of lissencephaly-associated genes is the alpha-tubulin (TUBA) subunit. Mutation in the *TUBA1* gene interferes with the efficiency of tubulin dimerization. Sporadic mutations in *TUBA3* produce human lissencephaly; depending on the missense change, this phenotype can appear as severe grade 3 classical lissencephaly with a posterior predominance or a grade 4–5 pachygyria. The *TUBA3* phenotypes include partial agenesis of the corpus callosum and brainstem hypoplasia, all of which point to defects in axonogenesis. Severe epilepsy was described in at least one patient with *TUBA3* mutation.

Genetic defects affecting cytoskeletal regulation and neuritogenesis – and probably synaptogenesis and intracellular protein trafficking – are major underpinnings of seizure disorders associated with cortical neuronal migration defects. Basic studies of mouse models, as well as clinical genotype–phenotype investigations, have revealed important insights into epilepsy pathogenesis associated with these structural abnormalities. Further investigation into the underlying mechanisms is required.

Microcephaly Genes and Deficits in GABAergic Interneurons

Another prevalent category of brain malformation is congenital microcephaly, defined as an occipital–frontal head circumference (OFC) measuring – two standard deviations (SD) below the median for age or smaller. Data from the Center for Disease Control indicate that congenital microcephaly from all causes occurs at a rate of 1.07 per 1000 live births, exceeding the rate of neural tube defects (spina bifida and anencephaly) in the United States. Although undoubtedly dozens of genetic defects are associated with microcephaly, many of the genes now identified are known to impact the cell cycle. For example, microchephaly because of loss of function in the *MCPH1* gene (microcephalin) is associated with neural cells 'hung up' in prophase, impairing passage through M-phase, and thus preventing the split of cells into two daughters. MCPH5 is caused by mutation in the *ASPM* gene – that produces abnormal spindles to interfere with M-phase. And MCPH6 is associated with a mutated centrosomal protein *CENPJ*. All of these phenotypes are relatively mild, in that children display mild mental retardation and a normal lifespan. If seizures do occur with these disorders, they are readily controlled with medication. The most serious consequences of the genetic losses, in this group of relatively highly functioning patients with microcephaly, are in cell proliferation; there appears to be less impact on cortical connectivity and excitability.

Other genes that drive the cell cycle are prime candidates for additional forms of microcephaly. In the mouse, inactivation of cyclin D1 (*Ccnd1*), which acts in G1-phase, produces mild microcephaly and more prominent small stature, with limited life span. Although there are signs of neurological dysfunction (including spasticity) in these animals, the brains are neuroanatomically intact, with no specific cell type deficits. Interestingly, mice deficient in cyclin D1 are relatively resistant to certain epileptogenic drugs like kainate, while they have the same seizure threshold to PTZ as wild type siblings.

In contrast, knockout of another G1-phase cyclin, cyclin D2 (*Ccnd2*), is associated with normal size and body mass but severe microcephaly that is gene dosage dependent; that is, heterozygous nulls have a brain size intermediate between wild type and homozygous null siblings. More important, while the total numbers of all neuronal types is reduced in *cyclin* $D2^{-/-}$ brains, there are selective deficits – of cerebellar granule neurons and cerebellar stellate interneurons in the molecular layer. In the cerebral cortex and hippocampus, there is a selective deficit in parvalbumin-expressing interneurons, but other interneuron cell types are spared, including somatostatin-expressing interneurons that arise from the same (MGE progenitor pool as parvalbuminergic cells. This selective deficit in GABAergic interneurons is sufficient to produce reduced seizure threshold and loss of the larger amplitude miniature postsynaptic currents (mIPSCs). Thus, at least some proliferation deficits may alter the cellular composition of cerebral and hippocampal structures to result in seizures.

Another microcephaly phenotype associated with seizures is the Amish-type lethal microcephaly (MCPHA), due to a defect in a mitochondrial dinucleotide carrier (DNC) protein. Affected children display α-ketoglutaric acidosis and their severe microcephaly is associated with increased cell death in brain, though the precise mechanism leading to reduced brain size has yet to be elucidated. Seizures are presumed at present to result from a metabolic imbalance altering neuronal homeostasis.

Still other forms of microcephaly occur in conjunction with other brain malformation types. For example, bilateral periventricular nodular heterotopia (BPNH) with microcephaly has been linked to the *ARGEF2* gene, which encodes brefeldin-A inhibited guanine exchange factor 2 (BIG2) protein that is required for vesicle trafficking out of the trans-Golgi apparatus. Knockdown of *ARGEF2* inhibits cell proliferation and transport of proteins like E-cadherin and β-catenin to the plasma membrane. Although identification of BIG2 implicates a defect in vesicle trafficking, the mechanism(s) leading to microcephaly and epilepsy in this patient group have yet to be discovered. Nevertheless, the relationship of *Lis1*, and potentially *DCX* as well as ARGEF2/BIG2, with intracellular protein transport suggests that this trafficking has an important role in molecular mechanisms leading to seizure disorders.

Future Directions for Research

The pathogenesis of seizures related to brain malformations is an important frontier that is still in its early stages of investigation. Perhaps the greatest insight yet from these studies is that the mechanisms leading to seizures are not primarily attributable to misplacement of neurons. Rather, epileptogenesis in cortical malformations has more to do with the cellular subtypes comprising the disrupted cytoarchitecture, and the impact of the mutations on the intrinsic physiology of neurons in these altered brains. Important issues arising from these studies include such questions as: How do altered cytoskeletal dynamics impact the cell number, type, and connectivity of neurons – including neurite outgrowth, growth cone motility, synaptogenesis, and synaptic plasticity? What is the role of protein trafficking in neurons and glia in cell function, and what is the impact of impaired protein trafficking on excitability? Detailed knowledge of these events may yield new approaches to seizure management that goes beyond a focus on transmitter/receptor interactions, but impacts events like receptor turnover or subunit composition.

We can expect future fascinating revelations from approaches like conditional knockouts in the mouse to probe the contributions of various brain regions to seizure phenotypes. Conditional knockout models will also yield insight into the timing of when these molecular processes are most critical to the pathogenesis of epilepsy. With such insights, it may then be possible to intervene in the progression of developmental events that contribute to epileptogenesis. For example, modulation of Rho-family GTPase activities during particular temporal windows may promote synaptogenesis, stabilize existing contacts or promote physiological pruning of synaptic contacts to improve seizure control, or promote better cognitive function in the setting of antiepileptic drug administration. Such studies represent a truly new frontier in epilepsy research.

See also: **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; **Genetics**: Cytoskeletal Genes and Idiopathic Epilepsies; Genetic Contributions to Brain Structure and Epileptogenesis; **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; **Neuropathology**: Neuropathology of Epilepsy; **Pediatric Epilepsy**: Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Genetics of Lafora and Juvenile Myoclonic Epilepsies

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Introduction

How do we go about finding the cure(s) for a fatal epilepsy like Lafora progressive myoclonus epilepsy (Lafora epilepsy or Lafora disease), a condition which is rare in the Western world but frequent in the Middle East and South Asia? Likewise, how do we find the cure for a nonfatal but life-long epilepsy like juvenile myoclonic epilepsy (JME), which is common worldwide? To find the cure for these epilepsies, the epilepsy-causing mutations in *Laforin/dual specificity phosphatase* which cause 58% of Lafora disease, and in *Malin E3 ubiquitin ligase* which cause 35% of Lafora disease, were identified. The epilepsy-causing mutations in *myoclonin/EFHC1*, which causes 9% of clinic cases of JME, were also isolated.

These three nonion channel genes cause two similar myoclonic epilepsies when they start during adolescence. Understanding the nature of these gene products has allowed us to clinically separate them at the onset and helped us solve two mysteries of brain biochemistry and clinical epileptology: (1) Why is glycogen absent in neurons in contrast to other cells of the human body? and (2) Why do patients with juvenile myoclonic epilepsy (JME), classified as idiopathic, have areas of brain cortex thicker than normal? The first two genes, *Laforin (dual specificity phosphatase)* and *Malin (E3 ubiquitin ligase)*, working in concert, purge neurons of glycogen and polyglucosan inclusions by polyubiquitination and proteasome-dependent degradation. Thus, there is normally no glycogen in nerve cells. Mutations in *Laforin* or *Malin*, when inherited as a recessive gene, cause insoluble poorly branched glycogen (polyglucosan) to accumulate in nerve cells, cause cell death, produce stimulus-sensitive myoclonias, grand mal and absence seizures, rapid neurologic deterioration and death. The third nonion channel gene, *Myoclonin/EFHC1*, causes chronic and life-long adolescent onset juvenile myoclonic epilepsy. *Myoclonin/EFHC1,* a developmental gene, prunes branches and leaves of dendrites and modulates dendritic R-type voltage-dependent calcium channels (VDCC). Mutations in *Myoclonin/EFHC1*, when inherited as a dominant trait, reduce its pruning functions, cause thicker brain cortex, and at the same time alter dendritic R-type VDCC functions, producing myoclonias and grand mal seizures. Unlike fatal Lafora epilepsy, JME has a good prognosis and responds to antiepileptic drugs that work on VDCC.

Methods

> . . .In linkage analysis the simplest situation is when unequivocal linkage can be demonstrated in a single large pedigree (with lod score >3). Lander and Schork, 1994.

Large (more than 35 members) families, consisting of at least 3 generations that had at least 9 members that were clinically affected with Lafora disease or JME, were used for family genetic studies and linkage analyses. In Lafora disease, homozygosity mapping facilitated location of a chromosome locus. Haplotypes and recombinations, together with positional cloning, then narrowed the size of the chromosome region to 1–2 cM and to kb. Genes in the reduced region were then sequenced, looking for mutations that segregated with affected members of multigeneration families. Functions of *Laforin/dual specificity phosphatase* and *Malin E3 ubiquitin ligase* were studied in a variety of preparations – whole mouse brains, differentiated mouse N2a cells, primary cultured mouse neurons and astrocytes, rabbit skeletal muscles and liver – from wild type mice and Laforin deficient KO mice. To study the functions of *Myoclonin/EFHC1,* primary cultured hippocampal neurons were used for apoptosis experiments, and baby hamster kidney (BHK) cells were used for patch clamps electrophysiological studies.

Background and Results

Laforin and Malin Gene Function in Lafora Disease Explain a Mystery of Brain Biochemistry: Why is Glycogen Absent in Neurons?

Lafora epilepsy is an autosomal recessive fatal epileptic encephalopathy like other progressive myoclonic epilepsies (PME). It is characterized by the triad of stimulus-sensitive resting and action myoclonus, epilepsy, and progressive intellectual, cerebellar and neurological deterioration. A constellation of symptoms separate Lafora disease (LD) from other PMEs, including: greater frequency of visual auras, the rapid course of the illness (with death occurring 10 years after onset of epilepsy), and excessive accumulation of abnormally branched insoluble glycogen (polyglucosan inclusion bodies) in brain, muscles, liver, heart, retina, peripheral nerves, and

skin. Lafora's disease is found in the Mediterranean countries of Southern Europe (Spain, Portugal, Italy, Herzegovina, Croatia, Slovenia, Bosnia, Albania, Greece), Northern Africa (Morocco, Algeria, Tunisia, Libya) and the Middle East (Syria, Israel, Lebanon, Turkey, Cyprus, Egypt). It is also found in Ukraine, Uzbekistan, Kazakstan, Turkmenistan, Azebajian, Armenia, Malaysia, Indonesia, India, Pakistan, and other countries where consanguinity occurs.

Although Gonzalo Rodriguez–Lafora first discovered the periodic acid-Schiff and Alcian blue staining of polyglucosan inclusion bodies in a boy with PME in 1911, the modern story of Lafora disease did not start until 1995 when the first chromosome locus (6q24) for Lafora disease was discovered by Serratosa *et al.* Positional cloning then led to the eventual identification of *Laforin/dual specificity phosphatase* in 1998 and *Malin/E3 ubiquitin ligase* in 2003. About 80–90% of patients with Lafora disease have mutations in EPM2A/*Laforin/dual specificity phosphatase* and EPM2B/*Malin/E3 ubiquitin ligase*. A third locus/gene is yet to be identified. Because the gene function was unknown, the gene was originally called EPM2A. Now we know that it encodes a *dual specificity protein phosphatase*, named *laforin*, that localizes in the rough endoplasmic reticulum. Laforin also contains an N-terminal domain that binds to glycogen and Lafora bodies (see **Figure 1**). The EPM2B or NHLRC1 gene encodes a protein ubiquitin ligase (E3 ubiquitin ligase) called malin that interacts with Laforin in the endoplasmic reticulum (Gentry *et al.*, 2005). Laforin is one of the substrates of malin, meaning malin ubiquitinates laforin. Because laforin and malin are recruited upon proteasomal blockade, both must be involved in protein clearance through the proteasomal system (Mittal *et al.*, 2007). By 2002, two mouse models of Lafora disease were prepared by scientists in Japan and Toronto: one in which the laforin gene was knocked out in the developing embryo and the other in which a mutation of *laforin* was inserted into the developing embryo. With the discoveries of its disease-causing genes and their functions also came explanations for neuronal cell death and associated rapid neurologic deterioration. The EPM2A/*Laforin/dual specificity phosphatase* knockout mice had neuronal degeneration before the formation of lafora bodies and the onset of myoclonic seizures. The transgenic mice line expressing a dominant-negative mutant of *EPM2A/Laforin/dual specificity phosphatase* developed Lafora bodies but not epileptic seizures or early neuronal death. These observations suggest that the formation of Lafora bodies, the onset of epileptic seizures, and cell death are independent phenotypes of disease pathways regulated by *EPM2A/Laforin/dual specificity phosphatase*. Because laforin and malin are both recruited to aggresome when proteosome functions are blocked, cell death in mutations of

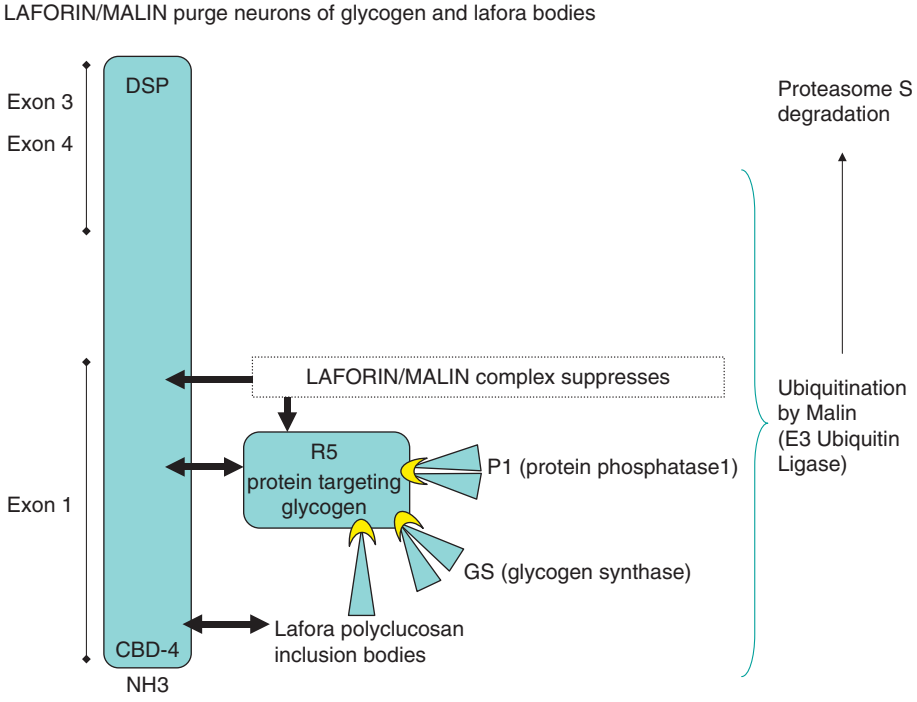


Figure 1 Laforin/malin complex purge neurons of glycogen and lafora bodies. Functional and interacting sites of EPM2A/Laforin with its dual specificity phosphatase (DSP) domain and R5, the protein targeting glycogen (PTG) scaffold in carbohydrate binding domain (CBD-4). Laforin and malin in concert suppress binding/targeting of protein phosphatase and glycogen synthase to R5, thus keeping glycogen synthase in an inactive phosphorylated state. Glycogen in turn inhibits laforin suggesting a feedback system. Malin polyubiquitinates laforin and Lafora bodies and eliminates the 'polyubiquinated laforin-Lafora polyglucosan inclusion and glycogen complex' through proteasome S degradation.

laforin or malin is now considered to be at least partly due to impaired proteolytic processes.

Since the discovery of *laforin* in 1998, six separate laboratories (from California, Indiana, Toronto, Madrid, and Barcelona) had been looking for the biochemical pathways that connect laforin and malin to glycogen and polyglucosan synthesis or degradation. We had hypothesized earlier that decreased *Laforin phosphatase* activities (when the gene was mutated) could lead to decreased dephosphorylation of glycogen synthase, turn glycogen synthase into an overphosphorylated state, and cause the accumulation of excessive amounts of an insoluble form of glycogen – polyglucosans. In this hypothesis of 'secondarily flawed' glycogen synthesis, there is an imbalance between branching enzymes and glycogen synthase activities, with glycogen synthase being favored to go into overphosphorylated drive.

Two earlier observations suggested a link between glycogen metabolism and formation of lafora bodies. The first observation is the G240S mutation in *laforin* which does not affect phosphatase activities or glycogen binding but impairs R5 binding of glycogen synthase. **Figure 1** shows the functional and interacting sites of *EPM2A/Laforin* with its dual specificity phosphatase (DSP) domain in exons 3 and 4 and R5, the protein targeting glycogen (PTG) scaffold in the carbohydrate binding domain (CBD-4) of exon 1. The G240S mutation in exon 1 of *laforin* meant that phosphatase activities can be normal but lafora bodies will still form if R5 binding of glycogen synthase is abnormal. The second observation is the serendipitous observations of Raben *et al.* in the GSL30 transgenic mice. GSL30 transgenic mice are mice that had acid alpha glucosidase knockedout and overexpressed glycogen synthase. Raben *et al.* was trying to replicate the fatal disease phenotype of Pompe disease in the acid alpha glucosidase knockout mice. However, the disease phenotype did not resemble the rapidly deteriorating muscle disease of Pompe disease. Thus, Raben *et al.* overexpressed glycogen synthase in the acid alpha glucosidase knockout mice in an attempt to accelerate the course of the disease. Instead, they were surprised to find accumulation of excessive amounts of poorly branched glycogen that resembled Lafora bodies in skeletal muscle.

Vilchez and colleagues addressed why glycogen is normally absent in neurons, and how poorly branched insoluble glycogen (Lafora inclusion bodies) forms and accumulates in neurons in Lafora disease. Using a neuronal cell model in culture, Vilchez *et al.*, from Guinovart's laboratory in Barcelona, showed that neurons have the enzymatic machinery for synthesizing glycogen. However, this machinery is suppressed by retaining glycogen synthase in the phosphorylated and inactive state. How is this suppression ensured? It appears that laforin and malin cause proteasome-dependent degradation of the receiving or binding protein of laforin called R5 or PTG (protein targeting glycogen in the carbohydrate binding domain of exon 1 of *laforin* (see **Figure 1**)). R5 or PTG (for protein targeting glycogen) is a scaffold adaptor protein that binds and targets glycogen to the glycogen binding site of *laforin* (see **Figure 1**). PTG also normally binds and brings the enzymes involved in glycogen synthesis to *Laforin* which is normally located in the vicinity of the endoplasmic reticulum inside neurons. This function includes bringing protein phosphatase 1 to glycogen synthase, thus activating this enzyme which synthesizes glycogen. PTG also normally brings glycogen synthase to laforin. The laforin–malin complex by degrading R5/PTG in essence block glycogen binding, protein phosphatase 1 activation of glycogen synthase and glycogen synthesis. Overexpression of R5/PTG leads to glycogen deposition in cultured neurons and apoptosis. Thus, the laforin–malin complex ensures a blockade of neuronal glucogen synthesis under normal conditions. In the presence of a mutation in either the laforin or the malin gene, excessive glycogen is deposited in neurons and apoptotic cell death results. In 2008, Worby *et al.* found similar results when they overexpressed PTG, *malin and laforin* in tissue culture cells. The expression of malin or laforin decreased PTG, stimulated glycogen accumulation by 25%, and coexpression of malin and laforin abolished PTG stimulated glycogen accumulation. *Malin* ubiquitinates PTG in a laforin-dependent manner, both in vivo and in vitro, and targets PTG for proteasome-dependent degradation.

The actual experiments proving the 'overphosphorylated glycogen synthase hypothesis' were recently provided in tissue culture and in the *laforin* deficient mice (Epm2a−/−) by experts in glycogen metabolism at laboratories in Indianapolis. Tagliabracci *et al* tested this concept of an overphosphorylated glycogen synthase in the mouse knockout model of Lafora disease (Epm2a−/−). Glycogen isolated from the KO mice had a 40% increase in covalent phosphate content in liver and a fourfold elevation in muscle. Recombinant *laforin* normally releases phosphate in vitro in a time-dependent reaction, with an apparent $K(m)$ for glycogen of 4.5 mg ml^{-1}. These investigators therefore proposed that mutations of laforin disable its carbohydrate/glycogen binding domain and also eliminate its ability to dephosphorylate glycogen. Excessive phosphorylation of glycogen leads to aberrant branching of glycogen, excess glycogen, and Lafora polyglucosan inclusion bodies.

Myoclonin/EFHC1 Gene Function in JME Explains a Contradiction in an Idiopathic Epilepsy: Why do some JME Patients have Areas of Thicker Brain Cortex?

At present, there is no cure for juvenile myoclonic epilepsy, a common and lifelong affliction. JME is the most common form of idiopathic generalized epilepsy and the

most common cause of primary grand mal seizures. JME accounts for 12–30% of all epilepsies in hospitals and clinics, while a house-to-house population survey estimates and undercounts JME at 3% of all epilepsies. The two most common forms or subtypes of JME are classic JME and childhood absence that evolves into JME. In classic JME, awakening myoclonias start when patients reach 13–15 years of age, and grand mal convulsions appear 2 years later. Absence seizures are not present in probands and do not appear as the sole phenotype in affected nonproband family members. When childhood absence evolves to JME, pyknoleptic absences with 3 Hz spike and wave complexes and 4–6 Hz spike or polyspike waves complexes persist into adolescence at which time myoclonias and grand mal convulsions start. Nonproband affected family members can have absence only, grand mal only, or absence and grand mal but they rarely have JME.

Neuropathology of some patients with JME reveals increased number of and diffusely distributed dystopic neurons in gray matter stratum moleculare and subcortical white matter of brains. Quantitative high-resolution voxel-based MRI detects such JME neuropathology as significantly larger, thicker, and increased cerebral cortical gray matter in mesial frontal lobes.

To determine the cause of JME, Suzuki *et al.* identified its disease causing gene, *Myoclonin/EFHC1*, in chromosome 6p12. Mutations accounted for 20% of our JME families. The ancestral origins of these families were from Spain mixed with native American Indians, e.g., Spanish-Amerinds who presently reside in Mexico and Los Angeles, California. Medina *et al.* showed that 9% of consecutive clinic patients with JME, including singletons and sporadic, have M*yoclonin/EFHC1* mutations. Singletons are patients who have no affected siblings but have one parent carrying a mutation. Sporadics are patients who have no affected siblings and have parents who do not have the disease mutation. True sporadic imply a 'de novo' mutation.

To explain displaced neurons in brain gray matter molecular layer on neuropathology, and MRI detected thicker cerebral cortical gray matter in mesial frontal lobes, Suzuki *et al.* transfected mouse hippocampal neurons in cell cultures with wild type *Myoclonin/EFHC1* and characterized neuronal differentiation and neurite outgrowth. Sixteen hours after transfection, GFP–EFHC1 positive neurons showed shorter neurites and fewer branches (in contrast to neurons transfected by pEGFP vector only. Forty-eight hours after transfection, all GFP-EFHC1 positive neurons exhibited signs of neurodegeneration and cell death, including shrinkage of cell body and fragmentation of processes. Control cultures transfected with pEGFP vector showed no features of degeneration. EFHC1 transfected cells were TUNEL positive, indicating apoptosis or programmed cell death, a genetically driven mechanism by which nature prunes cells during development.

To explain susceptibility to seizures in patients with mutations in *Myoclonin/EFHC1*, we looked at its calcium functions, since the myoclonin gene has one calcium binding motif in its EF-Hand component. Our collaborators at the RIKEN Brain Science Institute in Japan patch clamped baby hamster kidney (BHK) cells cotransfected with various types of VDCCs. Significant results were found when *Myoclonin/EFHC1* was cotransfected with R-type VDCC, calcium channels that are mainly in dendrites; *Myoclonin/EFHC1* significantly increased R-type VDCC currents. Mutations from human JME reversed the increased R-type Ca^{2+} currents.

These studies allowed us to hypothesize that *Myoclonin/EFHC1* is involved in pruning neurons and its dendritic tree, and in the enhancement of dendritic R-type VDCC currents. Mutations decrease *Myoclonin/EFHC1* pruning functions and produce the thicker cerebral cortical mantle detected by voxel-based quantitative MRI in JME patients. Mutations also alter calcium homeostasis in postsynaptic dendritic trees mediated by R-type VDCC, thus potentially explaining why JME patients are susceptible to myoclonias and grand mal convulsions when sleep deprived or exposed to alcohol. With our collaborators, we tested these hypotheses in mice and in *Drosophila melanogaster*, and confirmed that *Myoclonin/EFHC1* is a developmental gene involved in neuronal cell division, neuroblast migration and programmed cell death of neurons and its dendrites. We are now examining these same biological functions in the myoclonin deficient KO mice to see if we can link these functions to seizure susceptibility and epileptogenesis.

Present and Future Goals

Genotyping Adolescent Myoclonic Epilepsy Helps Treatment Choice

Genotyping adolescent myoclonic epilepsies will separate Lafora disease from JME. In their early stages, Lafora disease and JME are clinically similar (see **Table 1**). Both Lafora disease and JME start with expression of myoclonic, tonic clonic grand mal, and absence seizures at late childhood or adolescence. The identification of nonsense mutations in Lafora disease is important because nonsense mutations and their premature stop codon can be treated effectively with premature stopcodon readthrough drugs, such as gentamicin. Theoretically, treatment in Lafora disease is best started before neurologic deterioration ensues, before ataxia and dementia set in, before independent daily living activities are lost, and certainly before the need for gastrostomy is realized.

Can early Lafora PME be clinically distinguished from JME? Yes, a clinician experienced in PME can look for subtle differences between these epilepsies, but clinicians

Table 1 Lafora PME and JME are similar at onset of illness

Epilepsies	*Seizures*		*Age (yrs) at onset of neurological deterioration*						
	Age (yrs) at onset of Grand Mal, Myoclonic and Absence	Occipital Seizures	Mild cognitive decline	Mild ataxia	Severe ataxia dementia, preserved daily living activities	Impaired/loss of independent daily living activities	Anorexia, swallowing difficulties, gastrostomy	Respiration Assist; Mutism	Prognosis
Lafora PME									
(a) Laforin-deficient	6–19 yrs (80% at 10–12 yrs)	Yes (in 1/3)	10–14 yrs	10–14 yrs	17–20 yrs	16–20 yrs	18–20 yrs	20–25 yrs	Death in 2–10 yrs
(b) Malin-deficient	7–15 yrs	Yes (in 1/3)	16–27 yrs	16–27 yrs	26–32 yrs	26–32 yrs	26–37 yrs	26–37 yrs	Death in 10–30 yrs
Juvenile Myoclonic Epilepsy (JME)[a] (Herpin-Rabot-Janz type)	8–26 yrs (75% at 12–18 yrs); (Mean age at onset 14 yrs[b])	None	None	None	None	None	None	None	Good

[a]Some cases with myoclonus only.
[b]Usually triggered by sleep deprivation, menses, stress, fatigue, alcohol.

can still mistake one for the other early in their courses, especially when seizures are not stimulus-sensitive, action myoclonus is absent, and there are no occipital seizures. Genotyping will solidify and clarify diagnosis. Genotyping is crucial for early diagnosis in Lafora PME so treatment of nonsense mutations can begin.

Treatment – Curing Lafora Disease

Boosting protein synthesis from <1% to as little as 5% of normal levels reduces severity of disease phenotype.

Premature stopcodon readthrough drugs

Genotyping in Lafora disease will show if deletions, frameshifts, missense or nonsense mutations are present in laforin or malin. If nonsense mutations are present, intravenous gentamicin, a premature stopcodon readthrough drug, is clinically justified for 'compassionate use' in this fatal disorder. PTC 124, a new orally bioavailable nontoxic, small molecule that selectively induces ribosomal readthrough of premature but not terminal codons, and is superior in potency to gentamicin, is not yet commercially available. PTC124 coupled to a fusion protein could penetrate the blood–brain barrier and may be a future treatment.

Because Lafora PME is a lysosomal disease, polyglucosan inclusion bodies accumulate in liver, retina, cardiac muscles, esophageal skeletal muscles, and diaphragm muscles producing anorexia, malaise, visual problems, cardiac arrhythmias, swallowing and breathing difficulties. Boosting protein synthesis of laforin and malin, which function to purge polyglucosan inclusion bodies from cells, should slow the disease and alleviate the peripheral pathologies responsible for anorexia, malaise swallowing and breathing difficulties. High concentrations of intravenous gentamicin can also be expected to cross the blood–brain barrier – because the continuing and frequent seizures cause disruption of the blood–brain barrier, and because endothelium and neuronal cell death break open the blood–brain barrier. Selective and specific readthrough of disease-causing premature termination codons in endothelium and neurons should boost protein synthesis of laforin and malin, which purge the neurons of Lafora inclusion bodies and alleviate the central pathology of Lafora PME. Potential renal and otic toxicities produced by high concentrations of intravenous gentamicin limit their clinical use to invariably fatal diseases like Lafora PME.

Gene replacement therapy is the future

For deletions, frameshifts, missense or nonsense mutations in laforin or malin, gene replacement treatment would be the ideal treatment. Cornford and Hyman have successfully transported laforin across the blood–brain barrier of laforin-deficient KO mice after intravenous administration of an expression plasmid containing laforin packaged in the interior of neutral pegylated immunoliposomes (PIL). The external PIL is conjugated with OX26 monoclonal antibodies against transferrin receptor, which transports the vehicle across the blood–brain barrier. Presently, these investigators are administering PIL packaged with all four exons of laforin, with SV40 promotor, using mice epm2a polyclonal antibodies to confirm passage of epm2a through the blood–brain barrier in exon 4 KO homozygous null mutant mice. So far, laforin has been shown to rescue the pathology of LD in epm2a KO mice when administered in utero and in early postnatal months. Laforin replacement is now being studied in older mice with Lafora disease. In the near future, the same method of delivering epm2a/laforin can be applied to Laforin deficient humans.

See also: **Genetics**: Cytoskeletal Genes and Idiopathic Epilepsies; Epilepsy Genes that do not Encode Ion Channels; *GABRB3* and Childhood Absence Epilepsy; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future; Large-Scale Mapping and Epilepsy Gene Discovery; **Treatments (non-Antiepileptic Drugs)**: Immunoliposome Gene Therapy and its Potential use in Treatment of the Epilepsies.

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Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future

W N Frankel, The Jackson Laboratory, Bar Harbor, ME, USA

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Introduction

Broadly defined, a complex genetic trait is one which is present in families, but which is not governed by simple Mendelian inheritance (e.g., autosomal dominant or recessive) whereby the presence or absence of the single genetic variant is the sole determinant of a disease. Although there are several ways in which to violate Gregor Mendel's laws of inheritance, the concept 'complex genetic trait' usually refers to the effects of multiple genetic variants that are coinherited in an individual, and which together with nongenetic, environmental factors, are necessary to effect a biological outcome (**Figure 1**).

Several terms for 'complex genetic trait' have been used somewhat interchangeably in the scientific literature. The term 'polygenic' refers to traits that are determined literally by *many* genetic variants, and 'oligogenic' refers to traits that are determined by *several* genetic variants. The term 'multifactorial trait' is broader, and includes both genetic and nongenetic factors. In animal models, and increasingly in human, one also sees the abbreviation 'QTL' (quantitative trait locus). QTL was classically defined as a trait that is characterized by continuous, metric variation (e.g., height, weight, blood glucose) due to multiple factors. But since many disease-related traits are measured instead qualitatively (i.e., all-or-none traits such as diabetes, cancer, epilepsy, etc.), the term QTL is sometimes used to encompass discrete conditions as well.

How can multiple factors cause an all-or-none disease? The most commonly accepted model is referred to as 'liability-threshold' (**Figure 2**). That is, while disease outcome may be all-or-none, it is presumed that there are multiple factors 'under the hood' – namely, genetic variants and environmental effects which present a liability to the individual. Together, these factors conspire to lower the threshold for disease (or alternatively, to increase susceptibility). Of course, the effect of an individual gene may (if there was a way to measure it) ultimately be quantitative with respect to subsidiary aspects of the disorder. These underlying features, sometimes called sub- or endo-phenotypes, are usually more convenient to measure in metabolic and systemic disorders (e.g., T-cell counts in autoimmune diseases, blood glucose in diabetes, cholesterol in heart disease) than in disorders involving the intact central nervous system. In other words, full-blown disease is often the only endpoint that can be used to measure a trait such as epilepsy.

Finally, adding to the genetic complexity is a phenomenon known as locus heterogeneity – referring to the observation that individuals may have the same disorder but for different genetic reasons. Progress in unraveling the genetic basis of epilepsy is particularly hampered by this phenomenon. A family may indeed carry a single gene defect that underlies the disease, but locus heterogeneity makes finding the defect much more difficult because individual families are usually too small for unambiguous linkage and high resolution genetic mapping.

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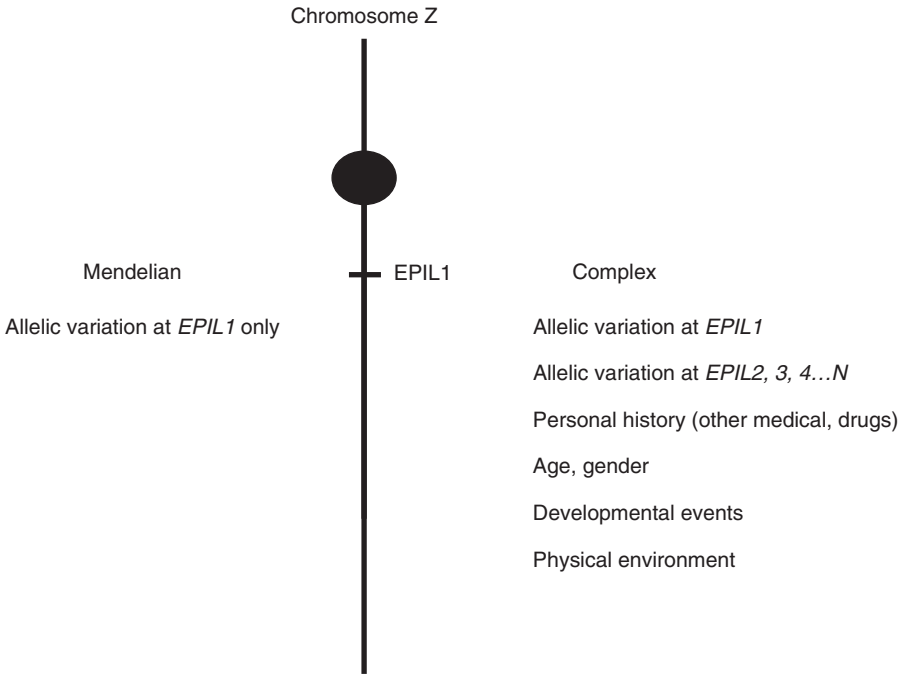


Figure 1 Hypothetical factors influencing clinical manifestation of fictitious epilepsy gene, *EPIL1* Mendelian versus complex trait.

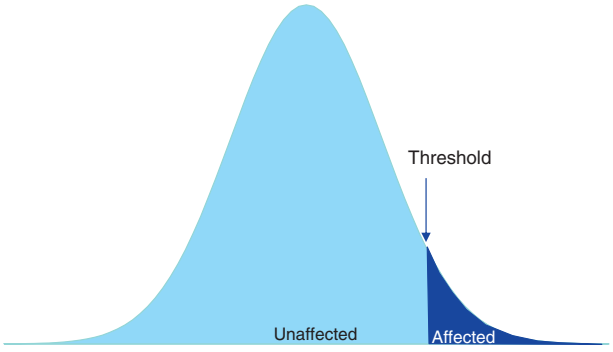


Figure 2 Threshold model for inheritance of discrete complex genetic traits.

The converse, phenotypic heterogeneity, is also a complication in epilepsy. Here, the same genetic variant may result in different outcome for different individuals. Naturally, until the genetic basis of a given individual's disorder is worked out, one cannot know whether phenotypic heterogeneity is due to variable gene expression or to other predisposing factors.

Background

Complex Genetics of Human Epilepsy

The population incidence of epilepsy in developed countries is about 1%. Many epilepsies are generalized, and without obvious physical or organic cause they have been termed idiopathic; other generalized epilepsies that have a suspected cause are termed symptomatic (or cryptogenic). It has long been suspected that idiopathic generalized epilepsies are genetic, since cases tend to cluster in families and do not have other obvious explanations. Indeed, the risk of having a seizure disorder is greater in first-degree relatives of individuals with epilepsy compared with the general population, but the increased risk is modest – only 2–4-fold. Nonetheless, from studies of monozygotic (MZ; genetically identical) versus dizygotic (DZ; fraternal) twins, the evidence for a genetic basis of epilepsy is very strong – for example, with respective casewise concordances of 0.62 (MZ) versus 0.18 (DZ). Furthermore, when considering certain subtypes of epilepsy (e.g., idiopathic generalized, symptomatic generalized, partial), the monozygotic twin concordances are even greater for both idiopathic and symptomatic epilepsies, but even partial epilepsies have a significantly higher concordance in monozygotic twins (MZ = 0.36 vs DZ = 0.05). Together with the observed familial aggregation, these data indicate a strong genetic component for all generalized epilepsies, and further suggest that even partial epilepsies have an appreciable genetic component. However, within most epilepsy families, the mode of inheritance is not straightforward; usually only a few individuals are affected, leaving little power for robust genetic analysis.

Genes for Genetically Complex Epilepsy in Human

Only a very small fraction of families inherit epilepsy in a Mendelian fashion, but almost everything that is known

about the genetic basis of inherited human epilepsy comes from the identification of genes in these few families. To date, approximately 20 genes for human idiopathic epilepsy are suspected or known. Most were identified by linkage analysis and candidate gene testing in pedigrees or small families that had a particular form of Mendelian epilepsy, such as benign neonatal convulsions, febrile seizures, juvenile myoclonic epilepsy, and rare partial epilepsies. Most of the identified genes encode ion channel proteins, others encode genes that likely regulate ion channels, but several encode other types of proteins, some with as-yet unknown function. One way to collect a sufficient number of subjects to confidently detect candidate genes for the more common, genetically complex epilepsies is by undertaking very large-scale genome-wide association studies (GWAS). Indeed, in 2007, GWAS facilitated the identification of genetic factors for several human complex traits, including discrete disorders such as Crohn's disease and diabetes, and quantitative traits such as height. However, it is unclear for epilepsy or other complex genetic disorders whether GWAS will prove to be a panacea or rather merely continue to chip away at the problem by identifying the 'low hanging fruit.' For example, a very good physiological candidate for childhood absence epilepsy is the *CACNA1H* gene, encoding a low-threshold voltage-dependent Ca^{2+} channel – a central player in generating the thalamocortical rhythms that are thought to be involved in absence seizure episodes. Initially, mutations were found in Chinese families. However, a later study examined a larger group of epilepsy patients, and identified a number of *CACNA1H* variants which, when tested in the laboratory, proved to encode functional differences at the protein level. These results suggested that *CACNA1H* variants may contribute to epilepsy more commonly in the population; however, such variants would have been impossible to detect statistically in a typical GWAS approach because they are relatively rare. This 'rare variant-common disease' conundrum does not mean that attempted large-scale GWAS for epilepsy will not be worthwhile, since more common functional variants may well exist. Rather, these results indicate that it is difficult to know how successful the approach will be.

With regard to ion channels as prime candidate genes for common epilepsies, a different approach is represented by the Ion Channel Project at the Baylor College of Medicine. This underlying hypothesis of this study is that common genetically complex epilepsies are largely due to unique (and presumably deleterious) combinations of ion channel variants that exist in the population. The investigators have begun sequencing 250 ion channel genes in several hundred IGE patients and normal controls. Although ion channel variants may not be the whole explanation for genetically complex epilepsy, it is a hypothesis well worth examining, given the role of ion channel defects in Mendelian epilepsies (as well as in animal models). It will be very interesting to learn whether unusual combinations of naturally occurring variants cluster significantly in epilepsy patients.

Methods

Approaches to identifying genetic variants that are associated with complex disease generally fall under the category of 'forward genetics,' which means simply that the molecular identity of the gene is not known ahead of time, and that genetic and genomic methods are used to find it. Typically, to identify genes for simpler genetic traits, one uses the approach of *positional cloning*, whereby the causative gene is identified by virtue of its position on a chromosome. Classically, this process involves 'walking' along the chromosome from a genetic marker on one side of the chromosomal interval associated with a disease, to the other side, characterizing and evaluating molecular variants (e.g., by using gene expression or structure analysis) along the way until one variant is found to be a strong possibility. That variant is then independently tested (e.g., by creating and testing a new allele, or performing a rescue experiment, or both). A variant of *positional cloning* is *positional candidate* – a slightly less systematic approach whereby the chromosomal interval or region is known, and researchers make educated guesses about the gene of interest based on the position or nature of a nearby gene; molecular variants of this 'guess' can then be evaluated.

In principle, finding genes for complex traits can also use positional cloning or candidates. However, the methods are technologically somewhat more challenging because of the ambiguous relationship between genotype and phenotype – that is, the property that defines complex traits as complex. Thus, a study population that is segregating one molecular variant that influences a particular phenotypic trait, is also segregating all the other variants, making it difficult to unambiguously define the genotype–phenotype relationship for a variant. Consequently, it is much harder (and takes quite a bit longer) to achieve a high-resolution genetic map for the trait at hand; in many cases, the best possible chromosomal intervals are still rather large. Because of this difficulty, several genetic and genomic 'add-ons' are often applied to forward genetics of complex traits. These additional features include: (a) treating the genetic mapping population more like an association study rather than as a linkage study (to disambiguate the genotype–phenotype relationship statistically, rather than empirically); (b) combining the genetic analysis (to narrow the position of the gene on the chromosome) with a genomic analysis – for example, to use microarrays or even proteomics tools to find all expression differences between two strains that differ for a trait, and then determine which ones map to the defining

chromosomal interval; and (c) incorporating the role of gene x gene interactions, or epistasis, into the analysis (i.e., when the consequences of inheriting one variant depends very much on whether another variant is coinherited).

Interestingly, in experimental organisms, the same kinds of mapping populations (e.g., backcross, intercross, recombinant inbred, and congenic strains) can be used to map both genetically simple and genetically complex traits. However, the parameters of the study (population size, density of genetic markers used, type and extent of analysis) are much more demanding for genetically complex traits.

Results

Rationale for Modeling Genetically Complex Human Epilepsy in Laboratory Rodents

Rodent genetic models contribute significantly to our understanding of basic mechanisms of human disease in just about every field of research – in autoimmune disorders, cancer, heart disease, and disorders of the central nervous system such as epilepsy. From a molecular perspective, this translation from rodent to human is not surprising, given the very high degree of conservation between human and rodent genomes. There is good reason to think that many core mechanisms are very similar, if not identical, between the two. When clinical manifestations of seizures appear to differ between rodent and human, that difference may reflect (at least in part) our inability to observe the relevant neurological signs at the same level of detail in mice as in patients. Perhaps, with practice, this difficulty may be overcome. However, there may be some aspects of human seizure disorders which mutant rodents might never be able to model; for example, their lifespan may be too short to develop the full spectrum of secondary pathologies of human temporal lobe epilepsy (TLE), even though some rodent models mimic the behavioral, physiological and pharmacological aspects quite well. Nevertheless, enlightening examples of conservation of higher-order function across mammalian orders do exist. For example, given that mice are nocturnal animals and they do not have even a developed frontal lobe, per se, it is not clear that we would predict that the mutation for Autosomal Dominant Nocturnal Frontal Lobe Epilepsy (ADNFLE) (a rare form of partial epilepsy in human) would be functionally conserved between humans and mice. However, the cognate mutant mice seem to model the human disorder reasonably well, as the authors of one study conclude: '. . .these mice cover the spectrum of behavioral and electrographic events seen in the human condition. . .'

This statement epitomizes the general approach to studying animal models of genetically complex epilepsy. They are studied because of obvious parallels with the human condition: the type of seizure, physiological and pharmacological properties – and the complex nature of inheritance. Since until recently it has not been possible to even contemplate mapping and identifying genes for complex traits in humans, rodent systems have been approached with the broader rationale of understanding the 'architecture' of epilepsy as a complex genetic trait. It is likely that the same genes, or at least the same molecular pathways, are affected in humans as in rodents – although this assumption remains to be demonstrated, mainly because so few human epilepsy genes are known.

However, even if we did not know anything about the genetic basis of human epilepsy, from a phenotype search of mouse mutation listings in the The Jackson Laboratory's Mouse Genome Informatics database (from which information about all published mouse mutations can be obtained) one infers that over 400 different genes have the potential to be human epilepsy genes (i.e., by causing spontaneous seizures in mice when mutated) or to lower the seizure threshold. Since most genes have not yet been mutated in mice, and since many that have been mutated represent complete gene knockouts (as opposed to subtle, but functional protein variants), it seems likely that the number of 'epilepsy genes' will be much higher than 400. Certainly, such predictions are consistent with the observed locus heterogeneity in human studies so far.

Beyond locus heterogeneity, however, the inheritance characteristics of rodent models are very likely to predict and validate what we can expect to find in genetically complex epilepsy in human. The remainder of this article considers several examples of what has been learned so far from complex genetic models of epilepsy in mice and rats, drawing from different types of seizure disorders.

Genetically Complex Models for Grand-mal Type Seizures in Mice

Several inbred mouse strains display complex partial seizures with secondary generalizations – grand-mal like episodes that mimic the behavioral and encephalographic features of temporal lobe epilepsy (TLE), a common form of human epilepsy. The strains EL, PL, and SWXL-4 exhibit recurrent seizures beginning at several months of age, following either routine handling (e.g., weekly cage changing) or more systematic, but still gentle physical stress. In 1991, one of the first efforts to map QTLs for any complex trait in mammals was done for epilepsy, by analyzing EL mice that were backcrossed to DBA/2J and ABP, unrelated strains that are relatively resistant to this type of seizure. These studies resulted in three dominant QTLs for seizure frequency (*El1*, Chr 9; *El2*, *Chr 2*; *El3*, *Chr 10*), accounting for a significant fraction of the seizure frequency in the backcross. Although the mapping itself became fairly straightforward with the advent of

genome-scanning technologies, (and many studies followed suit for different disorders and traits), the underlying genetic complexities of epilepsy soon became apparent. First, only one of the three initially mapped loci (*El1*) had an effect in crosses to both resistant strains. Second, in follow-up studies that used an intercross rather than a backcross (these two crosses give different mixtures of parental genotypes for a gene), only one of the three chromosomes showed any association (Chr 9), and further analysis suggested that this association was due to a fourth locus (termed *El4*) residing some distance away from *El1*. Despite this result, however, when the *El1*-, *El2*- and *El3*-containing chromosomal regions were transferred from the EL strain onto ABP (resulting in a congenic strain), two regions (*El1*, *El2*) had large effects on seizure frequency – validating the initial mapping. Last, and strikingly, in crosses between EL and a resistant strain (DDY) that is most closely related to EL, none of the previously mapped QTL had any measurable effect whatsoever! Instead, a new major locus, *El5* (Chr 14) was found, but only in an intercross population, not in a backcross! Together, these data suggest the frustrating conclusion that, despite the persistence of several trait loci in crosses and in congenic strains, none represented a core or essential seizure locus – that is, a locus that defines, or at least is always associated with, the seizure susceptibility of EL mice.

Similar studies involving another inbred mouse strain demonstrated more directly that complex diseases are defined by deleterious combinations of genes from the parents. SWXL-4 mice display seizures similar to EL, but while the precise genealogical origin of the EL strain is not known, SWXL-4 is one of a set of seven. Recombinant Inbred (RI) strains constructed to inherit a deliberate but arbitrary 50:50 mix of the two progenitor strains SWR and C57L. Interestingly, of each progenitor and all six SWXL RI strains, only SWXL-4 exhibited seizures – suggesting that the seizure disorder was a 'synthetic' trait caused by a combination of parental genes. This hypothesis was tested and proved by re-crossing the SWR and C57L, and determining that a fraction of hybrid progeny had high seizure frequency like SWXL-4. Again, however, while QTL associated with seizures were mapped in crosses of SWXL-4 mice, and a new seizure frequency locus (*Szf1*, Chr 7) was confirmed in a congenic strain, *Szf1* was not sufficient to explain why SWXL-4 has a serious seizure disorder. Furthermore, as was the case with at least one locus from EL mouse, *Szf1* itself was determined not to be one gene but to consist of several genes, that happened to be coinherited on the same chromosome and that interacted functionally with each other – another frustrating aspect of resolving some complex trait loci as has been noted in other animal models of complex inheritance.

Additional complexities in these and other models come from gene x environment interactions. Recently, some of the EL genetics were examined in a protocol that was particularly attuned to certain nongenetic variables. The authors were able to replicate and extend some, but not all the seizure loci detected previously; this study demonstrated that both native (e.g., age) and experimental factors (e.g., test conditions) can influence the outcome. Despite the attention to these finer points, the genetic map, and the essential complexity of the models, were not resolved sufficiently to allow breakthroughs at the molecular level – that is, to result in gene discovery.

In summary, these studies of grand-mal like seizures in inbred mouse strains illustrate the typical properties of complex genetic traits. If such mapping results were obtained in a human population and not in the laboratory where they can be relatively easily validated, one might dismiss most of the mapping results as 'false positives.' Instead, however, since many loci were replicable, the results illustrate defining characteristics of complex genetic traits: the impact of, and the ability to define, a susceptibility gene depends heavily on the presence of other genetic variants, and on environmental conditions. It is important to consider this concept in any future efforts to better resolve human complex traits.

Rodents with Genetically Complex Absence, or Petit-mal Seizures

Several strains of laboratory mice and rats display spontaneous absence, or petit-mal seizures, as evidenced by electroencephalographic (**Figure 3**), behavioral, and pharmacological features. As with other animal models of epilepsy, the degree to which these strains mimic the human condition is sometimes questioned. One property of rodent models of absence epilepsy is that frequency of the corresponding electrographic oscillation, known as a spike-wave discharge (SWD), is usually not 3–4 Hz as in human absence epilepsy, but rather 5–9 Hz. This difference is probably due to a species-specific property, perhaps related to the absence of thalamic interneurons in rats and mice which in human may serve to buffer the oscillation. Another difference between rodent and human conditions is that rodent absence episodes do not subside after adolescence; the reason for this difference is not known, but may be viewed as a pragmatic advantage, facilitating laboratory study. In other ways, the models discussed here have all the hallmarks of human absence epilepsy.

Two well studied rat models of genetically complex epilepsy are absence models: the GAERS (Genetic Absence Epilepsy Rat from Strasbourg) and the WAG rat strains. The physiology and pharmacology of GAERS have been studied for many years, while the WAG rat is a more recent subject. Initial genetic analysis has also been done in GAERS and WAG. In crosses between WAG and a nonseizing strain, parameters such as the frequency,

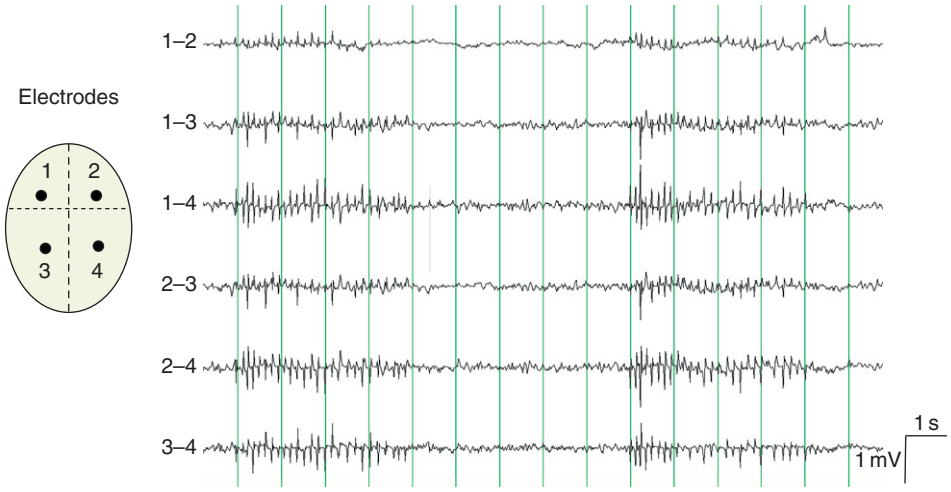


Figure 3 Spike-wave discharges in a mouse model of genetic absence epilepsy.

duration, and amplitude of spike-wave discharges were carefully measured, as well as the qualitative form and shape of the SWD themselves, and QTL analysis was performed. Investigators concluded that absence epilepsy segregates as a complex genetic trait in WAG, with underlying genes mapping to multiple regions of the genome, analogous to the situation with EL mice for convulsive seizures. A similar conclusion was drawn for GAERS, although with different chromosomal locations. While candidate genes reside in the vicinity of these QTLs, the critical intervals are still too large to provide useful hints about which genes encode the underlying variants.

A/J and C3H/HeJ are two inbred mouse strains that display genetically complex absence seizures, possibly representing slightly simpler forms of genetically complex epilepsy. Like GAERS and WAG, these strains do not have any other overt neurological abnormalities. They exhibit a modest incidence of seizure episodes, particularly during slow-wave sleep or in transitions to and from slow-wave sleep. Preliminary analysis in crosses between A/J and the normal strain (C57BL/6J) suggests oligogenic inheritance, with loci on Chromosome 4 contributing to the absence seizure phenotype (although genes have not yet been identified). Significant progress has recently been made in the C3H/HeJ model. Crosses between C3H/HeJ and C57BL/J revealed that a major locus, accounting for about half of the phenotypic variance, mapped to the *Gria4* gene on Chromosome 9, encoding a subunit of the AMPA receptor ion channel. A unique mutation was found in the *Gria4* gene, causing decreased expression of full-length RNA and protein. Further studies utilizing a man-made knockout (null) allele proved that *Gria4* gene defect was responsible for the epilepsy. Interestingly, however, the rate of seizures was much higher in outcrosses than in C3H/HeJ itself, and was not dependent on activity state. This result suggested that several recessive modifier genes together actually dampen the seizure phenotype in the C3H/HeJ inbred strain. In outcrosses between C3H/HeJ and other strains, these modifiers become 'unmasked,' allowing full expressivity of the *Gria4* deficiency; in this latter case, many mice exhibit hundreds of seizure episodes every hour, as opposed to 20–30 episodes per hour in C3H/HeJ itself. The identities of these modifier genes are not yet known.

The A/J and C3H/HeJ represent complex genetic traits that are not hopelessly complex, but not simple either. In the case of A/J, it apparently requires several susceptibility alleles to cause an appreciable frequency of absence seizures, whereas seizures in C3H/HeJ appear to be due to a more Mendelian-like single gene trait, with modifier genes that modify the phenotype.

Genetically Complex, Experimentally-Induced Seizures in Mice

A large body of literature considers the ability of adverse stimuli, including chemicals, electrical shock, and loud noises, to elicit seizures in rodents. In fact, experimentally induced seizures in mice and rats remain the primary basis of screens to evaluate the efficacy of anticonvulsant drugs. While most drug screening efforts utilize only one or two strains, the larger collection of strains that exist reveal a very broad spectrum of seizure response to adverse stimuli, suggesting that there is considerable genetic complexity in mice.

Several efforts have exploited strain diversity in order to study the genetic basis of seizure threshold response to stimuli in mice. The earliest studies in this area considered the susceptibility to audiogenic seizures. In 1946, investigators discovered that the DBA mouse strain is susceptible, and C57BL/6 is resistant, to wild (often lethal) tonic–clonic seizures when exposed to a loud doorbell hung on a galvanized drum. Decades later, extensive

surveys were done using calibrated stimuli to establish the parameters of audiogenic seizure induction in various inbred strains. In the late 1980s and early 1990s, recombinant inbred strains between the susceptible DBA/2J and resistant C57BL/6J progenitor strains were used to map genes that determined sensitivity to audiogenic seizures, resulting in the mapping of several presumptive loci, *asp1* (audiogenic seizure prone-1, Chr 12), *asp2* (Chr 4), and *asp3* (Chr 7). As was the case in EL and SWXL-4 strains, the underlying genes for these loci have not yet been identified, likely due to their complex inheritance.

However, thanks to perseverance by other researchers, there may be light at the end of the tunnel for identifying underlying seizure-susceptibility genes. The same progenitor strains (DBA/2J and C57BL/6J) showed a similar polar response to chemoconvulsants (e.g., pentylenetetrazole (PTZ), the GABA receptor antagonist, and kainic acid) and to electroconvulsive seizures, with DBA/2J being relatively seizure sensitive and C57BL/6J relatively resistant. QTL studies were done in an intercross between these strains; a locus on Chromosome 1 (*Szs1*) was found to be a major determinant of susceptibility, and other significant or suggestive QTLs were ultimately mapped as well (*Szs2*-*Szs13*), some of which have been replicated. Using a congenic strain and an elegant transgenic rescue strategy, investigators narrowed the critical interval for *Szs1* to only six possible genes. A strong candidate was identified in *Kcnj10*, encoding an inward rectifier K^+ ion channel, in which a nonconserved amino acid differs between parental strains. While definitive confirmation is still awaited, the human homologue, *KCNJ10*, has also been shown to harbor a coding variant related to human IGE.

Another example of imminent success comes from studies of mouse strain susceptibility to kainic acid seizure-induced hippocampal cell death. In these studies, the investigators looked beyond clinical phenotype towards possible mechanisms associated with neuronal loss following seizures (as seen in human epilepsies such as TLE). Genetic crosses were characterized between C57BL/6J (relatively resistant) and FVB/N (relatively sensitive) – focusing on cell death as the endpoint – and several QTL were found to contribute to the phenotype, with *Sicd1* (seizure induced cell death-1) on Chromosome 18 having the largest effect. The genetic effect of *Sicd1* was later confirmed using congenic strains, and a strong candidate gene, *Galr1* – encoding a galanin receptor, has emerged.

Although the aforementioned examples have come closest to unequivocal gene identification, numerous other studies have focused on genes causing varying seizure threshold responses in response to other substances. QTL mapping for susceptibility to β-carboline-, cocaine- and pilocarpine-induced seizures, as well as to seizures resulting from alcohol, phenobarbitol, and zolpidem withdrawal, have been carried out. Many of these QTLs were defined in crosses between DBA/2 and C57BL/6 strains; these have been the most popular mouse strains to use over the past decade or two, not only because they are genetically diverse from one another, but also because of the availability of specialized genetic or genomic resources for their study (e.g., RI strain sets, genome sequence, recombinant DNA libraries). However, it should be noted that there are well over 40 inbred mouse strains available to researchers. As mouse strains show wide diversity in seizure threshold paradigms, there are likely many interesting combinations to utilize for genetic studies, to model just about any aspect of seizure disorders or to study the genetics of antiepileptic drug action. Worldwide research community efforts, such as the Mouse Phenome Database, collect and compare phenotypic and genetic information in a large set of mouse strains, thus providing instigation for resource development that supports the study of genetic diversity in mice – which will make it easier to work with more exotic strains in future.

Directions for Future Research

Is Mendelian Epilepsy Really all that Genetically Simple? Studying Human in Mouse

More than 400 mouse mutations cause spontaneous seizures or alter the seizure threshold. Most are single gene, null mutations. One possible interpretation of these findings is that if these genes were mutated in human epilepsy, they would exhibit Mendelian, not genetically complex, inheritance. However, this interpretation is based on a superficial assumption. Most mouse mutations have been studied in mixed hybrids from only two parent strains: 129 (the strain in which most targeted germ-line mutations are created in mice), and C57BL/6 (a popular 'wild-type' strain, often used as a standard genetic background). Fewer studies have examined mutations on either the C57BL/6 or 129 background separately, and fewer still have gone farther afield to explore more strain diversity. Still, those few that have ventured away from C57BL/6 and 129 have noted some very striking differences in seizure incidence, severity or manifestation. For example, mice homozygous for null mutation in *Slc9a1* (encoding a sodium–hydrogen exchanger) were examined on the SJL/J strain (on which the mutation arose), the C57BL/6J strain (onto which it was transferred), and in first (F_1) and second (F_2) generation hybrids between the two parents. Mutants on all four backgrounds exhibit tonic–clonic seizures, but they also exhibit absence seizures that are only obvious in the F_2 hybrids. Another example involves the *Gad65* gene, which encodes one of two glutamate decarboxylase isoforms, the enzyme responsible for synthesizing GABA. Homozygous knockout mice had a much more severe phenotype when the 129-strain based mutation was crossed to the NOD strain background. A third study explored the seizure phenotype

of a gain-of-function transgenic mutation in *Scn2a*, encoding a sodium channel in which human mutations have been associated with Mendelian epilepsy (Generalized Epilepsy with Febrile Seizures Plus, GEFS+). On the SJL strain background, the seizures were very severe and occurred at a younger age than on other backgrounds.

In a final example, a rare variant of childhood absence epilepsy occurred in the gene *GABRG2*, encoding a subunit of a receptor for GABA, the primary inhibitory neurotransmitter in the brain. The same mutation was made in the mouse version of the gene, and 'knock-in' mice were created. Like the ADNFLE mice mentioned earlier, the resultant mice have bona-fide disease, in this case absence seizures (and, despite carrying a mutation that was found in human, have the 5–9 Hz rodent version of SWD). But most interestingly for the GABA story, the severity of disease was very dependent on mouse strain background – with C57BL/6 showing more resistance than DBA/2. This important example illustrates how mouse strain diversity can be exploited directly to gain insight into the regulation of a human mutation. Beginning to identify such genetic modifiers of seizure susceptibility in mouse is a major goal for current research, especially given that several such modifiers have already been found in other mouse models of disease.

The studies discussed here are just the tip of the iceberg, given that most of the genetic diversity in mouse strains is largely untapped. Conceptually, effects of genetic background on the functional expression of single gene mouse mutations will surely mimic the situation in the general human population, where the inheritance of an epilepsy variant is dependent upon the presence of other genes inherited. In fact, genes that cause Mendelian epilepsy in isolated families, which are less genetically diverse than the general population (although not to the extent of a congenic mouse strain or backcross!), may be associated with more genetically complex epilepsy in more typical, genetically heterogeneous families. Although the awaited GWAS and re-sequencing studies in human are welcome, and are bound to yield a measure of success, further understanding (and exploitation) of genetically complex epilepsy in laboratory animals promises to provide a critical understanding of how susceptibility to epilepsy is tweaked by nature. Such insight will certainly be of therapeutic value to human epilepsy.

See also: **Genetics**: Cytoskeletal Genes and Idiopathic Epilepsies; Epilepsy Genes that do not Encode Ion Channels; Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Genetics of Lafora and Juvenile Myoclonic Epilepsies; Large-Scale Mapping and Epilepsy Gene Discovery; Sixty Years in the Making: A Multifactorial Mouse Model of Seizure Susceptibility.

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Role of Genetic Factors in the Etiopathogenesis of Epilepsy-Related Brain Damage

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Introduction

Epilepsy, one of the most common neurological conditions, is a heterogeneous disorder defined by paroxysmal and abnormal discharges of neurons, resulting in recurrent attacks of seizures. Brain damage is caused by persistent and highly repetitive seizures associated with excitotoxic cell death mechanisms. Now it is accepted that epilepsy-associated damage is multifactorial, with genetic and environmental factors interplaying to determine disease onset and progression. Similar to all complex conditions, the genetic component of epilepsy is not limited to a single gene mutation. As a result, the design of genetic studies in complex diseases is challenging. Furthermore, our understanding of the molecular pathways that regulate cell death after seizure activity remain in its infancy – and largely lag behind work in other areas of brain injury. This delay in understanding is due, in part, to the difficulty in delineating changes in gene expression, which are causal for seizure-induced cell death; indeed, any changes detected may simply be secondary to the effects of the seizures themselves. A complementary approach to mechanistic-based studies has been to identify genes that may contribute to seizure-induced cell death through genetic manipulation in animal models of experimental epilepsy. The overarching hypothesis of our research is that a subset of endogenous genes expressed following seizure induction can determine whether or not cells will survive.

Animal modeling of seizure disorders has been instrumental in elucidating the pathophysiological mechanisms underlying epilepsy and in designing more effective therapies for seizure-induced damage. These models allow us to determine the nature of injuries that might contribute to the subsequent development of epilepsy, to observe and intervene in the disease process subsequent to an injury but prior to the onset of spontaneous seizures, and also to study the chronically epileptic brain in exquisite detail, using state-of-the-art physiological, pharmacological, molecular, and anatomical techniques.

To date, numerous studies have examined the neurological effects of seizure-induced cell death, but the elucidation of causal factors mediating susceptibility has proven to be more difficult. A contributing factor is the genetic variability even in genetically-modified animal models. In particular, strain-dependent variation among normal mouse strains has recently been recognized as a major confounding variable, influencing the interpretation of the neurochemical, pharmacological, and behavioral changes in genetically-modified mice. Our research has yielded a genetic differentiation between mouse strains that show no difference in the extent of seizure activity, but exhibit robust differences in susceptibility to seizure-induced excitotoxic cell death. As a result of this analysis, we have produced the *first* model that allows the investigator to test the hypothesis that different molecules are responsible for seizure sensitivity versus seizure-induced cell death susceptibility. The identification of specific genotypes with divergent responses to seizure-induced excitotoxic cell death will facilitate studies into the biological mechanisms underlying neuronal sensitivity. Subsequent identification of these epilepsy susceptibility genes may illuminate new pathways involved in epilepsy and epileptogenesis, and may also aid in the discovery of the common human genetic variation that plays a role in determining an individual's genetic susceptibility to epilepsy.

Background

Excitotoxic Cell Death: A Common Pathologic Mechanism in Neurodegenerative Diseases

It has been suggested that cell death in diverse neurological disorders, such as epilepsy, stroke, Alzheimer's disease, Huntington's disease, and Parkinson's disease, may occur in part from excessive activation of glutamate receptors, resulting in excitotoxic damage. Glutamate, the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS), plays a major role in synaptic transmission. However, when glutamate receptors are excessively activated, a form of cell death termed 'excitotoxicity' ensues. Excitotoxicity refers to a process of neuronal death initially triggered by elevated levels of excitatory amino acids, resulting in the opening of glutamate receptor-associated ion channels; this action at glutamate receptors causes prolonged depolarization of neurons, the subsequent influx of calcium, and the activation of calcium-dependent enzymatic and nuclear mechanisms of cell death. Although the cellular and molecular mechanisms underlying vulnerability to excitotoxic cell death are unknown, an understanding of the genetic characteristics and molecular mechanisms that trigger excitotoxic cell death may offer therapeutic strategies for neuronal damage and loss that occurs in these disorders.

Strain-Related Differences in Seizure-Induced Excitotoxic Cell Death

Similar to what has been observed for seizure sensitivity, genetic background plays a major role in determining the acute effects of seizures. Previously, we demonstrated that mouse strains show remarkable genetic differences in susceptibility to the neuropathological consequences of seizures in the kainate model of status epilepticus. In particular, the extent of seizure-induced cell death differs markedly between strains of inbred mice; importantly, these differences are not necessarily related to differences in the extent of seizure activity induced by kainate. Given that strains of mice differ markedly in their response to seizure-induced excitotoxic cell death, it is obvious that there must be a genetic basis for these differences that are passed on from one generation to the next. Thus, genetic techniques can be employed to define and understand these differences.

Functional Genetic Approaches to Identification of Candidate Genes Modulating Excitotoxicity

The well-accepted notion that overactivation of glutamate receptors can mediate cell death in several neurological diseases has prompted considerable effort to develop drugs to attenuate or abolish the excitotoxicity. In many biological systems, mutant organisms have proved to be powerful tools for advancing our understanding of the molecular pathways that could be considered as potential therapeutic targets. At present, gene function can be disrupted, attenuated, or enhanced in conditional and reversible fashion using a combination of gene targeting and transgenic approaches. It is becoming increasingly clear that several genes can modulate susceptibility to excitotoxic cell death, and much research has focused on identifying genes that may contribute to the pathogenic sequelae of neuronal damage. Traditionally, these studies can be divided into forward or reverse genetic approaches. Forward genetic approaches (from phenotype to gene) use genetic dissection (e.g., the identification and mapping of specific genes) to isolate mutants that affect a process of interest. In this process, genes are mutated at random. Thus, the resulting changes in the phenotype of a cell or organism are then attributed to the mutated genes, without any prior knowledge of the genes' function. Studies in our laboratory have utilized a forward genetic approach to dissect the genetic influences on differences in seizure-induced cell death between two strains of mice that are divergent for susceptibility to seizure-induced cell death.

Conversely, reverse genetic approaches (from gene to phenotype) involve the disruption of a gene of interest, and have been used extensively to determine the role of a particular gene within a specific pathway. In the mouse, a reverse genetic approach has been utilized extensively to identify the functional relevance of several genes in modulating susceptibility to excitotoxin-induced cell death. Classical reverse genetic approaches have involved the creation of knockout cell lines or organisms to eliminate the function of a gene of interest, or have involved transgenesis studies, in which a particular gene is overexpressed in a cell line or organism. Although the analysis of gene modulation in vivo, using knock-out, knock-in or transgenic mice, remains the gold standard for any target validation project, these approaches can be expensive and time-consuming. Further, gene expression is often poorly controlled (e.g., may be 'supraphysiological'), creating the potential for expression artifacts.

Methods

Experimental Genetic Approach: Identification of Quantitative Trait Loci (QTLs)

Genetic linkage is often used to elucidate the underlying genetic mechanisms for inherited traits and to find chromosomal locations for genes. The experimental dissection of quantitative traits is a multi-stage process, typically involving (1) coarse genome-wide linkage mapping, (2) fine mapping of specific regions, (3) recombination or association mapping to identify candidate genes, and finally (4) positionally cloning and characterizing quantitative trait loci (QTLs). The QTL method can identify chromosomal regions involved in regulating a trait in any animal system for which two key resources are available: genetically divergent strains and a linkage map covering all of the genome.

Linkage mapping of quantitative trait loci in mice begins by choosing parental inbred strains that are genetically variable for the trait of interest. Although not selectively bred for these characteristics, the FVB/N mouse has been found to be genetically predisposed to susceptibility to excitotoxicity, whereas the C57BL/6 mouse has been found to be genetically predisposed to be cell death resistant. As these two strains exhibit maximum differences in susceptibility to seizure-induced excitotoxic cell death, they represent the phenotypic extremes for this trait. There must be a genetic basis for these differences that are passed on from one generation to the next, and thus genetic techniques can be employed to define and understand these differences.

In an attempt to perform a genetic dissection of seizure-induced cell death through a linkage analysis approach, we created mouse hybrid populations from the crossbreeding of seizure-induced cell death susceptible (FVB/N) and seizure-induced cell death resistant (C57BL/6) mice. These two parent strains have different mean values for the trait of susceptibility to seizure-induced cell death. A mapping population was then derived by backcrossing the first generation (F1) progeny

to one or both parents. The intervals defined by ordered pairs of chromosomal markers are searched in increments, and statistical methods are used to test whether a QTL is likely to be present at the location within the interval or not. While the position of the peak statistically significant value gives the most likely position for at QTL, its exact location remains unknown. As a result, the identification of any QTL via coarse genome-wide linkage mapping is only the first step towards the ultimate goal of gene identification. The next step is the production of congenic strains, by which the existence of a QTL may be verified and the implicated chromosomal region be reduced to a size applicable to positional cloning of the causal gene.

Confirmation of the QTL and Fine Mapping

In organisms amenable to genetic manipulation, the effects of an individual QTL can be magnified by constructing strains that are genetically identical except for a defined region surrounding the QTL. A congenic strain is traditionally constructed by the serial backcrossing of a donor strain to a recipient strain. Chromosomal regions are monitored at each backcross for heterozygosity using polymorphic microsatellite markers. These strains constitute a permanent genetic resource from which multiple measurements of the trait phenotype can be obtained in order to increase the accuracy of determining the QTL genotype. The region surrounding the QTL can be a whole chromosome or a small interval (interval-specific congenic strains). After eight to twelve backcrosses, according to Mendelian laws, more than 99% of the genetic background is that of the recipient strain. On completion of the backcrossing, the final step of brother X sister mating fixes the strain so that the desired chromosomal region is homologous for the donor's alleles in one quarter of the offspring.

Once a phenotype has been anchored to a congenic segment, the investigator requires a fine saturated map of the region of interest in order to progressively narrow the area containing the disease-related gene, and thus to generate congenic sublines carrying smaller and smaller pieces of the original QTL. To fine-map this locus, we created congenic mice by introgressing (moving a group of genes from) segments of C57BL/6 (B6) chromosome 18 into the FVB/N (FVB) background, predicting that B6 alleles would further reduce susceptibility to seizure-induced cell death. This procedure has the further advantage of reducing a multifactorial trait into a simple Mendelian trait. Moreover, it allows the final cloning of the gene either by position or by candidate gene analysis.

Identification of Candidate Genes through Recombination Or Association Mapping

While QTL mapping cannot provide direct evidence for which specific genes are involved in a particular trait, mapping experiments can identify the probability that a gene of interest exists at the chromosomal location. Once a plausible candidate gene(s) has been identified in the QTL region, the next crucial step is to identify likely disease gene candidates. The analysis of candidate genes is a key step in strategies for disease gene identification and includes: (1) identifying candidate genes that might have a role in the etiology of the disorder; (2) identifying variants in or near those genes that might cause a change in the protein or its expression; or (3) assessing the association between a particular allele (or set of alleles) of a gene in population and the underlying phenotype; and then using statistical methods to determine whether there is a correlation between those variants and the phenotype. For example, for our study of seizure-induced excitotoxic cell death, we take advantage of previous studies have identified causative genes (in mutant mouse models and in familial human forms of epilepsy) that encode ion channels and neurotransmitter receptors. Once a candidate gene has been chosen, a decision must be made as to which type of polymorphism would be most useful for testing in an association study. Most of the DNA sequence variation in the human genome is in the form of single base pair changes in the DNA, known as single nucleotide polymorphisms (SNPs) based on their natural frequency and presence in both coding and noncoding regions, single nucleotide substitutions are probably the underlying cause of most phenotypic differences among humans.

Recent Results

Quantitative trait loci (QTL) mapping studies designed to localize seizure-induced cell death susceptibility genes in C57BL/6 (seizure-induced cell death resistant) and FVB/N (seizure-induced cell death susceptible) mice have detected a significant effect originating from distal chromosome 18. In order to confirm the presence and refine the position of the chromosome 18 QTL for susceptibility to kainate-induced cell death, reciprocal congenic strains between B6 and FVB mice were created using a DNA marker-assisted backcross breeding strategy, and mice were studied with respect to changes in cell death. A genomic interval delimited by marker D18Mit141 and D18Mit25 was introgressed for 10 generations. Comparison of cell death susceptibility between congenic and control (parental genetic background) mice indicated that genes influencing this trait were captured in nearly all strains. Thus, mice from strains having B6 alleles from chromosome 18 on a FVB genetic background exhibited significantly reduced cell death compared to control littermates (Figure 1) in contrast, congenic mice harboring FVB chromosome 18 alleles on a B6 genetic background exhibited significantly greater cell death compared to control littermates.

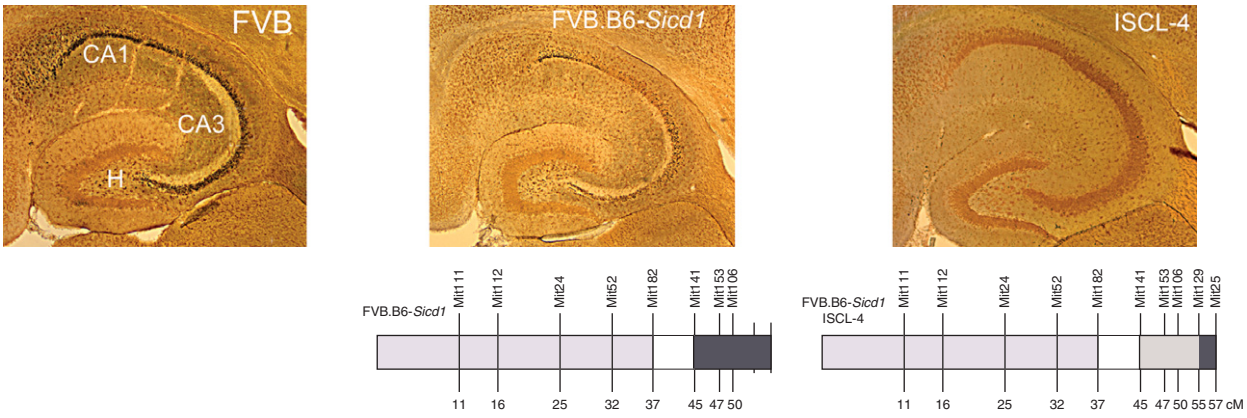


Figure 1 Kainate-induced cell death susceptibility is reduced in the FVB.B6-Sicd1 congenic strain and substantially reduced in interval-specific congenic line-4. Low-magnification Gallyas silver-staining of horizontal sections through the hippocampus in FVB/N (background strain), FVB.B6-Sicd1 congenic mice, and in ISCL-4 congenic mice 7 days following systemic administration of KA. Note that cell death is reduced in area CA3 and CA1 in FVB.B6-Sicd1 congenic mice and that little to no cell death is observed in ISCL-4. Schematic diagrams of chromosome 18 in FVB.B6-*Sicd1* congenic mice and in ISCL-4 congenic mice are shown below the corresponding photomicrograph. Lightly shaded regions reflect genomic contribution from the FVB strain and darkly shaded regions reflect genomic contribution from the B6 strain. Numbers below represent the genetic map position (http://www.informatics.jax.org/) and are given in cM. CA1 and CA3 denote the hippocampal subfields; H, dentate hilus.

As a means of refining this interval, we next created a series of overlapping, congenic, recombinant strains by breaking the QTL interval into subregions. The development of a series of strains termed, interval specific congenic lines (ISCLs) have been used extensively to refine the location of a QTL for identification and confirmation of candidate genes in a variety of other species. Each ISCL is recombinant at a specific sub-interval, thereby reducing the QTL to a much smaller interval than the original congenic strain. We produced four interval-specific congenic strains carrying differing intervals from chromosome 18 of B6 on an FVB-derived susceptible background. All of the ISCLs exhibited reduced cell death associated with the B6 phenotype, as compared with the parental FVB strain. However, the most dramatic reduction, a nearly four-fold reduction in the extent of seizure-induced cell death susceptibility, was expressed in ISCL-4 which carried a reduced B6-derived region of chromosome 18 on an FVB-derived susceptible background. In particular, seizure-induced excitotoxic cell death was significantly reduced in all hippocampal subfields (dentate hilus, area CA3, and area CA1) as compared with the FVB parental strain, the other ISCL strains, and the congenic strain (Figure 1). Consequently, the reduction in cell death may be attributed to the B6 alleles from this introgressed region. Moreover, the ISCL-4 strain further narrows the QTL interval to a small region of murine chromosome 18, and supports the proposition that the protective gene(s) is contained within this smaller interval – i.e., that ISCL-4 contains the putative gene(s) of the QTL for susceptibility to seizure-induced cell death.

As a means to verify that the reduced chromosome 18 locus significantly reduced susceptibility to KA-induced excitotoxic cell death, we took a candidate gene approach to identify the causal susceptibility gene(s) responsible for the effect. The genetic variation underlying this QTL could consist of polymorphisms in either the coding region (thus altering the amino acid sequence of the translated protein) or in the regulatory region (affecting expression of a gene). Among the many candidate genes present in this region, *galanin receptor 1* (*Galr1*) and *myelin basic protein* (*Mbp*) are the most compelling possibilities. Both of these genes have relevance to modulation of neuronal excitability and seizure threshold. To confirm the identity of any putative candidate genes, expression studies can be performed using qRT-PCR, and any gene expression differences observed would presumably be the result of changes in allele frequencies due to selection rather than a response to kainate treatment. We found that B6 mice, as well as the congenic mice carrying the B6-derived resistant allele at the region where *Galr1* is located on the FVB genetic background, showed significantly decreased basal hippocampal *Galr1* expression compared with the susceptible FVB control littermates. We speculate that the downregulation of *Galr1* observed in these mice supports its role as a modulator of neuronal excitotoxic cell death in the hippocampus.

Future Goals: Challenges for the Future

There are several inherent complexities in the investigation of genetic influences on protection against seizure-induced cell death. Firstly, epilepsy is a heterogeneous disorder characterized by variable clinical and pathological presentations. Thus, genetic dissection of such a

complex disease is a difficult task; there are likely to be contributions of several genes, each one exerting a small effect on the final phenotype. By definition, as most epilepsies are likely polygenic diseases, no single marker will ever allow a definitive diagnosis or certain risk prediction. However, it is hoped that specifiable combinations of such markers will be found, and will provide a reasonable basis for diagnosis or prognosis. Results of our work suggest that, although susceptibility to seizure-induced cell death may be affected by more than one locus, a few loci may account for much of the variation present in the trait of susceptibility at any given time.

Secondly, seizure-induced neuronal damage represents an extremely complex pathophysiological cascade of events that includes inflammation, oxidative stress, necrosis and apoptosis, calcium dysregulation, mitochondrial dysfunction, and excitotoxicity. As such, the factors resulting in damage associated with epilepsy are still largely undefined. Hypothesis-driven candidate gene studies are important, but with approximately 25 000 genes in the human genome it is often difficult to predict how variation in any one gene product will affect a particular phenotype. Thus, any attempt to link a gene with a specific pathophysiological occurrence that exerts influence on a specific disease or clinical outcome can be problematic. In the future, it will be of interest to determine the nature of DNA sequence variants, which give rise to alleles of quantitative effect, and eventually understand phenotypic evolution at the level of the individual loci. We have narrowed down the search for molecular variants responsible for quantitative genetic variation in susceptibility to seizure-induced cell death will; this work will provide a basis for further studies to estimate the frequency of these variants in different populations and species. Identified variants can also become key entry points from which to explore the network of genes, proteins, pathways, and systems important in the determination of susceptibility to seizure-induced excitotoxic cell death. Furthermore, as the presence of strong homologies between genes in mice and humans usually guarantee overlapping or even near identical functions, polymorphic genes in mice may also be polymorphic in humans.

Thirdly, the road from locus to gene identification can be difficult and cumbersome. It remains unclear whether the difficulty in moving from quantitative trait locus to quantitative trait gene is the result of complex gene-to-gene interactions (affecting gene expression throughout the genome), or is more reflective of the heterogeneous and complex nature of the epilepsy disorders (e.g., complex pathophysiology, large number of interacting proteins at different stages of the evolution of the disorder, etc.). Thus, future genetic research studies should focus on both 'top-down' approaches (such as genome scans) and 'bottom-up' approaches (such as candidate gene studies) to make sense of what is going on. As our understanding of gene function at present is too limited to let us predict a priori which genes are involved with a particular trait, a combined approach of mouse genetics and transcriptomics, together with studies involving humans patients with diverse epilepsy phenotypes, will yield the fastest progress.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Genetics**: Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future; Sixty Years in the Making: A Multifactorial Mouse Model of Seizure Susceptibility.

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Single Gene Mutations in Inherited and Sporadic Epilepsy

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Introduction

Epilepsy is a common neurological disorder affecting ~1% of the population. One-third of patients have an underlying physical cause such as trauma or stroke. For the remaining two-thirds of cases, no preexisting condition is recognized and these epilepsies are classified as 'idiopathic.' Genetic factors are thought to play a central role in idiopathic epilepsy. Common types of idiopathic epilepsy are likely to be polygenic, resulting from the combination of mild mutations in several different genes. A small proportion of epilepsies exhibits Mendelian inheritance and is caused by mutation of a single gene.

Background

We will review achievements of the past decade in identifying the molecular basis for many of the monogenic epilepsies. Identification of these genes relied heavily on tools generated by the Human Genome Project, including an abundance of markers for tracing the inheritance of diseases through large family pedigrees, and the complete human genome sequence for identifying positional candidate genes. The genes mutated in rare monogenic epilepsies define pathways that are also likely to play a role in the more common, polygenic epilepsies. Some of the identified epilepsy genes play important roles in neuronal signaling, including voltage-gated ion channels, neurotransmitter receptors, and ion channel-associated proteins (**Table 1**). We will examine the spectrum of single gene mutations and the mechanics leading to a common endpoint, the increased firing of excitatory neurons that underlies epileptic seizures.

Methods

Success in identification of genes responsible for monogenic epilepsies was obtained by classical genetic mapping in large families with inherited epilepsy. Examination of linkage with thousands of microsatellite markers and millions of SNP markers makes it possible to localize the disease gene in a large family to only a few megabases of DNA. Examination of the reference human genome sequence can then provide a list of candidate genes located within the regions of linkage, which can be amplified by PCR and sequenced to identify the pathogenic mutation. After potential disease-causing variants are identified, the effect on protein function must be determined, using functional assays specific for each candidate gene. Introduction of the human variant into the germ line of the mouse can be used to confirm the pathogenic effect. Ion channel mutations have been evaluated in transfected cells and in genetically engineered mice. Spontaneous mouse models have also been important in suggesting new candidate genes for human epilepsy.

Recent Results

Sodium Channel Mutations

Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials in neurons. The sodium channel consists of a pore-forming α subunit and accessory β subunits that modulate activity. The α subunits are large transmembrane proteins (~2000 amino acids) containing four homologous domains, each with six transmembrane segments (**Figure 1**). The four domains surround a central, sodium-permeable pore through which sodium ions flow down their concentration gradient after depolarization of the cell membrane. β subunits contain an extracellular IgG loop, a single transmembrane segment, and a short intracellular C-terminus (**Figure 1**). Association with β subunits influences cell-surface expression and kinetics of the α subunit. There are nine human α-subunit genes and four β-subunit genes, encoding channel proteins that differ in tissue specificity and biophysical properties. Mutations in three of these genes have been associated with epilepsy, the α-subunit genes *SCN1A* and *SCN2A* and the β-subunit gene *SCN1B*.

SCN1B

In 1998, the first sodium channel mutation was identified in an Australian family with generalized epilepsy with febrile seizures plus (GEFS+), a mild, dominantly inherited disorder. This syndrome is characterized by febrile seizures that persist beyond 6 years of age and may progress to afebrile, generalized seizure types. Only three additional mutations of *SCN1B* have been reported since the first report. The effect of the *SCN1B* mutations in functional assays is reduction of sodium channel activity. This partial loss-of-function mechanism is supported by the observation that heterozygotes for a mouse knockout allele of *Scn1b* exhibit spontaneous seizures.

Table 1 Single gene mutations responsible for inherited epilepsy

	Gene	*Chromosome*	*Syndrome*	*OMIM ref*[a]
Voltage-gated ion channels	*SCN1A*	2q24	GEFS+, SMEI	182389
	SCN1B	19q13	GEFS+	600235
	SCN2A	2q24	BFNIS, GEFS+	182390
	KCNQ2	20q13	BFNS	602235
	KCNQ3	8q24	BFNS	602232
	CLCN2	3q27	IGE	600570
Neurotransmitter receptors	*CHRNA4*	20q13	ADNFLE	118504
	CHRNB2	1q21	ADNFLE	118507
	CHRNA2	8p21	ADNFLE	118502
	GABRG2	5q34	GEFS+, FS, CAE	137164
	GABRA1	5q34	JME, CAE	137160
	GABRD	1p36.3	GEFS+	137163
Others	*EFHC1*	6p12	JME	608815
	LGI1	10q24	ADPEAF	604619
	MASS1	5q14	FS	602851

[a]Further information is available using the OMIM reference number in the OMIM database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM).

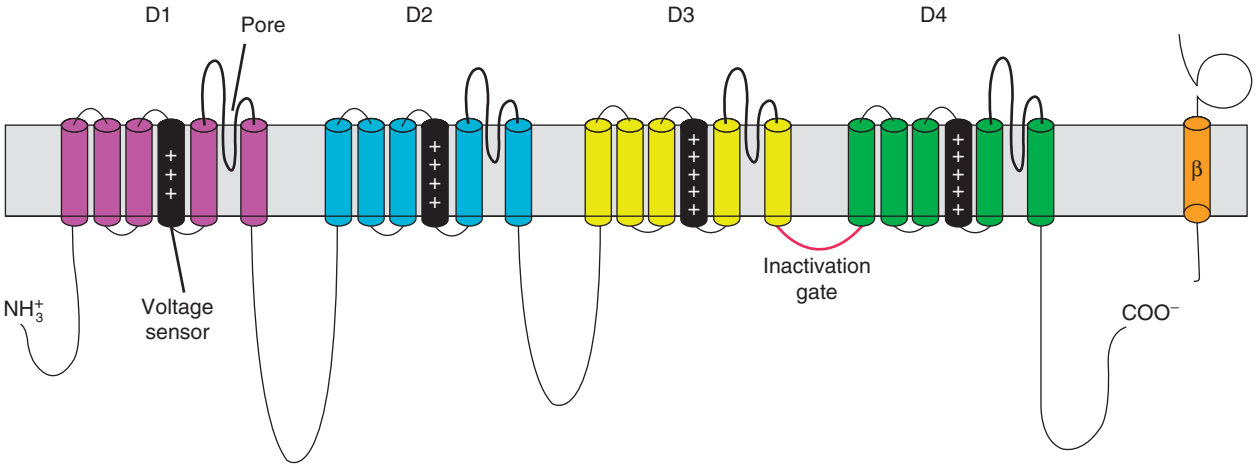


Figure 1 The sodium channel α and β subunits are transmembrane proteins. The four homologous domains of the α subunit are represented in different colors. Important functional domains are labeled.

SCN1A

In 1999, a second GEFS+ locus was mapped to chromosome 2q24 by linkage analysis in two large families. Our laboratory identified heterozygous *SCN1A* missense mutations in both families that change evolutionarily conserved amino acids: T875M in one family and R1648H in the other. Since 1999, 20 mutations of *SCN1A* in GEFS+ families have been reported (**Figure 2(a)**). Functional characterization of GEFS+ mutations in heterologous expression systems has revealed several different gain-of-function effects. The lack of a clear phenotype–genotype correlation may reflect the limitation of nonneuronal expression systems to evaluate mutations in neuronal sodium channel genes. The most reliable data on functional consequences of channel mutation can be obtained from recordings of neurons in mice engineered to carry human mutations.

In 2001, de Jonghe reported the surprising observation of *SCN1A* mutations in patients with a noninherited form of epilepsy, severe myoclonic epilepsy of infancy (SMEI). This infant-onset syndrome is characterized by generalized clonic, tonic–clonic, or hemiclonic seizures. In later life, patients may develop myoclonic, absence, or partial seizures. Poor response to antiepileptic drugs is common. Development is normal prior to onset, but disease progression is often accompanied by decline of psychomotor and mental development. Nearly 250 mutations of *SCN1A* have been identified in SMEI and borderline SMEI (SMEIb), accounting for the disease in ~70% of SMEI patients. SMEI mutations arise de novo in the affected child and are not present in the parents, with the exception of rare cases of parental mosaicism. The large proportion of SMEI patients with de novo mutations of *SCN1A* means that genetic screening of *SCN1A* is an effective diagnostic tool for children with SMEI.

Approximately half of SMEI mutations result in protein truncation due to nonsense, frameshift, or splice site

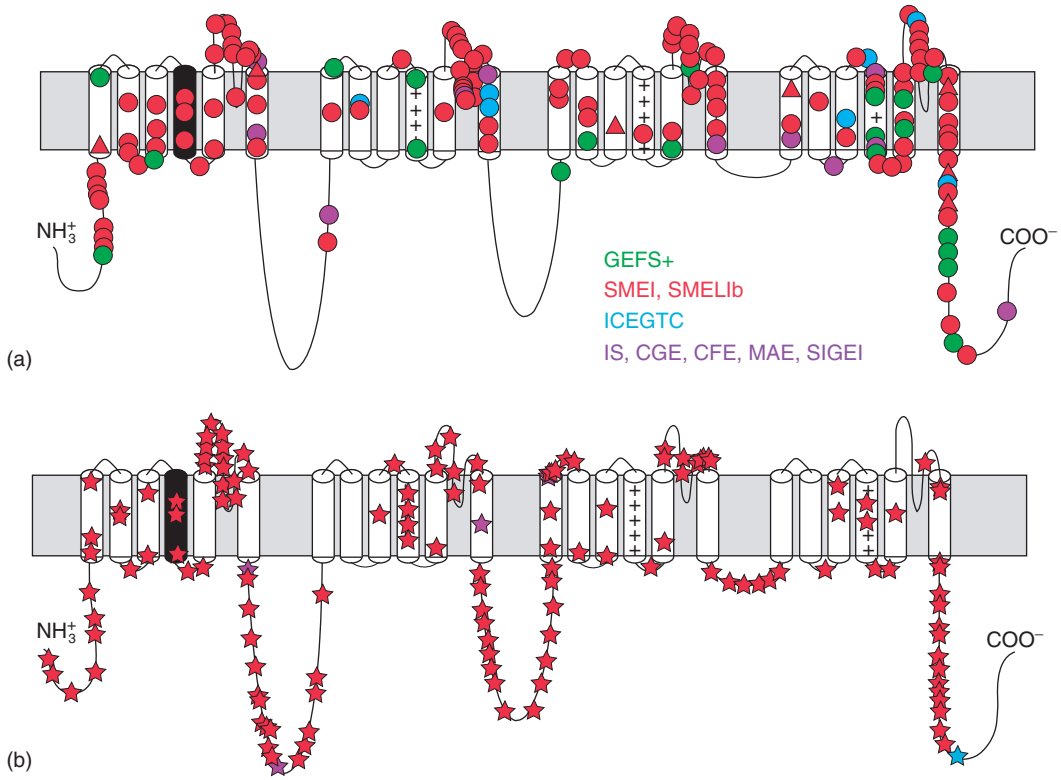


Figure 2 Epilepsy mutations in *SCN1A*. (a) Missense mutations (circles) and small in-frame deletions (triangles). (b) Truncations (stars) resulting from nonsense, splice site, and frameshift mutations. GEFS+, generalized epilepsy with febrile seizures plus; SMEI, severe myoclonic epilepsy of infancy, SMEIb, severe myoclonic epilepsy of infancy borderline; ICEGTC, idiopathic childhood epilepsy with generalized tonic–clonic seizures; IS, infantile spasms; CGE, cryptogenic generalized epilepsy; CFE, cryptogenic focal epilepsy; MAE, myoclonic astatic epilepsy; SIGEI, severe idiopathic generalized epilepsy of infancy.

mutations; these lesions are distributed throughout the protein domains (Figure 2(b)). Missense mutations of *SCN1A* in patients with SMEI are concentrated in the transmembrane segments of the protein, similar to GEFS+ mutations (Figure 2(a)). Deletions of individual exons and of the entire *SCN1A* gene have been reported. The large number of protein truncation alleles suggests that haploinsufficiency of *SCN1A* is responsible for the severe SMEI syndrome. Consistent with this model, mice heterozygous for a null allele of *Scn1a* exhibit spontaneous seizures and reduced lifespan. Recordings of cultured hippocampal neurons from these mice revealed reduced activity in inhibitory interneurons and normal activity in excitatory neurons, suggesting a cell-specific mechanism of reduced inhibitory input underlying seizures. Twenty missense mutations from SMEI patients have been characterized in mammalian expression systems, and 12 of these mutations lacked channel activity, consistent with a loss-of-function basis for this disorder.

A small number of *SCN1A* mutations have been identified in other types of infantile epilepsy, including idiopathic childhood epilepsy with generalized tonic–clonic seizures (ICEGTC), infantile spasms (IS), cryptogenic generalized epilepsy (CGE), cryptogenic focal epilepsy (CFE), myoclonic astatic epilepsy (MAE), and severe idiopathic generalized epilepsy of infancy (SIGEI) (Figure 2).

Vaccine-related encephalopathy and SCN1A

There has been considerable controversy regarding claims that routine vaccination can result in an encephalopathy syndrome that includes seizures and mental decline. Berkovic and colleagues recently tested DNA from patients who had been initially diagnosed with vaccination encephalopathy, due to seizure onset within 72 h of DPT or PT vaccination. Eleven of the 14 individuals tested were found to carry mutations of *SCN1A*. Two of the mutations have previously been seen in patients diagnosed with SMEI. In eight of these cases, parental samples were tested and found to lack the mutation, indicating that these were de novo mutations. The data indicate the fever associated with vaccination may have triggered the first episode of an underlying SMEI-like genetic disorder.

Neuronal specificity of SCN1A mutations

Neuron subtypes vary with regard to the relative level of expression of different ion channel genes. To explain the association of *SCN1A* mutations with hyperexcitability, it has been suggested that inhibitory interneurons might be

particularly dependent on this channel. In support of this hypothesis, recent immunohistochemical studies demonstrated that Nav1.1 is localized to the axon initial segments of parvalbumin-positive inhibitory interneurons in developing neocortex and hippocampus, while it has lower somatodendritic expression in excitatory pyramidal neurons. In *Scn1a* heterozygous null mice, a model for SMEI, inhibitory interneurons isolated from hippocampus displayed reduced current amplitude and reduced generation of action potentials, while excitatory pyramidal neurons did not differ from wild-type mice. In a mouse knockin model of the human SMEI mutation R1407X, Ogiwara *et al.* (2007) showed that parvalbumin-positive inhibitory neurons in neocortical slices displayed pronounced spike amplitude decrement during prolonged spike trains while neighboring pyramidal neurons did not differ from those in wild-type. These data demonstrate the importance of neuronal heterogeneity in determining the clinical manifestation of genetic mutations.

SCN2A

SCN2A is a sodium channel gene closely related to *SCN1A* and located in an adjacent position on chromosome 2q24. Since 2002, missense mutations of *SCN2A* have been identified in one patient with GEFS+ and nine patients with benign familial neonatal-infantile seizures (BFNIS), a syndrome of mild seizures that remit by the first birthday. Functional characterization of BFNIS mutations demonstrated gain-of-function effects that are predicted to increase sodium current and result in neuronal hyperexcitability. One truncation mutation of *SCN2A* was found in a patient with intractable epilepsy and mental decline, similar to SMEI. The mouse model Q54 carries a missense mutation of *Scn2a* that increases neuronal excitability and results in epilepsy. The dramatic loss of hippocampal neurons in the Q54 mouse after the onset of seizures resembles hippocampal sclerosis in patients with mesial temporal lobe epilepsy. The influence of strain background on the severity of seizures in Q54 mice is being used to identify genetic modifiers that could play a role in human epilepsy.

Potassium Channel Mutations

The potassium channels encoded by the genes *KCNQ2* and *KCNQ3* combine to form a heteromultimeric potassium channel responsible for M current. This neuronal current maintains the resting potential of the neuron and limits repetitive firing. Mutations in *KCNQ2* and *KCNQ3* were first reported in 1998 in families with benign familial neonatal seizures (BFNS), a mild, autosomal dominant disorder of generalized seizures during the first few days of life that remit within months. More than 60 mutations of *KCNQ2* have been identified in BFNS families, as well as a few de novo mutations in sporadic cases. Half of *KCNQ2* mutations lead to protein truncation, and exon duplications and deletions have been reported. Only four mutations in *KCNQ3* have been observed. The loss-of-function mutations of *KCNQ2* and *KCNQ3* in BFNS result in reduced M current, increased repetitive firing, and neuronal hyperexcitability.

Acetylcholine Receptors

The nicotinic acetylcholine receptor (nAChR) is a pentameric complex with three α and two β subunits. These presynapatic ligand-gated channels regulate excitatory neurotransmitter release. Mutations in the nAChR subunits *CHRNA4*, *CHRNB2*, and *CHRNA2* have been identified in families with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). This disorder is characterized by partial seizures originating from the frontal lobe that occur during nonrapid eye movement sleep. Analysis of ADNFLE mutations demonstrated reduction in the calcium dependence of acetylcholine release, leading to excess excitatory signaling.

GABA Receptors

GABA is the primary inhibitory neurotransmitter in the brain. The $GABA_A$ receptors are pentamers typically composed of two α subunits, two β subunits, and one γ, δ, ε, or π subunit. The subunits assemble to form a chloride-selective central pore. Upon binding GABA, the channel opens and the rapid increase in chloride conductance suppresses depolarization, resulting in reduced firing of neurons. Mutations in *GABRG2*, *GABRA1*, and *GABRD* have been reported in GEFS+, juvenile myoclonic epilepsy (JME), and childhood absence epilepsy (CAE). Several mutations result in loss of function of the $GABA_A$ receptor, leading to reduced inhibition and net hyperexcitability.

Chloride Channels

CLCN2 encodes the voltage-gated chloride channel ClC-2, which is expressed in neurons that are postsynaptic to GABAergic inhibitory synapses. Maintenance of a low intracellular chloride concentration by ClC-2 is necessary for the inhibitory response to GABA. Mutations in *CLCN2* have been associated with several idiopathic generalized epilepsy (IGE) subtypes, including JME, CAE, and EGMA (epilepsy with grand mal upon awakening). However, in families with monogenic IGE, there is variable expressivity, suggesting that genetic modifier alleles may influence the clinical phenotype. Functional studies of *CLCN2* mutations indicate that reduced ClC-2 function

leads to hyperexcitability of the cell postsynaptic to GABAergic synapses.

LGI1

LGI1 (leucine-rich, glioma-inactivated 1) is a subunit of the presynaptic voltage-gated potassium channel (Kv1) complex. Mutations in *LGI1* are found in autosomal dominant partial epilepsy with auditory features (ADPEAFs), with auditory simple partial seizures with onset in the second or third decade of life. Loss of function of *LGI1* results in elevated rapid inactivation of the Kv1 channel, prolonged action potentials, and neuronal hyperexcitability.

EFHC1

The EFHC1 protein contains a Ca^{2+}-sensing EF-hand motif and modulates R-type calcium current by interaction with the C-terminus of Ca(v)2.3. Mutations of EFHC1 have been identified in nine families with JME. The mechanism for seizure induction is not clear.

MASS1/VLGR1

MASS1 (monogenic audiogenic seizure-susceptible) was first identified by positional cloning of the mouse *Frings* mutant with audiogenic seizure susceptibility. *MASS1* encodes a calcium-binding seven transmembrane G-protein-coupled receptor, designated VLGR1 (very large G-protein coupled receptor). A nonsense mutation in *MASS1* was found in one family with febrile and afebrile seizures. The relationship to neuronal firing is not clear.

Future Goals

A major goal of future research will be to determine the role of these monogenic disease genes in common varieties of epilepsy. This determination will require large-scale genomic sequencing in patients with sporadic, nonfamilial disease. In a test of large-scale 'medical re-sequencing' in progress at the Baylor Genome Center, exons from 250 channel genes are being sequenced in epilepsy patients and controls. This project previews the type of diagnostic testing that is likely to become available within the next 5 years, replacing single gene analysis with a comprehensive evaluation of the overall channelome in each new patient with a seizure disorder.

The 'channelome,' the total ion channel content of a specific neuron, determines neuronal excitability and firing patterns. From a systems biology perspective, it is becoming apparent that neuronal firing patterns are determined by the sum of genetic variation at all of the ion channels expressed within each neuron subtype. Two recent examples illustrate the interaction between genetic variation affecting different ion channel genes that are expressed in the same cells. In one case, mice were generated that carried two subclinical mutations, one in *Kcnq2* (V182M) and one in *Scn2a* (Q54); the combination resulted in severe, early onset seizures. In another case, seizure severity was *reduced* when a pathogenic mutation in *SCN1A* was combined with heterozygosity for a null allele of *Scn8a*. The differences in the channel content of different types of neurons discussed earlier underlie these examples. Null alleles of *Scn1a* reduce the activity of inhibitory interneurons in the hippocampus, while null alleles of *Scn8a* have a greater effect on the activity of excitatory neurons. Similarly, mutations in the related channel *SCN9A* have different impact in DRG and visceral sensory neurons, due in part to the expression of *SCN10A* in the DRG neurons only. In the future, newly diagnosed patients with seizure disorders will be genotyped for hundreds of candidate genes, and individualized therapies tailored to the overall channelome will become possible.

See also: **Genetics**: *GABRB3* and Childhood Absence Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Ion Channel Mutations in Familial Eplepsy; Sodium Channel Mutations that Cause GEFS+.

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Sixty Years in the Making: A Multifactorial Mouse Model of Seizure Susceptibility

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Introduction

Inbred strains of mice have long been established to provide research scientists with standardized tools for designing and conducting a wide variety of experiments related to human health and disease. The fundamental characteristics of inbred mouse strains not only facilitate studies of biological processes in whole organisms, but also provide a means to understand the complex genetic architecture that contributes to the determination of their anatomy, physiology, and behavior. It is hypothesized that aspects of genetic influence on complex traits and behaviors of mice – as they are mammals – are relevant to those of humans. And despite the obvious limitations inherent in extrapolations from mouse to man, numerous examples of important associations between mice and men have been documented with respect to genetics and behavior.

Background

C57BL/6J and DBA/2J Mice

Of all of the many mouse strains that have been used to study experimental seizure susceptibility, none has been used more extensively than the C57BL/6J (B6) and DBA/2J (D2) inbred strains. B6 is the most common inbred strain of mice used for research (as measured by literature citations) and was used to generate the publicly available sequence of the mouse genome. D2 was the first inbred strain established and remains one of the most important strains of mice utilized for research purposes. Thus, these two inbred mouse strains hold special importance among the larger panel of common mouse strains used in biomedical research.

The robust difference in seizure susceptibility between B6 and D2 mice was first evidenced over 60 years ago. By measuring the propensity of mice to experience a maximal seizure (defined essentially as involving tonic extension of hind limbs) in response to a loud sound, it was shown that D2 mice exhibit a very high degree of susceptibility relative to B6. An electrophysiological correlate of this so-called 'audiogenic' seizure can be demonstrated through measurements of auditory-evoked brainstem potentials, which showed greater neuronal recruitment to acoustic stimuli in higher-order auditory brain centers of D2 mice compared to B6. Initial studies of audiogenic seizures were extended to show that the susceptibility of D2 mice is influenced significantly by age, with animals becoming resistant to audiogenic seizures by the time they are 3–4 weeks old. A confounding factor in testing susceptibility to audiogenic seizures is age-related hearing loss, a strain-specific phenomenon that occurs early in life in D2 mice compared to B6. Importantly, however, while D2 mice acquire resistance to audiogenic seizures with age, they remain more susceptible to most other forms of experimentally induced seizures compared to B6 mice (as well as many other inbred strains). Such experimentally induced seizures include those induced by pentylenetetrazol (PTZ) and electroshock stimulation, as well as other physical methods of seizure induction such as elevated atmospheric pressure. Comparisons of adult B6 and D2 mice with regard to their seizure susceptibility have documented differential strain responses to a wide variety of chemical convulsants, including drugs that diminish GABAergic activity such as bicuculline and PTZ, and drugs that enhance glutamatergic activity such as kainic acid (KA) and NMDA. D2 mice are also more susceptible than B6 to seizures induced by less conventional chemoconvulsants such as beta-carobolines. Taken together, studies of acute seizure susceptibility suggest that there are fundamental differences in the regulation of neuronal excitability between B6 and D2

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C57BL/6J and DBA/2J Mice

Of all of the many mouse strains that have been used to study experimental seizure susceptibility, none has been used more extensively than the C57BL/6J (B6) and DBA/2J (D2) inbred strains. B6 is the most common inbred strain of mice used for research (as measured by literature citations) and was used to generate the publicly available sequence of the mouse genome. D2 was the first inbred strain established and remains one of the most important strains of mice utilized for research purposes. Thus, these two inbred mouse strains hold special importance among the larger panel of common mouse strains used in biomedical research.

The robust difference in seizure susceptibility between B6 and D2 mice was first evidenced over 60 years ago. By measuring the propensity of mice to experience a maximal seizure (defined essentially as involving tonic extension of hind limbs) in response to a loud sound, it was shown that D2 mice exhibit a very high degree of susceptibility relative to B6. An electrophysiological correlate of this so-called 'audiogenic' seizure can be demonstrated through measurements of auditory-evoked brainstem potentials, which showed greater neuronal recruitment to acoustic stimuli in higher-order auditory brain centers of D2 mice compared to B6. Initial studies of audiogenic seizures were extended to show that the susceptibility of D2 mice is influenced significantly by age, with animals becoming resistant to audiogenic seizures by the time they are 3–4 weeks old. A confounding factor in testing susceptibility to audiogenic seizures is age-related hearing loss, a strain-specific phenomenon that occurs early in life in D2 mice compared to B6. Importantly, however, while D2 mice acquire resistance to audiogenic seizures with age, they remain more susceptible to most other forms of experimentally induced seizures compared to B6 mice (as well as many other inbred strains). Such experimentally induced seizures include those induced by pentylenetetrazol (PTZ) and electroshock stimulation, as well as other physical methods of seizure induction such as elevated atmospheric pressure. Comparisons of adult B6 and D2 mice with regard to their seizure susceptibility have documented differential strain responses to a wide variety of chemical convulsants, including drugs that diminish GABAergic activity such as bicuculline and PTZ, and drugs that enhance glutamatergic activity such as kainic acid (KA) and NMDA. D2 mice are also more susceptible than B6 to seizures induced by less conventional chemoconvulsants such as beta-carobolines. Taken together, studies of acute seizure susceptibility suggest that there are fundamental differences in the regulation of neuronal excitability between B6 and D2

mice. This hypothesis is supported by behavioral studies that test aspects of neuronal excitability such as acoustic startle responses, which are more readily elicited in D2 mice compared to B6. Interestingly, in contrast to results with other drugs, B6 mice have been reported to be more susceptible than D2 with respect to seizures induced by the administration of cocaine.

Brain Structure and Function

Given the robust difference in susceptibility to experimental seizures that has been documented between B6 and D2 mice, many studies have been based on the hypothesis that insight into mechanisms of seizure susceptibility will be gained by comparing parameters of brain structure and function between the two strains. Thus, over the years, numerous anatomical, physiological, and biochemical studies have been conducted using B6 and D2 mice. These are summarized to indicate their scope and allow an appreciation of the general nature of the differences observed.

Adenosine triphosphatases

One of the first clues to a potential mechanism involved in B6 and D2 seizure susceptibility came from studies of the fundamentally important brain enzyme system, Na-K-ATPase. Early work documented lower brain levels of Na-K-ATPase in both juvenile (21-day-old) and adult (110-day-old) D2 mice compared to same-age B6 mice. Two other related cellular enzyme activities, carbonic anhydrase and HCO_3-ATPase, distinguish juvenile B6 and D2 mice, but are equivalent in older mice, suggesting a potential relevance specifically to changes in audiogenic seizure sensitivity in D2 mice. Strain differences in Na-K-ATPase function are also observed in response to the anticonvulsant drug phenytoin, further supporting a potential relationship of this enzyme to strain differences in neuronal excitability. However, despite some promising leads, a clear mechanistic association between ATPase activities and seizure susceptibility in B6 and D2 mice has not been forthcoming.

Neurotransmitters

Differential function of neurotransmitters may play a role in strain-specific seizure responses and measures of all the major neurotransmitter systems of B6 and D2 mice have been compared at some level. However, the nature of this work has been largely correlative and the cause/effect studies between brain neurochemistry and seizure susceptibility are lacking. Temporal and spatial resolutions of neurochemical analyses have been a major limiting factor as well. Nonetheless, many neurochemical differences have been documented between B6 and D2 mice and it is possible that some of them reflect differences in seizure susceptibility. Such differences, summarized below, may be used ultimately to validate future studies of the mechanism.

Acetylcholine

With respect to cholinergic neurotransmission, significant strain differences have been reported in acetylcholine turnover and cholinergic receptor binding. Also, significantly fewer forebrain cholinergic neurons are present in B6 compared to D2 mice, a finding that has been investigated more in relation to strain differences in learning and in responsiveness to antipsychotic drugs than to seizure susceptibility. Nonetheless, the role of differential cholinergic function in the determination of differential B6 and D2 seizure responses cannot be excluded on the basis of available data.

Glutamate and GABA

Amino acid neurotransmitter function has long been documented to play a role in neuronal excitability and the generation of seizure activity. Studies of glutamatergic systems have revealed profiles of brain kainate and AMPA receptor binding that show several minor but significant differences between adult B6 and D2 mice in the striatum and cortex, with more major strain differences in glutamate receptors being associated with aging. However, despite comprehensive receptor profiling, the involvement of glutamate receptors in differential strain seizure susceptibility is not established. GABAergic systems have also been studied in several ways in B6 and D2 mice and have also been suggested to be involved in strain-specific seizure susceptibility. However, the analysis of GABA receptor binding has yielded conflicting results, with some studies reporting similar measures between the strains and others reporting differences. Minor differences in GABA levels, GABA uptake processes, and GABA turnover have also been reported. Nonetheless, as is the case with glutamate, it has not been firmly established whether any of these differences in parameters of GABAergic function are relevant to strain-specific seizure susceptibility. The prominent role of glutamate and GABA in determining neuronal excitability supports the need for additional investigation of these neurotransmitter systems in B6 and D2 mice.

Monoamines

Extensive comparisons have been made of the three main monoamine neurotransmitters in the central nervous system, serotonin, norepinephrine, and dopamine, between B6 and D2 mice; however, like studies of the other neurotransmitter systems, they have not led to a unifying hypothesis regarding the neurochemical basis of differential seizure susceptibility. The strains have similar serotonergic system profiles, although there is evidence that cortical serotonin receptor density distinguishes them and it is possible that this is related to differences in seizure susceptibility. Noradrenergic systems are also characterized by a few minor but statistically significant differences between strains. However, of all the major neurotransmitters, the system that best distinguishes B6

and D2 strains is the dopamine system. There are many differences between B6 and D2 mice reported for parameters of dopaminergic neurotransmission, including immunohistochemical measures of the dopamine transporter protein, the vesicular monoamine transporter type 2 protein, and tyrosine hydroxylase. Such differences are consistent with reported dopaminergic underactivity throughout the brain in B6 mice relative to D2. Although strain differences in dopaminergic measures have been suggested to influence strain-specific behavior related to drugs of abuse such as cocaine and ethanol, there is as yet no direct evidence for influences on seizure susceptibility.

Cell culture

Insight gained from whole animal studies has been supplemented through the use of in vitro methodologies. In one approach, cultured astrocytes from B6 and D2 mice have been used to examine basic parameters of cell function and have revealed higher sodium and chloride levels in astrocytes derived from B6 mice compared to those derived from D2. In vitro studies using cultured astrocytes also confirm higher Na-K-ATPase activity in B6 compared to D2 mice, but show further that these differences may not be sufficient to produce fundamental differences in cell activity. A novel potential mechanism with respect to seizure susceptibility emerged during the study of polyamine uptake and catabolism to GABA, which when altered, differentially influences the GABA/glutamate ratio, and thus potentially, the balance of excitation and inhibition, in B6 and D2 mice.

Neuroanatomy

Anatomical differences in the brain have also been documented between B6 and D2 mice and it is possible that such factors may be involved in determining differential seizure thresholds. Studies of brain size suggest both gross and microscopic strain differences. Thus, B6 mice have larger brains than D2 mice and they also have thicker layers in the cerebellum. Region-specific differences between B6 and D2 mice have also been documented with regard to anatomical features of the hippocampus and olfactory bulbs. Strain differences in long-term potentiation have been reported and suggested as functional correlates of differences in hippocampal and amygdalar architecture. Functional studies based on the audiogenic seizure phenotype have uncovered strain differences in the amplification of signals at different levels in the auditory pathway. These alterations may be related to morphological changes that occur both peripherally and centrally.

Hippocampus

The special role of the hippocampus in the control of seizure activity has focused considerable attention on this brain region in B6 and D2 mice. Differences in levels of hippocampal signal transduction molecules such as protein kinase C exist between B6 and D2 mice, but they have not been associated directly with differences in seizure susceptibility. Hippocampal slice electrophysiology is generally consistent with relative strain susceptibilities reported in vivo such that application of KA produces bursts of high frequency repetitive spikes in CA1 neurons in slices from juvenile D2 mice, whereas only single spikes are elicited from B6 CA1 neurons. Responses of CA1 neurons in slices from adult B6 and D2 mice are similar upon exposure to KA; however, measures of CA1 responses to a wider variety of chemical stimuli including glutamate, NMDA, and potassium show differences between the strains for both juveniles and adults. Measures of synaptic potentiation reveal that D2 mice have a significantly reduced paired-pulse inhibition of the population spike (a measure of polysynaptic inhibitory feedback) within the dentate gyrus and also show a deficit in the maintenance of potentiation of EPSP and population spikes compared to B6.

Genetic Studies

As can be appreciated from the sections above, considerable research effort has been expended to gain an insight into the neurobiology of differential seizure susceptibility in B6 and D2 mice through direct comparisons of various phenotypic traits. Limited insight has been gained into the biology of seizure mechanisms using this research strategy; however, the correlative nature of such studies does not permit the establishment of cause and effect relationships. Further restrictions imposed by the lack of resolution at both cellular and developmental levels also contribute to the limitations of previous phenotypic studies. Identification of the genetic determinants of a phenotypic trait provides direct insight into the biological mechanisms of trait expression. Unlike studies that are focused on specific transmitters, brain regions, or pathways, genetic studies using B6 and D2 mice offer unbiased opportunities to identify key molecules and systems that specifically define differences in seizure susceptibility.

One of the earliest studies of genetic influences on the difference in seizure susceptibility between B6 and D2 mice was based on a test involving the exposure of animals to environments in which the atmospheric pressure is gradually increased and a generalized seizure is thus elicited. In such a paradigm, susceptibility of 5–10-week-old D2 mice relative to B6 mice was modeled using classical genetic crosses between the strains, and shown to result from the influence of multiple genetic loci, one of which was suggested to have a major effect. Characterization of the model as polygenic prompted a quantitative trait loci (QTL) mapping study, the results of which localized one of the genes involved to a segment of chromosome 17 near the histocompatibility complex. Although the high atmospheric pressure model of seizures

has not been pursued, genetic analysis of other seizure phenotypes in B6 and D2 mice based on QTL mapping has proved to be a valuable means to exploit this animal model in the interest of gaining direct insight into the biology of seizure susceptibility.

Methods

QTL mapping is a method utilized to define the general chromosomal position of genes or genetic variants that influence the magnitude of a measurable trait. Most measurable or quantitative traits are considered complex in that they are determined by the influence of multiple genes that function independently and that also interact with each other and with the environment. Conceptually, mapping genes for complex traits is straightforward, involving correlation between trait values and genotypes at DNA markers spaced evenly at intervals across the genome (in individual organisms from a genetically variable population). However, in practice, many factors influence the likelihood of successful mapping, which is gauged not only by the detection of QTLs but also more importantly by the identification of underlying quantitative trait genes (QTGs). It is noteworthy that while QTLs have been mapped for hundreds of multifactorial traits in mice, only recently have some of these QTLs been resolved at the level of single genes. QTL methods are optimized if initial mapping populations are derived from a pair of inbred strains whose respective trait values are extremely discordant, residing at opposite ends of the phenotypic continuum – such as is the case with seizure susceptibility traits in B6 and D2 mice. This condition increases the likelihood that genes of detectable effect are involved and can be identified using currently available methods. Thus, in this sense, the B6 and D2 strains are an ideal pair for use in QTL mapping studies of seizure susceptibility.

Recent Results

QTL Mapping Studies

Audiogenic seizures

Similar to the high atmospheric pressure seizure phenotype described above, audiogenic seizure susceptibility in B6 and D2 mice has also been characterized as a polygenic trait. The rudimentary nature of mouse genetic maps in use at the time of early QTL studies did not allow comprehensive genome analysis. Thus, initial mapping was based on studies of a panel of BXD RI strains in which the strain distribution pattern at a relatively small number of DNA markers and gene variants (and at several other loci where genotype was inferred from the analysis of other traits unrelated to seizures) was correlated with audiogenic seizure susceptibility. Using this approach, genetic influences on audiogenic seizure susceptibility are shown to arise from chromosomes 4 and 12 (**Table 1**). Interestingly, Ca-ATPase activity, which is inversely correlated with audiogenic seizure susceptibility, is also linked to these loci. As no genes encoding Ca-ATPase subunits are located on either chromosome 4 or 12, the 'audiogenic seizure prone' (Asp) loci may involve an effect on regulation of gene expression from a trans-acting factor. Alternatively, it is possible that regulation of Ca-ATPase activity is an indirect or 'downstream' functional effect of the locus. Full dissection of audiogenic seizure susceptibility in immature B6 and D2 mice awaits completion, and thus, specific causative genetic variation remains to be identified in this seizure model.

Pharmacological studies

Most of the genetic studies in B6 and D2 mice have focused on paradigms involving the use of pharmacological agents to elicit seizures and quantify seizure susceptibility. Two general types of approaches have been utilized as screening tools in this regard. One involves scoring mice for seizure susceptibility following systemic administration of traditional convulsant agents such as KA and PTZ. The other involves scoring mice for susceptibility to withdrawal seizures induced by handling, following systemic administration of central nervous system depressants such as ethanol and barbiturates. A major difference between these pharmacological models and the audiogenic model is that the former involve study of adult mice whereas the latter involves juveniles.

The first full genome scan conducted to map loci for susceptibility to chemoconvulsant seizures utilized a population of B6 × D2 F2 intercross mice and challenged them with a single injection of KA. Two sets of QTLs are revealed by this paradigm. The first set is 'susceptibility' loci – that is, loci that influence whether or not mice will experience a KA-induced seizure – and the second set is 'severity' loci – that is, loci that influence the magnitude of the seizure response in mice that exhibit KA-induced seizures. The strongest locus in the model, which accounts for the greatest amount of the genetic variance, is in the telomeric region of chromosome 1 (**Table 1**). As expected, greater susceptibility is associated with the D2 allele. Susceptibility loci of weaker effect exist on chromosomes 4, 5, 11, 15, and 18 with susceptibility from chromosomes 4 and 11 arising somewhat unexpectedly from B6. Seizure severity loci are harbored on chromosomes 5, 7, and 15 (**Table 1**) with greater severity at the first two of these loci also associated with B6 alleles.

QTLs that influence 'ethanol-withdrawal' seizures in B6 and D2 mice are detectable on chromosomes 1, 4, and 11 by scoring hybrid progeny for seizures induced by handling the mice 2–12 hours following a single high dose of ethanol. This is consistent with QTLs for KA seizure susceptibility in that there is an overlap between confidence intervals for the chromosome 1 and 4 KA and ethanol withdrawal QTLs, and in that the susceptibility

Table 1 Summary of seizure susceptibility QTLs mapped in B6 and D2 mice[a]

Chr.	*Pos. (cM)*[b]	*Seizure phenotype*	*Susceptibility allele*	*Source*
1	41	Pentylenetetrazol	D2	Ferraro *et al*. (1999)
1	96	Handling-induced	D2	Buck *et al*. (1997, 1999)
1	89	Kainic acid	D2	Ferraro *et al*. (1997)
1	100	Pentylenetetrazol	D2	Ferraro *et al*. (1999)
1	88	MEST	D2	Ferraro *et al*. (2001)
2	15	MEST	D2	Ferraro *et al*. (2001)
2	36	Handling-induced	D2	Buck *et al*. (1997, 1999)
2	86	Handling-induced	B6	Buck *et al*. (1997, 1999)
2	106	MEST	D2	Ferraro *et al*. (2001)
3	14	Pentylenetetrazol	B6	Ferraro *et al*. (1999)
4	18	Pentylenetetrazol	D2	Ferraro *et al*. (1999)
4	45	Kainic acid	B6	Ferraro *et al*. (1997)
4	45	Audiogenic	D2	Neumann and Seyfried (1990)
4	45	Handling-induced	D2	Buck *et al*. (1997, 1999)
5	8	MEST	D2	Ferraro *et al*. (2001)
5	26	Kainic acid	B6	Ferraro *et al*. (1997)
5	26	Pentylenetetrazol	B6	Ferraro *et al*. (1999)
6	38	Pentylenetetrazol	D2	Ferraro *et al*. (1999)
7	35	Audiogenic[c]	D2	Neumann and Collins (1991)
7	57	Kainic acid	B6	Ferraro *et al*. (1997)
9	42	Cocaine	B6	Hain *et al*. (2000)
10	40	MEST	D2	Ferraro *et al*. (2001)
11	20	Handling-induced	B6	Buck et al. (1997, 1999)
11	20	MEST	B6	Ferraro *et al*. (1997)
11	66	Kainic acid	B6	Ferraro *et al*. (1997)
12	18	Audiogenic	D2	DiRocco *et al*. (1998)
13	45	MEST	D2	Ferraro *et al*. (2001)
14	30	Cocaine	B6	Hain *et al*. (2000)
15	14	Kainic acid	D2	Ferraro *et al*. (1997)
15	7	MEST	D2	Ferraro *et al*. (2001)
15	52	MEST	D2	Ferraro *et al*. (2001)
15	60	Cocaine	D2	Hain *et al*. (2000)
18	49	Kainic acid	D2	Ferraro *et al*. (1997)
18	49	Pentylenetetrazol	D2	Ferraro *et al*. (1999)

[a]QTLs are included if they meet criteria for genome-wide significance (Lander and Kruglyak, 1995) or if there is a correspondence of suggestive QTLs from multiple studies.
[b]QTL position refers to the position of the most significant single marker analyzed in the original genome scan or the position given to the QTL in the public database at http://www.informatics.jax.org/.
[c]This QTL was mapped in a cross of A/J and D2 inbred strains.

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allele on chromosome 1 is D2-derived and that on chromosome 11 is B6-derived. Like the KA QTL, the chromosome 1 QTL effect is robust for ethanol withdrawal seizures and none of the other loci have larger effects. It is possible that KA and ethanol QTLs on the telomeric aspect of chromosome 1 represent the same genetic influence. The chromosome 4 ethanol withdrawal QTL overlaps the chromosome 4 KA QTL; however, it is derived from the D2 strain suggesting a distinct genetic factor and/or mechanism. Contrary to the correspondence observed between the location of the KA and ethanol QTLs on chromosomes 1 and 4, the location of the QTLs on chromosome 11 are somewhat different, being centromere for ethanol withdrawal and telomeric for KA (Table 1). These findings suggest that the chromosome 11 QTLs for KA and ethanol are mediated by independent influences. Other provisional QTLs for handling-induced ethanol withdrawal seizures map to chromosome 2 (Table 1).

Confidence intervals of QTLs that mediate differences between B6 and D2 mice in susceptibility to PTZ-induced seizures overlap with those for KA and ethanol handling-induced (Table 1). Importantly, the strongest genomic influence localizes again to the telomeric region of chromosome 1, corresponding closely with the position of KA and ethanol QTLs. Other significant and suggestive QTLs for PTZ seizures map to chromosomes 1 (centromeric), 3, 4, 5, 6, and 18. Loci on chromosomes 5 and 18 overlap with and show inheritance patterns consistent with those reported in the KA screen (Table 1). The position and inheritance pattern of the chromosome 4 QTL are consistent with the locus detected in the ethanol withdrawal screen (Table 1).

Another withdrawal model utilizes a phenotype based on handling-induced seizure severity quantified following injection of pentobarbital. Applying this model to BXD RI strains, B6 × D2 F2 intercross mice and short-term selection lines permitted mapping of another series of seizure susceptibility QTLs. Like other pharmacological studies, the strongest influence in pentobarbital screens localizes to the telomeric region of chromosome 1 (Table 1) suggesting that it represents the effect of the same genetic variation detected in screens with KA, PTZ, and ethanol. In addition, QTLs for pentobarbital withdrawal seizure susceptibility on chromosomes 2, 4, and 11 overlap with those from paradigms described above. The chromosome 2 QTL intervals (centromeric and telomeric) overlap with QTLs from the ethanol screen, the chromosome 4 QTL interval overlaps with QTLs from the PTZ and ethanol screens, and the chromosome 11 QTL interval overlaps with QTLs from the KA and ethanol screens (Table 1). Thus, there is overall good correspondence between mapping results involving phenotypes based on the susceptibility of B6 and D2 mice to chemically mediated seizures. However, an important caveat, is that the overlap of QTL intervals between studies conducted with different but related paradigms does not guarantee that the influence of the same genetic variation is operative at each such locus, even when the studies utilize the same strains of mice.

Electroshock seizure threshold

Potentially confounding factors in the use of drugs to enhance the excitability of the nervous system and predispose to seizure activity relate to pharmacokinetics. Issues of drug disposition within whole animals may include some that are specific to particular drugs and some that are relevant to multiple drugs. A QTL mapping procedure for seizure susceptibility that circumvents the use of pharmacological agents involves measurement of electrical seizure threshold. Interestingly, QTLs for maximal electroshock seizure threshold (MEST) in B6 and D2 mice map to some of the same regions of the genome as identified in pharmacological studies. Most notably, as in studies that utilize drugs to study seizure susceptibility, a telomeric chromosome 1 locus explains the greatest amount of the variance and represents the prime genetic determinant of the large difference between MEST in B6 and D2 mice. Statistically significant MEST QTLs also exist on chromosomes 2, 5, and 15 in regions that overlap with QTLs detected in the pharmacological screens. With regard to chromosome 15, two significant MEST QTLs exist, one closer to the centromere and the other closer to the telomere. The centromeric locus overlaps closely with a chromosome 15 QTL detected in the KA screen (Table 1). The MEST QTL on chromosome 5 overlaps with chromosome 5 QTLs detected in the KA and PTZ screens; however, the susceptibility allele for the MEST chromosome 5 QTL is contributed by the D2 strain whereas the QTL for susceptibility to KA and PTZ seizures is contributed by B6, suggesting that they reflect the effects of different gene variants and/or mechanisms. Finally, the centromeric chromosome 2 QTL for MEST overlaps roughly with QTLs detected in screens involving ethanol and pentobarbital, as does the telomeric chromosome 2 QTL; however, susceptibility at the latter is contributed by B6 for MEST but by D2 for the depressant drugs (Table 1).

Genetic Studies: QTL Confirmation and Analysis

The polygenic determinism of the seizure paradigms applied to B6 and D2 mice involves QTLs that are shared by virtue of overlapping genetic map positions between all or several of the paradigms, as well as QTLs that appear to be specific for individual paradigms. Regarding shared QTLs, the hypothesis that the same genetic variants influence responsiveness in different kinds of seizure screens allows QTLs to be prioritized for further analysis since genes whose variation influences diverse seizure phenotypes may encode proteins that play a fundamental role in neurotransmission and may be most relevant to the initiation and/or propagation of seizure activity. This perspective

has provided the impetus for creating specialized congenic strains to analyze seizure QTLs in B6 and D2 mice. As described below, significant progress has been made towards identifying the genes and their variation that underlie seizure related QTLs on chromosomes 1 and 4; however, it is apparent that these influences together explain only a portion of the overall difference in seizure susceptibility and that more influences remain to be identified.

Congenic strain studies

Several seizure susceptibility QTLs mapped in the B6 and D2 model have been confirmed by creating and studying strains of mice that are congenic between B6 and D2, that is, strains in which a defined segment of the genome (representing the QTL interval) is transferred from one strain to the other via standard backcrossing and DNA marker-based selection of progeny. QTLs are confirmed when transfer of the genomic segment produces a predictable alteration in the phenotype of the congenic strain. Such predicted phenotypic changes have been documented by using congenic strains to study seizure-related QTL intervals on chromosomes 1, 4, and 5 – regions of the genome that have been shown to harbor B6–D2 seizure-related QTLs in multiple studies employing diverse experimental seizure screens (**Table 1**). The QTL on chromosome 11 has been further dissected and confirmed by haplotype analysis in a hybrid population of D2 and D1 strains of mice; however, direct confirmation of this locus in the B6–D2 model has not been reported.

Chromosome 4 QTL

The chromosome 4 QTL has been confirmed and narrowed to a 1 cM segment of DNA using a series of interval-specific congenic strains assessed with screens for ethanol and pentobarbital withdrawal seizure susceptibility. Analysis of genes within the critical interval has led to the nomination of variation in the promoter sequence of *Mpdz* as the cause of the effect. The *Mpdz* promoter sequence variation is associated with responsiveness to a variety of chemoconvulsants and with mRNA and protein expression across a panel of inbred strains. The product of the *Mpdz* gene belongs to a large family of proteins that have a PDZ domain. The PDZ domain is a common structural domain of 80–90 amino-acids found in the signaling proteins of bacteria, yeast, plants, and animals. PDZ is an acronym combining the first letters of three proteins – postsynaptic density protein (PSD95), *Drosophila* disc large tumor suppressor (DlgA), and zonula occludens-1 protein (zo-1) – which were the first discovered to share the domain. PDZ-domain proteins have diverse cellular functions related to molecular trafficking, anchoring of transmembrane proteins to the cytoskeleton, and maintenance of signaling complexes. It is not yet established how Mpdz variation, or differential expression of the Mpdz protein, influences seizure susceptibility in the B6 and D2 model.

Chromosome 1 QTL

The telomeric chromosome 1 QTL has also been dissected and refined using a series of congenic strains that have overlapping segments of the critical interval. Refinement of the QTL map position helped to identify a non-synonymous SNP in the inward-rectifier potassium channel gene *Kcnj10*, and this led to the nomination of *Kcnj10* as the QTG at the telomeric chromosome 1 locus. *Kcnj10* encodes an inward rectifier potassium channel subunit that is highly expressed in glial cells that are responsible for buffering extracellular potassium in the nervous system during action potential firing. The Thr(B6)262Ser (D2) protein coding substitution occurs in the C-terminal portion of the protein that is critical for determining channel density in the cell membrane as well as the appropriate clustered pattern of distribution. Bacterial artificial chromosome (BAC)-mediated transfer of a construct containing the B6 allelic version of *Kcnj10* (and five other tightly-linked genes) increases MEST and thus can 'rescue' a seizure-susceptible phenotype (defined by low MEST) in a gene-dosage dependent manner. Such evidence provides support for the hypothesis that *Kcnj10* is a seizure-related QTG in the B6 and D2 model with functional consequences determined by the Th262Ser amino acid variation. Another interpretation of these results is that overexpression of *Kcnj10* (or another gene harbored on the BAC) per se is responsible for the observed phenotypic rescue, and this possibility warrants further investigation.

Future Directions – Relevance to Human Epilepsy

Elucidation of the specific genes and their variation that define seizure susceptibility QTLs in B6 and D2 mice is given added importance by the possibility that such factors will provide insight into the biological basis of susceptibility to epilepsy in humans. Unraveling the complex genetic influences in common forms of human epilepsy is a daunting task that, to date, has been undertaken primarily by means of candidate gene association and some linkage studies. On the horizon are whole-genome association studies, large-scale expression and proteomics studies, and studies on the epigenome. All of these approaches are aided by data that help to prioritize candidate genes and specific kinds of variation within them for confirmation and validation.

The characteristics of B6 and D2 mice described in this chapter do not model epilepsy per se but rather reflect susceptibility to acute experimentally induced seizures. Despite this obvious difference, it is hypothesized that human epilepsy also involves alterations of seizure susceptibility in which neurons and their networks, predisposed by a pattern of variation among potentially many genes, exhibit paroxysmal hyperexcitability and mediate specific

seizure events. Translation of findings from the B6 and D2 genetic models to human epilepsy has begun to substantiate this hypothesis. Thus, provisional identification of variation in *Kcnj10* as the causative factor underlying the telomeric chromosome 1 QTL led to studies of this gene in human epilepsy. Association analysis using patients with generalized epilepsy identified the significant influence of a nonsynonymous SNP (predicting Arg271Cys) on *KCNJ10*. Interestingly, the human KCNJ10 variant is only several residues away from the variant in B6 and D2 mice and may have a similar end result on protein function. The significant genetic association results were confirmed in a second study by an independent laboratory using a larger patient population, and thus the KCNJ10 discovery represents one of the only replicated genetic associations in the field of human epilepsy research. This work provides a new inroad for understanding and treating human epilepsy.

Based on such results, it can be argued that genetically complex animal models of seizure susceptibility, as reflected in the B6 and D2 inbred strain model, are useful tools with which to gain insight into common forms of human epilepsy. In fact, such models may offer greater potential utility compared to single gene models that are based on spontaneous or induced mutations, since they may be more 'naturalistic' and may more closely reflect the multifactorial nature of most cases of human epilepsy. Thus, the continued dissection of the B6 and D2 model, as well as models developed using other common strains of mice, will help to further elucidate the combination of multiple gene effects, multiple environmental effects, and their interaction that defines the complex architecture of human epilepsy.

Acknowledgement

The author's research is supported by a grant (NS040554) from the National Institutes of Health.

See also: **Genetics**: Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Genetics of Lafora and Juvenile Myoclonic Epilepsies; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future; Role of Genetic Factors in the Etiopathogenesis of Epilepsy-Related Brain Damage.

Further Reading

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Sleep-Related Epilepsies and Mutations in Acetylcholine Receptors

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Introduction

Epilepsies are not a single disorder but rather a highly heterogeneous group of neurological conditions that differ in etiology, genetic background, treatment options, prognosis, and recurrence risks. Overall, epilepsies have a lifetime cumulative incidence of 3% in Western populations. One simplified classification would be to differentiate between epilepsies in which seizures are the main or only symptom and those in which seizures are just one symptom among others. The reasons why individuals develop epilepsy, or disorders in which seizures are a frequent feature, are numerous. Examples for mostly nongenetic causes of repetitive seizures are trauma, tumor, and infections (e.g., neurocysticercosis, a tapeworm infection that is a major cause of epilepsy in developing countries worldwide). On the other end of the spectrum are epilepsies that are caused by single gene mutations and are characterized by Mendelian inheritance patterns. Most epilepsies do not belong to either of these groups but are due to a mixture of genetic and nongenetic factors. The genetic factors in the common forms of epilepsies (such as juvenile myoclonic epilepsy, childhood absence epilepsy, juvenile absence epilepsy, and grand mal epilepsy on awakening) most often show only a low penetrance effect with respect to the phenotype. It can be assumed that several susceptibility genes are involved in each patient, and that the interaction among these gene loci is multiplicative rather than additive. Some genes might determine the seizure threshold by influencing neuronal excitability, while other susceptibility genes might be responsible for the age of onset and therefore the seizure subtype. The identification of such genes would be helpful not only for the understanding of epileptogenesis, but also for the development of new prevention and treatment strategies. However, low penetrance genes are notoriously difficult to identify in the genome. To get an idea about the type of genes that are involved in epilepsy, we decided to search first for genes underlying monogenic epilepsies. We made this decision 15 years ago, and the first gene that we discovered under this strategy was the α4-subunit (*CHRNA4*) of the neuronal nicotinic acetylcholine receptor (nAChR) that can cause autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). As it turned out, this gene – and the gene family to which it belongs – still fascinates researchers from various fields.

Background

ADNFLE: What is the Phenotype?

When patients are telling the story of their nocturnal frontal lobe epilepsy, one repeatedly hears that the diagnosis was only made when, retrospectively, the seizures had already occurred for months, or even years. This situation is mainly explained by the fact that the seizures are mostly occurring at night, and that late childhood to early adulthood is the main age of onset. Sometimes in children, the abnormal nocturnal episodes are recognized for what they are when the individual shares a room with a sibling or parent on vacation; in adults, this recognition may not occur until the individual starts living together with a partner. Even then, it is not uncommon for the seizures to be misdiagnosed as night terrors, somnambulism, or benign nocturnal parasomnias. A correct and early diagnosis is also hampered by the fact that frontal seizures are notoriously difficult to detect on EEG. Interictal EEG abnormalities are rarely seen in ADNFLE, and only some patients show infrequent ictal epileptiform activity in the EEG. The clusters of brief motor seizures occur mostly out of non-REM (rapid eye movement) sleep (typical for dream-poor sleep phases). Patients can have frequent recurring arousals per night, followed by seizure episodes of 2–20-s duration. Some seizure episodes last longer, and in sleep-walking periods, patients might move around, talk unintelligibly, or scream for 3 min or more. Many patients report nonspecific aura phenomena, including epigastrial, sensory, or psychic symptoms that sometimes precede their seizures. Typically, the seizures start with gasps, grunts, or some vocalizations followed by thrashing hyperkinetic activity or tonic stiffening of the limbs, often with superimposed clonic jerking. The consciousness is only infrequently impaired; indeed, patients often report that they have been conscious during the seizure. Seizure frequency can be highly variable even within the same family. In some of the patients, seizures are frequent and tend to cluster, while in others, seizure occurrence is more sporadic; sometimes (for unknown reasons) decade-long periods of spontaneous remission occur before the seizure episodes occur again. Most patients experience a decrease of seizure frequency in later adulthood. Physical examinations and brain imaging results are usually normal. Seizures are often, but not in all patients, readily controlled by antiepileptic drugs, especially carbamazepine.

ADNFLE: Hunting for the First Gene

In the first half of the last decade of the twentieth century, positional cloning of a gene required much more effort than what is required now (i.e., since the sequencing of the human genome has been completed). Once a gene was linked to a certain genomic region, either by linkage studies or through the finding of an unusual structural chromosomal abnormality in a patient, the hunt started for the gene of interest – which was often buried among many mostly unknown genes. This part of the cloning process could take years, in some cases even decades. Thus, we were more than lucky that we had already started to catalogue the genes located in chromosomal region 20q13.3 when Berkovic and colleagues mapped the first gene for ADNFLE to this region in early 1995. This mapping enabled us to join forces, and together, our groups were able to identify a mutation in the *CHRNA4* gene as the underlying cause in the large ADNFLE family that helped to map the disorder to 20q13.3. It quickly became obvious that *CHRNA4* is not the only gene for ADNFLE, but that some of the other subunits of the same receptor can also cause sleep-related epilepsy. Interestingly, several years after the discovery of the first epilepsy gene, a second epilepsy gene was located in close vicinity to *CHRNA4*. This second gene, *KCNQ2*, which codes for a voltage-gated potassium channel, is responsible for another rare monogenic type of epilepsy – benign familial neonatal convulsions (BFNCs). Thus far, nobody has been able to find a convincing functional explanation for this colocalization of two distinct epilepsy genes to the tip of the long arm of chromosome 20.

The Nicotinic Acetylcholine Receptor

Together with glycine, $GABA_A$, and serotonin receptors, the nAChRs are members of the large family of ligand-gated ion channels of which muscle nAChRs are the prototype. Five (identical or different) homologous subunits are assembled and arranged around a central axis, forming a cation-selective ion pore. Each subunit is composed of four transmembrane domains, an extracellular N-terminal domain, and a short intracellular loop between transmembrane domains III and IV. The extracellular domain carries the acetylcholine binding site that, in the assembled receptor, is located at the boundary between subunits, while the four transmembrane domains delineate the axial cation-specific channel or ion pore. The extracellular domain and the four-transmembrane domain-complex are topologically distinct but are coupled allosterically to each other. A chemical signal represented by a local increase of extracellular agonist concentration can be converted by the nAChR into an electrical signal generated by the opening of the ion channel.

Methods

Linkage and Candidate Gene Approaches

Most of the known epilepsy genes have been discovered using a combination of linkage analysis and candidate gene approach. Linkage analysis takes advantage of the numerous genetic polymorphisms that are precisely mapped in our genome. Linkage can be suspected if such a polymorphism cosegregates with the disorder in one large or several smaller families. The distance between the genetic polymorphism and the actual epilepsy gene can than be estimated through the observed recombinations between polymorphism and disorder. Once the recombination rate is (close to) zero, all genes in this chromosomal region can be screened for mutations systematically or some of them can be selected by a candidate gene approach. Ion channels are known to play an important role in neuronal excitability, thus they are obvious candidates for epilepsy genes, a hypothesis that had by now been confirmed by the discovery of several different epilepsy genes. If strong candidate genes exist, it is even possible to use the candidate gene approach directly without performing linkage analysis first.

Functional Studies

Ion channels such as the nAChR provide the possibility to study the effects of mutations found in patients in easily accessible model systems such as *Xenopus* oocytes or HEK cells. Once the mutation is identified in the patients' DNA sample, it can be artificially introduced into a plasmid containing the respective subunit DNA and transcribed. After the injection of the wild-type or mutated mRNA into the experimental cell system, the receptor itself is assembled by the host cell and expressed at the surface – where it can be studied by either whole-cell or single-channel recording techniques. Differences in ion channel properties between wild-type and mutated receptors – such as increased or decreased agonist responses, changes in the duration of opening or closing times, or altered calcium permeability – will then allow a first glimpse at the basic pathogenetic mechanism(s) underlying epilepsy of interest.

Recent Results

The Genes Underlying ADNFLE

CHRNA4 and CHRNB2

Uncovering the genetic basis of ADNFLE took a lot of time, and the story is far from over. To date, mutations in three genes (*CHRNA4*, *CHRNB2*, *CHRNA2*) have been identified in families with nocturnal frontal lobe seizures (**Figure 1**). All three genes encode subunits of the nAChR, which is involved in a wide range of physiological processes and behavioral traits. The first ADNFLE mutation, a

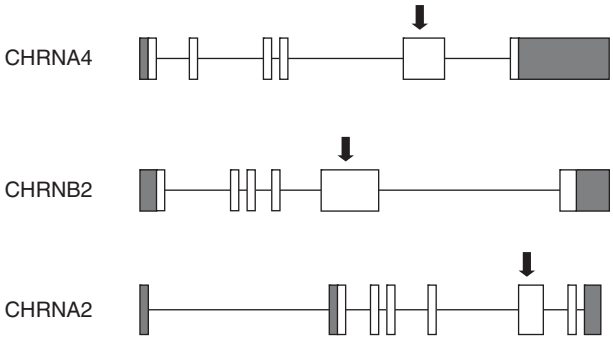


Figure 1 Schematic representation of nAChR subunit genes *CHRNA4*, *CHRNB2*, and *CHRNA2*. The positions of mutations associated with sleep-related epilepsy are indicated by arrows.

S248F amino acid exchange within transmembrane domain II of *CHRNA4*, was identified in 1995. The identification of *CHRNB2* as the second ADNFLE gene was published 6 years later, and so far four mutations have been described for each of these genes. Six of the eight mutations are located within the transmembrane domain II of either *CHRNA4* or *CHRNB2*, and two others were found in transmembrane domain III of *CHRNB2*. All attempts to find mutations in other parts of these genes were unsuccessful, and after more than 12 years of looking hard for such a mutation, nobody really expects to find one anymore (or at least not in association with a clinical phenotype of 'classical' ADNFLE). Why ADNFLE mutations are clustered in this small part of the genes can be explained from a functional point of view as follows: Both the second and the third transmembrane domain contribute to the walls of the ion channel. Thus, it seems that only mutations that have a direct effect on the ion pore are able to cause ADNFLE. One is even tempted to speculate that not all, but only certain, amino acid residues within these transmembrane domains make good candidates for ADNFLE mutations. This speculation is based on the observation that the number of known mutated amino acid residues is rather small, and that some of these amino acids are altered by mutations that occurred independently in unrelated families. The independent occurrence of the same mutation in families from different parts of the world offers the opportunity to study the effects of the genetic and ethnic backgrounds on mutation penetrance and clinical phenotype.

CHRNA2

A third nAChR gene, *CHRNA2*, has been recently reported as mutated in an Italian family with nocturnal seizures that were often accompanied by fear sensations, protruding tongue movements, and nocturnal wandering. Compared with the nAChR subunits *CHRNA4* and *CHRNB2*, much less is known about the expression pattern and functional role of *CHRNA2* in brain. Like *CHRNA4* and *CHRNB2*, *CHRNA2* mRNA has been shown to be ubiquitously expressed in mammalian brain. Highest expression levels were found in thalamus, an expression pattern that is theoretically consistent with a role of *CHRNA2* in epilepsy, since the thalamus is thought to be one of the most important brain structures with respect to epileptogenesis. The Italian family, and the mutation found in its affected members, differ from the earlier described ADNFLE families in some aspects. Fear sensations, choreoathetoid tongue movements, and nocturnal wandering have also been observed in ADNFLE patients, but seemed to occur more frequently in the Italian family. Furthermore, the missense mutation found in this family affects an amino acid residue that, due to its location in transmembrane domain I, is not likely to contribute to the structure of the ion pore. Unlike transmembrane domain II, only one-third of this domain is thought to contribute to the ion pore walls. Transmembrane domain I is linked covalently to the receptors agonist-binding site and is therefore believed to play an important role in the regulation of channel functions. When the *CHRNA2* mutation was first published in 2006, we had already collected a large sample of families with nocturnal frontal lobe epilepsy but no detectable mutations in either *CHRNA4* or *CHRNB2*. Thus, we tried to find mutations in the *CHRNA2* transmembrane regions in our family sample, but without success. Additional families with *CHRNA2* mutations are needed to decide if mutations in this gene are an extremely rare cause of ADNFLE or if these mutations give rise to a sleep-related epilepsy syndrome different from ADNFLE.

Are there Mutation-Specific Associated Features in ADNFLE?

As described earlier, the diagnosis of ADNFLE is not always a straightforward one. Thus, it is not surprising that patients frequently receive other diagnoses such as sleep disorders or a psychiatric diagnosis. It had been previously thought that the psychiatric diagnoses were a 'last resort,' because the epileptic disorder underlying the patient's symptoms had not been recognized. However, there is mounting evidence that the high frequency of psychiatric diagnoses in ADNFLE patients might not be an artifact. Comparison of the clinical phenotypes between ADNFLE families with different nAChR mutations suggests that at least some nAChR mutations not only cause epilepsy but are also associated with additional neurological and psychiatric features or cognitive deficits. A good example supporting this hypothesis is presented by the *CHRNA4* mutations 776ins3 and S252L. The 776ins3 mutation is a 3-bp insertion present in 11 members of a large Norwegian ADNFLE family. At least 6 of the 11 mutation carriers not only had epilepsy but also presented with serious psychiatric problems, mostly schizophrenia, negative symptoms of schizophrenia, severe apathy, or unclassified psychosis. This phenotype suggests that the 776ins3 mutation may be a risk factor for psychiatric

disorders. The S252L amino acid exchange is present in two unrelated families of Korean and Japanese origin, respectively. In both families, the seizures not only were often difficult to control with antiepileptic drugs (many patients required a combination of two or more antiepileptic drugs in order to decrease seizure frequency) but were also associated in most patients with mental retardation and/or behavioral problems. The differences between the two S252L-families, with respect to their geographic origin and their genetic background, strongly suggests that the associated cognitive deficits are caused by the mutation rather than by unrelated genetic or environmental factors.

About the Role of nAChR in Brain

Depending on their subunit composition, the different subtypes of homo- or heteropentameric nAChRs not only vary with respect to their expression patterns in brain but also display different biopharmacological channel properties. The primary nAChR brain subtype is the $\alpha4\beta2$ receptor, which is present in two different stoichiometries: $(\alpha4)_2(\beta2)_3$ or $(\alpha4)_3(\beta2)_2$. The nAChRs contribute to a wide range of brain activities and influence a number of physiological functions. Their binding sites are the targets of natural ligands like acetylcholine, but also of widely used drugs such as nicotine. They play a major role in higher cognitive functions and dysfunctions. In particular, the loss of cortical nAChRs is a neurochemical hallmark of Alzheimer and Parkinson diseases. Most nAChRs are believed to be located presynaptically and have a neuromodulatory role consisting of an enhancement of the release of GABA, glutamate, dopamine, nor epinephrine, serotonin, or acetylcholine. If present at postsynaptic locations, nAChRs have been shown to mediate fast excitatory synaptic transmission. Even an extrasynaptic localization has been demonstrated for nAChRs, although the functional significance of these finding remains unclear. So far, it is not known exactly where the mutated nAChRs involved in ADNFLE pathogenesis are preferentially localized, and by which mechanisms they are able to raise seizure susceptibility in the patient brains. The surprising, and so far not very well understood, observation is that a nAChR subtype that ubiquitously present in brain is able to cause a partial type of epilepsy rather than a generalized one. But as summarized in the following paragraph, recently published experiments with genetically altered mice have given us some insight into the pathomechanisms underlying epileptogenesis in ADNFLE.

ADNFLE Mutations Patch-Clamped

Functional studies with recombinant nAChR receptors carrying different ADNFLE mutations were performed to analyze the similarities and differences in the receptors' biopharmacological profiles. Coexpression of the *CHRNA4* subunit with the S248F mutation and of the wild-type *CHRNA4* subunit in *Xenopus* oocytes or HEK cells were followed by patch-clamp analysis. These experiments showed that acetylcholine-evoked currents of amplitudes comparable to those of wild-type receptors were obtained from receptors containing the mutated subunit, but that the mutation-containing receptor had a higher sensitivity for the natural agonist. Receptors carrying the 776ins3, S252L, or the V287M mutation also displayed increased acetylcholine sensitivity, but to different degrees, than did the S248F mutation. A reduction of calcium permeability was observed for the mutants S248F and 776ins3, but not for the S252L mutation. Taken together, a gain-of-function effect seems to be the main functional feature common to all known ADNFLE mutations. It is tempting to speculate that the particular functional signatures of each mutant contribute to the earlier described observations of associated neurological features in ADNFLE, while the gain-of-function effect might be responsible for the epilepsy phenotype.

What Stories are Mouse and Other Models Telling?

To examine the possible mechanisms underlying ADNFLE pathogenesis, several groups have already engineered mice carrying nAChR mutations. Two mouse strains that harbor either the S252F or the 776ins3 mutation on one of their *CHRNA4* alleles showed abnormal cortical EEG patterns, interictal spiking, and recurrent seizure activity. Furthermore, mice heterozygous for these mutations exhibited a markedly increased sensitivity toward nicotine-induced seizures and (on average) longer seizure durations than their wild-type litter mates. These results confirm that ADNFLE mutations are dominant with respect to the wild-type allele, and that they are able to cause hyperexcitability and spontaneous seizures. The EEG and electrophysiological data obtained from these mouse models were consistent with a model of epileptogenesis in which acetylcholine is one of the regulators of cortical GABAergic transmission. In such a model, the gain-of-function activation of mutant nAChRs would cause increased GABAergic inhibition. In the normal brain, asynchronous firing of pyramidal cells (e.g., in cortical layer II/III) would be synchronized after recovery from inhibition. In the mutant, an increase in inhibition – and therefore in network synchrony – could cause the pathological hypersynchronization (simultaneous firing of too many neurons at once) that gives rise to a seizure (**Figure 2**).

At first glance it might seem surprising that increased inhibition could be the basic mechanism underlying seizure generation. But just imagine that many balloons are held under water and somebody lets them all go up at once.

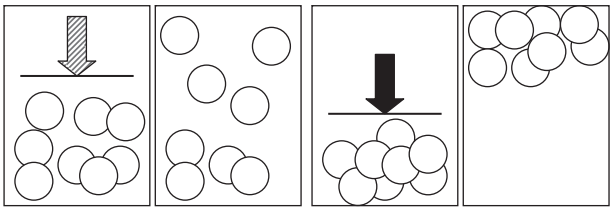


Figure 2 Model of GABAergic inhibition controlled by nAChR activation. Pyramidal cells inhibited by GABAergic interneurons are represented by balloons. Left side: inhibition of neuronal networks; right side, recovery from inhibition; (a) wild type and (b) mutant.

They would be creating one huge wave instead of many small ripples. The synchronizing effect of GABAergic inhibition and nAChR activation has already been demonstrated in several studies. For example, it has been shown that two pyramidal cells that are connected to a single interneuron synchronize their firing after recovery from inhibition. In the hippocampus, the activation of interneurons by acetylcholine first causes inhibition and than rebound spiking. So far it remains unknown why the increase of inhibition caused by mutated nAChRs only infrequently gives rise to seizures – i.e., what the triggering factors for seizures in ADNFLE patients are. One of these triggering factors is obviously to be found in the regulation of the sleep–wake cycle, since most seizures occur during non-REM sleep. These mechanisms are not easy to investigate in mice heterozygous for the aforementioned nAChR mutations, however, since these animals show nearly continuous abnormal EEG activity that makes it difficult to distinguish between the different sleep phases.

Challenges for the Future

Epilepsies as Channelopathies

In 1995, the year *CHRNA4* was identified as the first epilepsy gene, nobody could have foreseen how tedious the process of identification of additional epilepsy genes would be. Nevertheless, the genes that already have been found demonstrate how important is the role of ion channels in epileptogenesis. A great variety of different ion channels regulates brain excitability and prevents hypersynchronization of neuronal networks. Mutations in any of those ion channels might change the delicate balance between excitatory and inhibitory input, resulting in recurrent firing, hyperexcitability, and finally, seizures. Thus, it should not come as a surprise that one of the most important pathogenetic mechanisms in human epilepsy is based on mutations in various ion channel genes. Our growing knowledge about the genes that can underlie the various types of epilepsies will help future attempts to create a more precise classification of this heterogeneous group of disorders, and might also facilitate the development of new antiepileptic drugs for a more effective treatment of patients. To reach these goals, current research needs to focus not only on the identification of new epilepsy genes and their functional analysis, but also on the equally important task of finding additional mutations in known genes to establish a detailed knowledge of genotype–phenotype relationships.

See also: **Genetics**: Clinical Genetics of Epilepsy; Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Ion Channel Mutations in Familial Eplepsy; **Sleep**: Brain Mechanisms Linking Epilepsy to Sleep.

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Relevant Websites

http://www.ncbi.nlm.nih.gov/sites/entrez – Life Sciences Databases at the National Center for Biotechnology Information.

http://www.ilae-epilepsy.org/ – Homepage of the International League against Epilepsy.

GLIA/ASTROCYTES

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Astrocytic Regulation of Neuronal Excitability

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Introduction

Injuries to the brain, which can stimulate epileptogenesis, typically lead to a variety of changes. One major consequence of brain injury, which is often overlooked, is a change in structure and function of the supporting glial cells. Specifically, astrocytes, a subtype of glial cell, exhibit a hypertrophy of their processes and an altered expression of some of their proteins. When glial cells exhibit these alterations, the process is referred to as reactive astrocytosis. This naming stems from the original belief that these changes represent a response of the glial cell to primary changes occurring in the neuron. However, during the past two decades there has been a revolution in our thinking about the roles of glial cells in the brain. As a result, we are re-examining reactive astrocytosis and asking whether these modified glial cells may in fact regulate neuronal function, and may contribute critically to precipitating seizure disorders.

In this review, much of our attention focuses on the basic biology of astrocytes and how they are involved in a signaling dialog with neurons. However, we also discuss how these signals are altered in the epileptic brain – when astrocytes exhibit reactive astrocytosis, and the possibility that certain actions of some anticonvulsants, as well as the ketogenic diet, may be mediated through the astrocyte to reduce neuronal excitability.

We discuss, for example, the now-accepted concept that astrocytes release chemical transmitters that can change the excitability of the neuron. Astrocytes are known to release transmitters with excitatory actions – glutamate and D-serine. In contrast, by releasing ATP, which is hydrolyzed in the extracellular space to adenosine, astrocytes can exert inhibitory actions on neurons mediated by A1 receptors. Additionally, astrocytes regulate GABAergic synaptic inhibition by providing glutamine to neurons, which acts as a critical precursor for the synthesis of presynaptic GABA. We consider the recent notion that the expression of enzymes essential for adenosine and GABA-mediated inhibition are altered following injury, leading to the potential for reduced inhibition in the brain.

Background – The Tripartite Synapse

Electron micrographs show that many synapses are also associated with a process of the astrocyte. Because presynaptic, postsynaptic, and astrocytic processes are associated with one another, this structure has been termed the Tripartite Synapse. In stratum radiatum of the hippocampus, over 50% of the excitatory synapses are associated with an astrocyte. Clearly, one astrocyte has the potential to regulate numerous synapses. Indeed, in the same region, one astrocyte contacts over 100 000 synapses, and in the cortex it has been estimated that one astrocyte contacts up to 600 different dendrites. Though it has been recognized for many years that astrocytes have privileged access to the synapse, we are only just beginning to understand the diversity of signals released and taken up at these 'tripartite' synapses.

One important role for astrocytes is in the uptake of neuronally released chemical transmitters. Glutamate that is released at excitatory synapses is taken up primarily by astrocytic transporters. Studies in knockout mice lacking one of these transporters, GLT-1, demonstrated that this

glial transporter is responsible for the majority of glutamate clearance; mice lacking this receptor develop spontaneous seizures and hippocampal pathology resembling that of patients with temporal lobe epilepsy (TLE).

Astrocytes express a high density of K^+ channels that are conductive at the resting membrane potential. It is thought that these channels are important to buffer extracellular K^+ thereby preventing excess build up of extracellular K^+ following repetitive neuronal activity.

In addition to clearing K^+ and chemical transmitters, astrocytes can also respond to neurotransmitters with elevations in their internal Ca^{2+} signals. For example, stimulation of whiskers, which evoke neuronal activity in the barrel cortex, also evoke astrocytic Ca^{2+} signals in this brain region. Such Ca^{2+} signals result from the release of Ca^{2+} from internal stores that are triggered by metabotropic receptors, which induce the accumulation of the second messenger IP_3. Astrocytes express receptors for most transmitters that then lead to alterations in the levels of Ca^{2+} as well as other second messengers. Elevation of internal Ca^{2+} can, in turn, lead to the release of chemical transmitters from the astrocyte. In this article we use the term gliotransmitter to identify chemical transmitters released from astrocytes. Astrocytes release glutamate (which acts on neuronal NMDA receptors) and D-serine (which is an endogenous coagonist for the glycine binding site of NMDA receptors). Additionally, astrocytes release ATP, which is hydrolyzed by extracellular ectonucleotidases to adenosine.

Rather than trying to discuss actions of each of these gliotransmitters, we limit our discussion to two areas: First, we provide some examples of the roles of these gliotransmitters in normal physiology; then we contrast these functions with their potential role in the epileptic brain. Reviews concerning gliotransmission have been published recently (see Further Reading).

Methods

Studies of synaptic transmission and astrocytes rely on a combination of techniques that are rooted in electrophysiology and imaging. The study of neurons and synaptic transmission relies on the use of the patch clamp technique, in which glass pipette is used to record the ion currents that flow across the cell membrane. For these recordings, a pipette is filled with a physiological saline solution and is connected to a circuit for the measurement of current. An extremely tight electrical and mechanical seal is formed between the tip of the pipette and the cell membrane, then negative pressure is used to rupture the membrane within the pipette tip and obtain electrical access to the cell interior. The patch clamp methodology allows high fidelity recordings of electrical activity.

For many years, the possible role of astrocytes in brain function was overlooked because they do not exhibit the all-or-none electrical discharge that was believed to be the sine qua non of brain function. However, the development of high-resolution imaging microscopes that could observe changes in the fluorescence of labels provided the initially surprising result that these glial cells exhibit biochemical excitability. Chemically synthesized dyes, the fluorescence of which changes when they bind Ca^{2+}, are loaded into astrocytes. Then a two-photon microscope is used to observe the fluorescence of these dyes within cellular compartments. This approach can be used to image cells in vivo. To perform such studies, mice are immobilized under the microscope and, after making a small hole in the skull, a microscope lens is used to look into the cortex to examine changes in fluorescence. These fluorescence signals are then used to determine changes in free Ca^{2+} concentration.

Results

The Astrocytic Adenosine Cycle

The astrocyte contains the necessary machinery for the tonic control of the extracellular adenosine that acts on A1 receptors – which in turn leads to inhibitory effects in the central nervous system. There is a constant tone of extracellular adenosine that continuously activates A1 receptors, leading to a persistent A1-dependent presynaptic inhibition of excitatory transmitter release. Though this tone is not debated, the predominant source of this purine has only recently been identified. There are at least two potential sources of extracellular adenosine – either through adenosine loss from cells through equilibrative nucleoside transporters (ENTs), or an accumulation resulting from the hydrolysis of ATP that is released from cells. In support of the former is the notion that increased metabolic activity of cells will lead to an accumulation of intracellular adenosine, which will in turn passively result in an extracellular accumulation. However, because the addition of ENT inhibitors leads to elevated levels of extracellular adenosine, this is unlikely to be the predominant pathway of adenosine accumulation under physiological conditions.

Recent studies have shown that selective expression in astrocytes of a SNARE domain of a vesicle protein, which inhibits regulated exocytosis, leads to the loss of the continuous A1 receptor-mediated inhibition of synaptic transmission. This result shows that the source of adenosine that mediates this inhibition is the astrocyte – and that its release (or release of a precursor) is likely to be via an exocytotic pathway (rather than through the loss of adenosine through ENTs). Further studies demonstrated

that the expression of the SNARE domain inhibited ATP release, and that this nucleotide was in turn hydrolyzed to adenosine. Once adenosine accumulates extracellularly, how and where is it metabolized? Adenosine is taken up into the astrocyte by ENTs, where it is metabolized either by adenosine deaminase to inosine, or by an astrocyte-enriched enzyme, adenosine kinase, which phosphorylates adenosine to AMP (which is then subsequently phosphorylated to ADP and ATP). Because adenosine kinase is astrocyte-specific and the source of adenosine controlling A1 receptors is astrocytic, the pathways described above result in an adenosine cycle, in which ATP is released by astrocytes into the extracellular space and converted to adenosine, which is taken up by the astrocyte where it is converted back to ATP.

Although this discussion highlights the importance of astrocytes in the control of extracellular adenosine, it is important to understand that ATP is also released from nerve terminals, and that this ATP can be converted to adenosine. However, recent studies suggest that neuronally derived adenosine is more likely to activate A2A receptors.

Astrocyte-derived adenosine is not merely released at a background rate to exert a continuous adenosine tone. As discussed earlier, astrocytes can respond to neuronal activity, when neurotransmitters activate astrocytic metabotropic receptors. Indeed, stimulation of the Schaffer collateral pathway of the hippocampus induces astrocytic Ca^{2+} elevations, and this metabotropic pathway leads to elevated adenosine accumulation lasting for 5–15 min beyond a brief period (1 s) stimulus train. In the hippocampus, there is a well-documented form of heterosynaptic depression that is mediated by adenosine. Independent studies, using astrocyte-selective molecular genetics and dialysis of a Ca^{2+} chelator directly into the astrocyte, have shown that presynaptic neural activity can induce elevated release of ATP from astrocytes, which after hydrolysis to adenosine causes the A1 receptor-dependent heterosynaptic depression of neighboring unstimulated synapses. Taken together, the results of these studies demonstrate that the astrocytic adenosine cycle exerts a powerful non-GABAergic mechanism of inhibition in the CNS.

Glutamine Synthetase and GABA-Dependent Inhibition

GABA plays an essential role mediating inhibition in the nervous system. As a consequence, GABA receptors have been targets for the development of anticonvulsant drugs. However, the importance of the astrocyte in regulating presynaptic GABA pools has only recently been appreciated. Glutamine synthetase, an astrocyte-enriched enzyme, is responsible for inactivating glutamate through its conversion to glutamine. Glutamine is transported into neurons, where it is converted to glutamate by phosphate-activated glutaminase (PAG); in GABAergic neurons, this glutamate is subsequently converted to GABA by glutamic acid decarboxylase (GAD). Although studies have not been reported using tissue from epileptic subjects, a recent brain slice investigation has identified glutamine synthetase as a key enzyme controlling the functional presynaptic pool of GABA. When glutamine synthetase is chemically inhibited, there is a reduction in the presynaptic pool of GABA, which results in a reduction in the filling of vesicles with this inhibitory transmitter. Importantly, while glutamine synthetase is inhibited by methionine sulfoximine (MSO), addition of exogenous glutamine restores inhibition; pharmacological inhibition of neuronal transporters for glutamine leads to a similar reduction in GABA released. Therefore, on the time scale of minutes, the efficacy of GABAergic synaptic transmission critically requires an astrocytic source of glutamine.

It is self-evident that reduced levels of GABA will impact on the efficacy of neuronal inhibition, but they may also have effects of astrocytic signal transduction – e.g., through metabotropic $GABA_B$ receptors, which are highly expressed in astrocytes (as well as in neurons). Intriguingly, knockout of either $GABA_B$ receptor subunit produces a serve epileptic phenotype, which is lethal in some mouse strains; the relative roles of neuronal and astrocytic $GABA_B$ receptors in this phenotype remain to be explored.

Reactive Astrocytosis Disrupts the Adenosine and Glutamine Cycles

Given the importance of the astrocyte for controlling inhibition mediated by A1 receptors and by GABA, it becomes important to determine whether the adenosine and glutamine cycles are impaired in brains of epileptic patients. In patients with medial temporal lobe epilepsy, astrocytes become reactive and there is a dramatic loss of neurons in area CA1. In such sclerotic tissue, the expression of glutamine synthetase is dramatically reduced. Whether this reduction is a consequence of the neuronal loss, or whether initial reductions in glutamine synthetase led to reduced GABAergic inhibition which triggered neuronal demise, is not known. However, this observation highlights the potential importance of glutamine synthetase in epileptogenesis, and identifies a potential therapeutic site of intervention for restoration of GABAergic inhibition.

In addition to glutamine, the astrocytic adenosine cycle is impaired in tissue isolated from rodents exhibiting spontaneous recurrent seizures. Following status epilepticus,

the astrocytic expression of adenosine kinase is enhanced. The consequence for the A1-dependent inhibition is dramatic. Because adenosine kinase phosphorylates cytosolic adenosine, enhanced adenosine kinase expression leads to the depletion of intracellular adenosine, and as a consequence there is an increased gradient for extracellular adenosine to enter the astrocyte. Therefore, elevated adenosine kinase results in reduced extracellular adenosine and reduces the tonic inhibition of excitatory synaptic transmission mediated by presynaptic A1 receptors.

Because reactive astrocytosis occurs within days of an initial brain injury (and continues during the period of epileptogenesis), it is tempting to propose that alterations in enzyme expression within reactive astrocytes lead to reduced GABAergic and adenosine-dependent inhibition in the brain, and that the reduction in these forms of inhibition contributes to the chain of events that results in the reorganization of structure and function that supports the generation of seizures.

Astrocytes Excite Neurons – A Basis for the Modulation of Epilepsy?

Some of the first demonstrations that astrocytes release glutamate showed that this glutamate can act on NMDA receptors, and that the affected neurons can generate slow depolarizations similar in form to paroxysmal depolarizing shifts (PDS). Given the importance of the PDS to the generation of epileptiform activity, it has been proposed that excitatory glutamate-mediated signaling from astrocytes may underlie pathological events in the nervous system, including the generation of epileptiform activity.

In order to discuss the relevance of astrocyte-mediated neuronal excitation by glutamate, it is necessary to recount some of the supporting evidence for the presence of this important pathway in the nervous system. Initially in cell culture studies, astrocytic Ca^{2+} signals were shown to lead to delayed neuronal Ca^{2+} elevations that were shown to be mediated by the release of glutamate from the astrocyte. Subsequent studies have shown that Ca^{2+} is the causal stimulus for the release of glutamate. There are numerous pathways that have been shown capable of releasing glutamate from astrocytes, including release through hemi-channels, P2X receptors, and via exocytotic vesicular release of transmitter.

When glutamate is released from astrocytes, it can have several actions on neurons, including the regulation of NMDA receptor function, the control of neuronal excitability, and the modulation of synaptic transmission. A provocative study provided evidence supporting the idea that by releasing glutamate, astrocytes are able to trigger epileptiform events. Support is provided by the independent demonstration that astrocytic glutamate release can cause PDS-like depolarizations. Because of a lack of astrocyte-selective tools, it is experimentally challenging to directly test this hypothesis. However, since astrocytic glutamate predominantly signals through NMDA receptors, pharmacological studies indicate that this glial glutamate is not necessary for ictal epileptiform activity – although it is possible that ictal activity is modulated by a glial source of glutamate. Further support for an astrocytic contribution to epilepsy-related events is provided by the observation that selective chemical inhibition of astrocytic Ca^{2+} signals in vivo leads to a protection of neurons from the delayed neuronal death that follows status epilepticus. We await tools that allow the selective inhibition of astrocytic Ca^{2+} signals and/or the release of glutamate from these glial cells, to test the importance of glial modulation of neuronal excitability in epilepsy.

Directions for Future Research – Could treatments be Targeted through the Astrocyte?

Astrocytes offer the potential for an entirely new set of molecular targets for the development of therapeutics for a variety of disorders, including epilepsy. Perhaps some of the existing anticonvulsants do act through glia. In support of this possibility is the observation that valproate, gabapentin, and phenytoin all reduced astrocytic Ca^{2+} signals in vivo during epileptiform activity, and that when astrocytic Ca^{2+} signals are directly stimulated (using focal application of ATP), these same anticonvulsants reduced this glial Ca^{2+} signal.

In recent years, the ketogenic diet has won increasing clinical acceptance for the management of seizures in patients whose seizures are refractory to antiepileptic drugs, or, in whom pharmacologic intervention has evoked unacceptable side effects. This regimen entails the administration of 80–90% of calories as fat (with the remainder derived primarily from protein). Most patients demonstrate ketosis (3–5 mmol l^{-1}; normal 0.1–0.3 mmol l^{-1}) within a few days of diet initiation. Many patients enjoy an attenuation of seizure frequency and severity, and they do not suffer the obtundation that typifies the response to most medications. The precise mechanism for the therapeutic response is not known with certainty, and it is probable that ketosis evokes multiple changes in brain biochemistry, physiology, and gene expression. Several laboratories have noted alterations of brain glutamate handling in response to ketosis. For example, ketosis appears to favor flux through the glutamine synthetase pathway, thereby enabling more efficient

'detoxification' of glutamate and providing GABAergic neurons with an abundant supply of glutamine, a favored precursor to GABA via the glutamate decarboxylase pathway. Indeed, GABA concentration is increased in the cerebrospinal fluid of patients who receive the ketogenic diet.

Adenosine is considered an endogenous anticonvulsant. However, given the variety of systemic actions of A1 agonists, as well as the soporific effects of A1 receptor activation, it is not feasible to use such compounds as anticonvulsants. However, given that novel receptors have now been identified in astrocytes, it may become feasible to activate glial signaling pathways with novel drugs in order to induce the glia-dependent accumulation of adenosine in select regions of the brain, and either prevent epileptogenesis or inhibit seizures in the epileptic brain.

See also: **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Glial Modulation of Excitability via Glutamate and GABA Transporters; Peritumoral Epilepsy; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain.

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Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis

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Introduction

Epilepsy is among the most common neurological disorders, affecting about 1% of people, and is associated with significant morbidity and mortality. Since only a limited number of patients with epilepsy are candidates for surgical therapy, the majority of epilepsy patients are treated with antiepileptic drugs (AEDs), which are primarily designed to suppress seizures by directly controlling excitability of neurons. Unfortunately, seizure control is poor in about one-third of epileptic patients; seizures in these patients are refractory to currently available AEDs. To develop more effective therapies for epilepsy with potential novel mechanisms of action, it is important to better understand the basic cellular and molecular mechanisms underlying epileptogenesis.

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Glial Modulation of Excitability via Glutamate and GABA Transporters

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Introduction

Fine tuning of glutamatergic excitatory and GABAergic inhibitory neurotransmission is a prerequisite for optimal, normal functioning of the central nervous system (CNS). Termination of the transmission processes mediated by glutamate and GABA is controlled by a number of high-affinity transporters for these amino acids, transporters that are expressed in both neurons and astrocytes. The latter cell type has a major role in this fine-tuning process. Any disturbance in glial transporter function will lead to an imbalance in the equilibrium between excitation and inhibition, leading to seizure activity and epileptic episodes.

Disinhibition, resulting from altered GABAergic neurotransmission, has been associated with a number of neurological disorders, including epilepsy, migraine headaches, chronic pain, anxiety, and sleep disorders. Abnormal excitatory neurotransmission, resulting from excessive synaptic glutamate levels, has been associated with a number of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease, and neuronal damage associated with cerebral ischemia. In addition to a role in these and other neurodegenerative disorders, alterations in glutamate transporters also contribute to the development of seizures and epilepsy. Thus, efforts to regulate the function and trafficking of GABA and glutamate transporters will likely have important implications for the treatment of a number of these neurological disorders and diseases. Ongoing efforts to gain a greater understanding of the factors that control their cellular expression, function, and regulation at the cellular and network level are critical, and will play important roles in the development of a new class of therapeutics for a number of devastating neurological conditions. Indeed, a number of antiepileptic drugs exert their action by modulating these transport processes.

Background

Characteristics and Nomenclature of Glutamate and GABA Transporters

Cloning strategies have led to the identification of five high-affinity glutamate transporters, the operation of which depends on an intact membrane potential as well as a transmembrane sodium gradient. The prevalent cellular localization of the individual transporters is summarized

in Table 1. Quantitative immunogold analysis has provided evidence that the majority of the transport capacity resides in astrocytes, a conclusion confirming that of classical studies of the kinetics of glutamate uptake in neurons and astrocytes. The determination of the presence and functional importance of a presynaptic neuronal glutamate transporter has been somewhat elusive, but it appears likely that such a process does play a functional role. It is, however, clear that the ability of astrocytes to keep the extracellular glutamate concentration in the low micromolar range is of fundamental importance for the optimization of glutamatergic activity. Malfunction of this process will lead to, e.g., seizure activity, excitotoxic neuronal damage, and cell death.

In analogy to the glutamate transporters, cloning efforts have revealed the existence of four GABA transporters, one of which is highly homologous to the transporter for the osmolyte, betaine. The nomenclature and cellular localization of these transporters in rat and mouse brains are summarized in Table 2. It is unfortunate and confusing that nomenclature has developed such that rat GAT-2 and GAT-3 correspond to the mouse GAT3 and GAT4, respectively. Like the glutamate transporters, GABA transport is sodium dependent; in addition, these transporters exhibit chloride dependency. In contrast to the glutamate transporters, it appears that the major sites for GABA transport are expressed in GABAergic nerve endings. Nevertheless, astrocytic GABA transport plays an important role for the function of GABAergic neurotransmission, since astrocytic GABA uptake will reduce the amount of GABA available for recycling into the presynaptic nerve endings for subsequent transport into the vesicular GABA pool. It is now recognized that this balance between neuronal and astrocytic GABA transport plays a significant role in the prevention of seizure activity.

Pharmacological Characteristics of Glutamate and GABA Transporters

Throughout several decades, the pharmacological properties of glutamate transport have been extensively studied. Table 3 provides examples of the most commonly used pharmacological tools to investigate glutamate transporter function. The most important development in this regard has been the identification of DL-threo-β-benzyloxyaspartic acid (TBOA), which, in contrast to all other inhibitors, does not act as a transporter substrate. Thus, it is possible to block glutamate uptake without generating release of glutamate by heteroexchange. Studies with TBOA have unequivocally shown that an intact glutamate transport system is imperative for the prevention of excitotoxic neuronal damage, and that inhibition of glutamate transport will generate seizure activity.

Similarly, a vast number of studies have been devoted to investigating the pharmacological properties of GABA transporters. Table 4 provides a summary of GABA analogs that have been identified as inhibitors of the cloned GABA transporters, and that inhibit GABA transport into neurons and astrocytes. The identification of pharmacological tools that differentially affect neuronal and astrocytic GABA transport has led to the notion that inhibitors of astrocytic GABA transport may be of interest as anticonvulsant agents (see the following sections). This view is based on the idea that astrocytic GABA uptake may result in a drain of GABA from the neurotransmitter pool. Moreover, the development of subtype selective GABA transport (GAT) inhibitors should facilitate functional studies of these transporters and provide insights that may be of value for the development of antiepileptic drugs – e.g., with mechanisms of action analogous to that of tiagabine, which selectively inhibits GAT1.

The methods employed in the studies described in the remainder of this overview are described in detail in the primary literature referenced at the end of this chapter. Please refer to these for an in-depth description of the methods employed in the summarized studies in the following sections.

Methods

The techniques and methods, used to develop and characterize pharmacological agents that may selectively inhibit glutamate or GABA transporters, have been previously described and can be found in the citations included in Further Reading section.

Results

Glutamate Transporters

As discussed earlier, extracellular levels of glutamate are maintained at relatively low levels by the presence of high-affinity, high-capacity transporters localized on neurons and glial. The strategic location of various glutamate transporters on neurons and glia functions to

Table 1 Nomenclature and principal cellular localization of glutamate transporters

	Transporter subtype				
Systematic name	EAAT-1	EAAT-2	EAAT-3	EAAT-4	EAAT-5
Original name	GLAST	GLT-1	EAAC-1	EAAT-4	EAAT-5
Brain area location	Cerebellum	Cortex	Uniformly	Cerebellum	Retina
Cellular location	Glia	Glia	Neurons	Purkinje cells	–

prevent neuronal cell death as a result of overactivation of excitatory neurotransmitter receptors. To date, the numerous attempts to reduce excitatory neurotransmission by blocking postsynaptic glutamate neurotransmitter receptors have not been highly successful in identifying clinically effective neuroprotective therapies. Recently, investigators have been evaluating two other equally attractive options for regulating extracellular glutamate levels: blocking glutamate release and/or enhancing glutamate uptake. A number of different anticonvulsant drugs that display sodium channel blocking properties (e.g., phenytoin, carbamazepine, and lamotrigine) have been observed to reduce glutamate release by blocking depolarization of the presynaptic terminal. It is not clear whether this effect of sodium channel blockers is solely responsible for their anticonvulsant action, as this class of drugs has been shown to have other effects that limit seizure spread from the seizure focus.

In contrast to the efforts to prevent glutamate release, several different approaches have been employed (with varying levels of success) to test the hypothesis that increasing glutamate transport – from the extracellular to the intracellular compartment – will result in a therapeutic effect. These experimental approaches include efforts to study (1) transgenic mice that overexpress glutamate transporters, (2) pharmacological upregulation of transporters, and (3) use of viral vectors to increase expression of glutamate transporters in various cell types. The results from these studies have been encouraging, and suggest that the presence of increased transporter function can lead to reduced cell death and less severe seizures. For example, in the double transgenic mouse model created from crossing an ALS mouse model with a mouse overexpressing EAAT2, animals are able to maintain their grip strength longer than in the ALS model, and display delayed motor neuron loss and a slightly greater lifespan than the nonoverexpressing ALS mutant. Moreover, overexpression of EAAT2 results in reduced seizure severity and improved neuronal survival following status epilepticus (induced by either kainic acid or pilocarpine). A number of antiepileptic drugs have been found to increase glutamate transporter expression. For example, zonisamide has been reported to increase EAAC1 protein levels in the hippocampus and cortex in the $FeCl_3$-induced seizure model. This effect, coupled with a reduction in GAT1 GABA transporter levels, may contribute to the anticonvulsant action of this broad-spectrum anticonvulsant drug. In addition, topiramate has been shown to increase GLT-1 protein levels in cocultures of rat astrocytes and neurons; this result may explain the ability of this anticonvulsant to decrease elevated glutamate levels in the spontaneously epileptic rat, as well as cerebrospinal fluid glutamate levels in human traumatic brain injury.

Table 2 Nomenclature and principal cellular localization of GABA transporters from rat and mouse brain

Nomenclature and cell localization				
Species/cell type				
Rat brain	GAT-1	BGT-1	GAT-2	GAT-3
Mouse brain	GAT1	GAT2	GAT3	GAT4
Neurons	+++	+	+	+
Astrocytes	+	++	++	+++

Number of '+' indicates relative expression levels.

Table 3 Frequently used pharmacological agents acting on glutamate transporters EAAT-1,-2, and-3

Inhibitors of transport		
Transporter subtype	Substrate	Nonsubstrate
EAAT-1	D-asp; THA, t-2,4-PDC	TBOA
EAAT-2	DHK; D-asp; THA	TBOA
EAAT-3	D-asp; THA; t-2,4-PDC	TBOA

THA, Threo-hydroxy aspartate; PDC, Trans-pyrrolidine-2,4-dicarboxylate; DHK, Dihydrokainate; TBOA, Threo-benzyloxyaspartate; D-asp, D-aspartate.

Table 4 Summary of frequently used pharmacological agents (GABA analogs) acting on GABA transporters

	Transporter					
GABA analog	*Neuron*	*Astrocyte*	*GAT1*	*GAT2*	*GAT3*	*GAT4*
Nipecotic acid	+	+	+	–	+	+
THPO	(+)	+	+	–	–	–
Exo-THPO	(+)	+	+	–	–	–
N-methyl-ExoTHPO	–	+	+	–	–	–
DPB-Nip.	+	+	+	–	+	+
DPB-THPO	+	+	+	–	+	+
DTB-Nip. (Tiagabine)	+	+	+	–	–	–
DTB-ExoTHPO (EF1500)	+	+	+	–	–	–
DTB-Me-exoTHPO (EF1502)	+	+	+	+	–	–
SNAP-5114	–	+	–	(+)	+	+

Nipecotic acid, substrate; Others: nonsubstrates.

In addition to these anticonvulsant drugs, treatment with ceftriazone (a member of the β-lactam class of antibiotics) leads to increased expression of GLT-1 transporters and glutamate uptake (for review, see Sheldon and Robinson in Further Reading). This effect of ceftriaxone is of particular interest because it results in a marked and significant disease-modifying effect in models of ALS. For example, ceftriaxone promotes neuronal survival in vitro in a model of motor neuron degeneration, and increases survival and decreases loss of muscle strength in vivo in a mouse model of ALS. Moreover, EAAT2 overexpressing HEK cells appear to protect cultured motor neurons from glutamate-mediated toxicity. It is also of interest to note that a similar treatment protocol with ceftriaxone increased the seizure threshold for generalized clonic and generalized clonic–tonic seizures induced by the chemoconvulsant pentylenetetrazol in two separate mouse strains, albeit strain, sex, and age differences were observed (Jelenkovic *et al.*, 2008). Unfortunately, the authors of the manuscript did not confirm the presence of upregulated glutamate transporter protein, and any final interpretation of their results, as it relates to an improved removal of extracellular glutamate, will necessarily have to await additional confirmation. In addition to epilepsy and ALS, overexpression of glutamate transporters has been found to be neuroprotective in various in vitro and in vivo models of ischemia.

These few examples clearly exemplify the promise underlying this novel strategy to improve the treatment options for a number of devastating neurological disorders. However, it is important to note that every word of promise usually comes with a word of caution; i.e., the upregulation or overexpression of glutamate transporters could negatively affect the outcome under conditions of energy failure. For example, glutamate transporters can operate in reverse direction, and thereby cause an increased release of glutamate, under conditions of energy depletion. As such, their reversal during acute energy failure could contribute to increased neuronal death. Nonetheless, manipulation of glutamate transporter function is an important and exciting new approach to the treatment of neurological disorders that warrants further investigation.

GABA

As mentioned earlier, compounds that selectively block GABA uptake have been demonstrated to possess anticonvulsant activity in both animals and humans. The most successful example of this strategy is the GAT1-selective inhibitor, tiagabine. Tiagabine emerged from a mechanistic-based drug discovery program at Novo Nordisk to identify orally active anticonvulsant derivatives of nipecotic acid. Tiagabine is currently utilized for the add-on treatment of partial seizures in both children and adults. Unfortunately, tiagabine use is not without significant adverse events when used in high doses. These include agitation, sedation, and psychotic-like episodes in patients predisposed to psychiatric illness. As discussed earlier, tiagabine is a selective inhibitor of GAT1, which is preferentially localized on neurons as opposed to astrocytes. Whether or not the adverse effect profile of tiagabine is related to its selective inhibition of GAT1 is an issue that has yet to be resolved, but the possibility has led to the suggestion that inhibitors of non-GAT1 GABA transporters may afford some benefit over that of tiagabine and other GAT1-selective inhibitors. Interestingly, a number of nonselective GABA transport inhibitors have been experimentally demonstrated to possess anticonvulsant activity. Of these, one particular molecule – EF1502 (*N*-[4,4-bis(3-methyl-2-thienyl)-3-butenyl]-4-(methylamino)-4,5,6,7-tetrahydrobenzo[*d*]isoxazol-3-ol, which is substituted with the tiagabine side chain – displays significant inhibitory effects on both GAT1 and GAT2 transporters. Combination isobolographic studies conducted with EF1502 and tiagabine demonstrated that inhibition of GAT2 by EF1502 contributes to the potent and broad-spectrum anticonvulsant profile of systemically administered EF1502 in rodent seizure and epilepsy models. Moreover, the combination of EF1502 with tiagabine did not increase the behavioral toxicity of either drug alone. This latter finding suggests that inhibitors of the GAT2 transporter may offer some advantage over pure GAT1-selective inhibitors. The extrasynaptic location of non-GAT1 transporters, coupled with the pharmacological activity of EF1502, demonstrates the important role of extrasynaptic GABA transporters in controlling neuronal function.

Future Directions

Recent advances in our understanding of the expression pattern of neuronal and glial glutamate and GABA transporters have suggested that these transporters play an important role in a number of neurological and psychiatric disorders. The ability to increase the expression of glutamate transporters with a broad-series of β-lactam antibiotics represents an interesting and novel finding and has broad implications for the treatment of ALS, epilepsy, and other CNS disorders.

The fact that the non-GAT1 transporters located on glial and extrasynaptic membranes play a functional role in the control of neuronal excitability demonstrates that these transporters are potentially important targets for future drug design. Moreover, it is also important to note that the development of selective inhibitors of glial GABA uptake could one day play an important role in enhancing extrasynaptic GABA-mediated inhibition.

The successful translation of ongoing efforts to better understand the factors that control the regulation and expression of glutamate and GABA transporters will depend heavily on the design and synthesis of selective

agents that target these transporters, and the willingness of investigators, drug companies, and funding agencies to invest in the clinical trials that will ultimately determine the utility of this approach for the treatment of epilepsy and other neurological disorders.

See also: **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Peritumoral Epilepsy; Properties of Glia in Epileptic Brain; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Transporters**: Glutamate Transporters in Epilepsy.

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Peritumoral Epilepsy

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Introduction

Each year ~100 000 patients are newly diagnosed with tumors in the central nervous system (CNS) in the United States. According to data from the Central Brain Tumor Registry of the United States (CBTRUS), ~18 500 of these are considered primary brain tumors, i.e., arising initially in the brain; interestingly, the vast majority are of glial origin. These glial-derived tumors are collectively called gliomas and include oligodendrogliomas, astrocytomas, and glioblastomas. These tumors share a number of genetic alterations with other cancers, but also differ in some important aspects. These differences include, for example, their inability to spread via hematogenous routes and their physical containment within the bony cavity of the brain. Biological adaptations that allow gliomas to develop with these constraints may, in part, contribute to the high likelihood with which these cancers produce seizures in patients. Although most brain tumors, including metastatic tumors, can produce seizures, the causal relationship between tumor biology and peritumoral seizures is best understood in glial-derived tumors, which are the principle topic of this article.

Background

Gliomas and Epilepsy

Gliomas arise de novo within the CNS. Although they are generally believed to derive from glial cells or their progenitor cells, their etiology is still in question. Some gliomas are considered benign and can be treated, but the majority of gliomas form aggressive and highly malignant tumors that are largely unresponsive to treatment. Surgical resection is typically unsuccessful as tumor cells tend to invade the normal brain parenchyema diffusely and create satellite tumors. Upon resection of the main tumor, regrowth typically occurs within just a few months. Unfortunately, chemotherapy provides only limited benefit to patients, in part because the blood–brain barrier makes effective delivery difficult and in part also because gliomas tend to be quite resistant to commonly-used chemotherapeutic drugs. Consequently, the outlook for patients with malignant gliomas remains dismal, with average patient survival of less than one year even with all currently available treatments.

In 30–50% of patients, the diagnosis of a primary brain tumor is preceded by epileptic seizures. This percentage

'detoxification' of glutamate and providing GABAergic neurons with an abundant supply of glutamine, a favored precursor to GABA via the glutamate decarboxylase pathway. Indeed, GABA concentration is increased in the cerebrospinal fluid of patients who receive the ketogenic diet.

Adenosine is considered an endogenous anticonvulsant. However, given the variety of systemic actions of A1 agonists, as well as the soporific effects of A1 receptor activation, it is not feasible to use such compounds as anticonvulsants. However, given that novel receptors have now been identified in astrocytes, it may become feasible to activate glial signaling pathways with novel drugs in order to induce the glia-dependent accumulation of adenosine in select regions of the brain, and either prevent epileptogenesis or inhibit seizures in the epileptic brain.

See also: **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Glial Modulation of Excitability via Glutamate and GABA Transporters; Peritumoral Epilepsy; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain.

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Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis

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Introduction

Epilepsy is among the most common neurological disorders, affecting about 1% of people, and is associated with significant morbidity and mortality. Since only a limited number of patients with epilepsy are candidates for surgical therapy, the majority of epilepsy patients are treated with antiepileptic drugs (AEDs), which are primarily designed to suppress seizures by directly controlling excitability of neurons. Unfortunately, seizure control is poor in about one-third of epileptic patients; seizures in these patients are refractory to currently available AEDs. To develop more effective therapies for epilepsy with potential novel mechanisms of action, it is important to better understand the basic cellular and molecular mechanisms underlying epileptogenesis.

Traditionally, glia have been viewed as playing secondary supportive roles in the brain, and have received relatively little attention in contributing to proposed mechanisms of epileptogenesis. However, in recent years, accumulating evidence has suggested that glial cells have more fundamental roles in modulating and participating in neuronal and synaptic physiology, and can directly influence epileptogenesis. Astrocytes, in particular, have been shown to be involved in the reuptake of synaptically-released neurotransmitters and in the regulation of extracellular ion concentrations. Functions that were previously thought to be exclusively carried out by neurons, such as the release of local chemical signals, have also been reported for glial cells ('gliotransmitters'). In addition, although glia cannot generate typical action potentials, they possess numerous neurotransmitter receptors and ion channels, and have their own mechanisms of expressing excitability in the form of calcium waves, which can spread between astrocytes to trigger the release of gliotransmitters. Given this previously underappreciated diversity of glial functions in controlling neuronal physiology and excitability, it is now critical to reexamine the potentially important roles that glia may play in the pathophysiology of epilepsy.

Tuberous sclerosis complex (TSC) is one of the most common genetic causes of epilepsy, affecting ~1 in 6000 individuals worldwide. TSC is an autosomal dominant disorder, which results from a germline mutation in either the *TSC1* or *TSC2* gene. As the protein products of these two genes, hamartin and tuberin, are regulators of cell growth and proliferation, TSC involves hamartoma or tumor growth in multiple organ systems. Neurological involvement, including epilepsy, mental retardation, and autism, usually represent the most significant health concerns affecting patients with TSC. Epilepsy occurs in at least 80% of TSC patients, and typically involves multiple seizure types, including infantile spasms, which are often refractory to currently available AEDs and surgical treatments. Pathologic examination of the brains from affected individuals frequently reveals the presence of dysplastic areas or hamartomas (tubers), which positively correlate with the severity of cerebral dysfunction and seizures and are often identified as sites of epileptic foci in TSC brains. Tubers consist of focal regions containing increased numbers of astrocytes as well as 'giant cells,' which exhibit features of both neurons and glia. The observation that patients with TSC develop nonneoplastic tubers with abnormal glial features, as well as neoplastic growths composed of glia (subependymal giant-cell astrocytomas, SEGAs), suggests that brain dysfunction in TSC – including epilepsy – might result in part from primary defects in astrocyte function. In this regard, an improved understanding of the role of astrocytes in epileptogenesis in TSC may be important for developing more effective treatments for epilepsy in TSC patients.

Background

A number of recent studies have begun to investigate potential glial-mediated mechanisms of epileptogenesis. Because glial abnormalities may not only promote epileptogenesis, but also may result from seizures ('astrogliosis'), it can be challenging to demonstrate a clear cause (versus effect) role for astrocytes in epilepsy. A number of approaches have been utilized for identifying pathophysiological alterations of glia that might be responsible for, or contribute to, the initiation of seizures. One approach has involved studying glia cells in human brain tissue resected from patients with epilepsy, including patients with TSC. However, information from human tissue studies is often limited, because they almost always involve patients with chronic intractable epilepsy who are taking multiple AEDs. As a result, it is difficult to determine whether the glial abnormalities observed in such studies are a primary cause of the epilepsy or are secondary to other factors, such as the long-standing effects of AED treatment or seizure activity itself.

Clinically-relevant small-animal models offer an alternative approach to more directly elucidate the role of glia in epileptogenesis in TSC. The Eker rat, which harbors a spontaneous heterozygous *Tsc2* gene mutation, was initially described almost 50 years ago and has probably been the most commonly-used animal model of TSC. However, while the Eker rat is an excellent model for other features of TSC, such as renal disease, the brain is only modestly affected. In this regard, Eker rats exhibit relatively mild neuropathological abnormalities and do not appear to develop spontaneous epilepsy, limiting their utility for studying mechanisms underlying epileptogenesis. Conventional knockout mice with homozygous *Tsc1* or *Tsc2* gene inactivation die in midembryogenesis, and mice heterozygous for *Tsc1* or *Tsc2* gene inactivation develop tumors in other organs, but exhibit minimal neurological phenotypes. Since conventional *Tsc* knockout mice have limited utility for studying epilepsy, we and others have developed conditional knockout mice, in which *Tsc* gene inactivation is targeted to specific cell types within the brain. Given the prominent glial abnormalities observed in brain specimens from TSC patients, we generated conditional knockout mice with inactivation of the *Tsc1* gene primarily in glia ($Tsc1^{GFAP}$CKO mice). These mice develop spontaneous recurrent seizures and represent a very attractive model for investigating the role of astrocytes in epileptogenesis in TSC.

Methods

$Tsc1^{flox/flox}$-GFAP-Cre ($Tsc1^{GFAP}$CKO) mice were generated in our laboratories using Cre-LoxP methodology. To inactivate *Tsc1* gene expression in glial fibrillary acidic

protein (GFAP)-positive cells (glia), we developed GFAP-Cre mice in which Cre-recombinase was expressed under the control of a human GFAP promoter. These GFAP-Cre mice were crossed with *Tsc1*$^{flox/flox}$ mice containing LoxP sites targeted to the *Tsc1* gene (**Figure 1(a)**). Characterization of these mice with LacZ transgene expression, Cre-recombinase staining, and Cre reporter mice indicates that Cre recombination first occurs around embryonic day

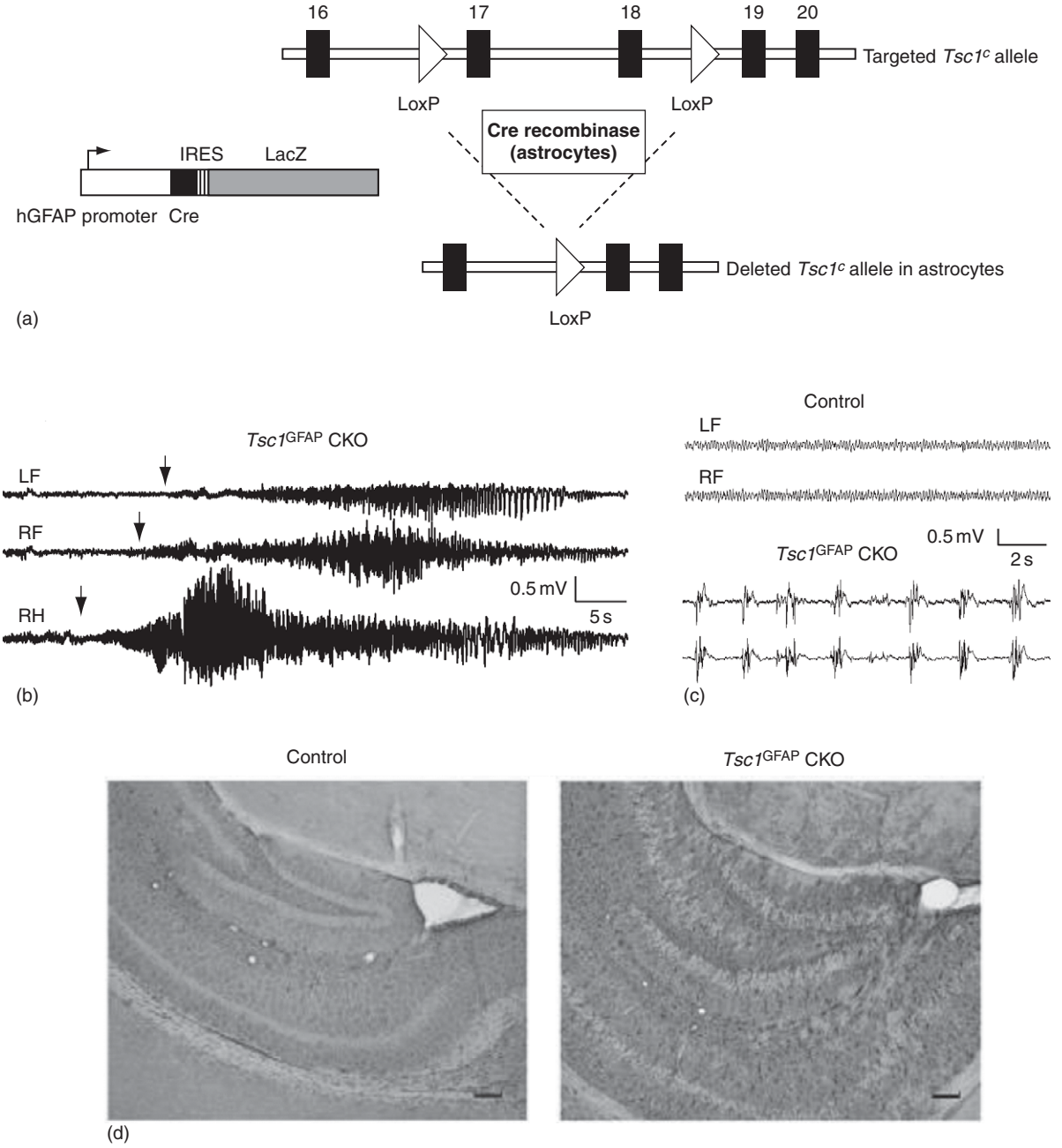


Figure 1 *Tsc1*^{GFAP}CKO mice exhibit progressive seizures and astrocyte proliferation. (a) Schematic diagram of the deletion of *Tsc1* exons 17 and 18 in glia mediated by the GFAP-Cre transgene. Arrowheads indicate the LoxP sequences. (b) Ictal EEG pattern of a typical seizure in *Tsc1*^{GFAP}CKO mice. Seizure onset began in the hippocampus before spreading to cortical surface electrodes. Arrows denote EEG seizure onset in each electrode. LF, left frontal; RF, right frontal; RH, right hippocampus. (c) Interictal EEG baseline in control and *Tsc1*^{GFAP}CKO mice. Control mice show a normal theta rhythm. *Tsc1*^{GFAP} CKO mouse typically develop a burst-suppression pattern with age. (d) Progressive astrocyte proliferation in *Tsc1*^{GFAP}CKO mice at 6 weeks of age compared with a control mouse, as assayed by GFAP staining. Magnification is the same for both images (scale bar = 200 μm). Adapted from Uhlmann EJ, Wong M, Baldwin, *et al*. (2002) Astrocyte-specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Annals of Neurology* 52: 285–296, with permission.

14.5 in GFAP + cells in the developing brain. *Tsc1*$^{flox/+}$-GFAP-Cre and *Tsc1*$^{flox/flox}$ mice have been found to have no abnormal phenotype and are used as controls for most experiments.

A range of electrophysiological recording methods have been used to characterize the seizures developing in *Tsc1*^{GFAP}CKO mice. Video-EEG monitoring has been used to record seizures as well as interictal EEG abnormalities. Epidural screw electrodes were surgically implanted under isoflurane anesthesia and secured with dental cement for long-term video-EEG recordings. EEG data were recorded with standard AC amplifiers and saved digitally, along with simultaneous video data. Extracellular and whole-cell recordings from hippocampal or cortical brain slices have been performed to characterize synaptic and neuronal physiology, as well as astrocyte physiology, using standard techniques. DIC-IR optics was used to assist with obtaining whole-cell patch-clamp recordings from directly identified neurons and astrocytes in slices. For more detailed pharmacological studies, patch-clamp recording have also been performed on cultured cells.

Tsc1^{GFAP}CKO mouse brains have also been used for histology and Western blot analysis. Histological studies have included traditional Nissl or hematoxylin and eosin staining as well as immunohistochemistry for a variety of molecular markers, such as cell death indicators (TUNEL, caspase-3, Fluoro-Jade), neurotransmitter receptors, transporters, and ion channels, and intracellular signaling molecules. Similarly, Western blot analysis for the expression of a variety of proteins has been performed using standard techniques.

Recent Results

The behavioral phenotype of *Tsc1*^{GFAP}CKO mice has been well characterized. From birth to at least one month, *Tsc1*^{GFAP}CKO mice are indistinguishable from control littermates in both appearance and behavior. However, after the first month, *Tsc1*^{GFAP}CKO mice become less active and tend to assume a retracted posture. The most prominent phenotypic feature of *Tsc1*^{GFAP}CKO mice is the development of epileptic seizures, which appear to start around 1 month of age. Seizures are initially rare, but become progressively more frequent with age, eventually leading to death at about 3 months. In developmental video-EEG studies, no seizures were recorded at 4 weeks of age in about two-thirds of mice, and the seizures were clinically subtle with a seizure frequency of 1.0 ± 0.6 seizures per day. By 9 weeks, all mice had clinically-obvious seizures and the average seizure frequency increased significantly to 9.5 ± 3.5 seizures per day. On video analysis, most seizures were characterized by a brief period of tonic stiffening of the trunk or extremities without loss of upright posture, followed by rearing and forelimb clonus, resembling limbic seizures of rodents. On EEG, most seizures appeared to have simultaneous onset in both hemispheres. However, some seizures that were recorded using hippocampal depth electrodes showed earlier onset in the hippocampus compared with the neocortex, indicating that some seizures may originate focally in the hippocampus (**Figure 1(b)**). Correlating with the increased seizure frequency as a function of age, the interictal EEG was normal at 4 weeks, but then developed progressive loss of normal background rhythms and increasing epileptiform spikes, culminating in a burst-suppression pattern by 9 weeks of age (**Figure 1(c)**). All *Tsc1*^{GFAP}CKO mice died, by 3–4 months of age, within the context of having frequent seizures. In addition to the obvious seizure phenotype, detailed behavioral testing at ~1 month of age also showed significant cognitive deficits in hippocampal-dependent learning tasks, such as water maze spatial learning and conditioned fear paradigms.

Consistent with the role of *Tsc1* as a negative growth regulator, histopathological examination of brains from *Tsc1*^{GFAP}CKO mice (using hematoxylin and eosin staining) revealed a striking, diffuse increase in size in all areas of the brain, which was first apparent at about 1 month of age and progressively increased with age. Immunohistochemical staining with GFAP showed a robust increase in astrocytes, which correlated with the increase in brain size (**Figure 1(d)**). By 6 weeks of age, the increase in GFAP-immunoreactive cells was approximately five-fold greater than control littermates. Immunofluorescence with a marker for proliferating cells was also increased in *Tsc1*^{GFAP}CKO brains, indicating the existence of increased astrocyte proliferation (as opposed to reactive astrocytosis). Intriguingly, development of increased astroglial proliferation roughly correlated with the onset of spontaneous seizures. No cortical tubers or gross abnormalities of neocortical lamination were observed. However, there was evidence of neuronal disorganization, particularly in hippocampus, where there was marked dispersion of pyramidal neurons in the stratum pyramidale, especially in CA3 and CA4.

Given the robust, progressive seizure phenotype and corresponding histological changes primarily in astrocytes in *Tsc1*^{GFAP}CKO mice, we next explored the potential mechanisms by which an astrocyte defect might predispose to neuronal hyperexcitability and seizures (**Figure 2**). As astrocytes play an important role in regulating extracellular glutamate homeostasis, we initially focused on the hypothesis that impaired glutamate transport by astrocytes may result in elevated extracellular glutamate concentrations, predisposing to neuronal hyperexcitability, excitotoxic cell death, and seizures. Expression of GLT-1 and GLAST, two astrocyte-specific

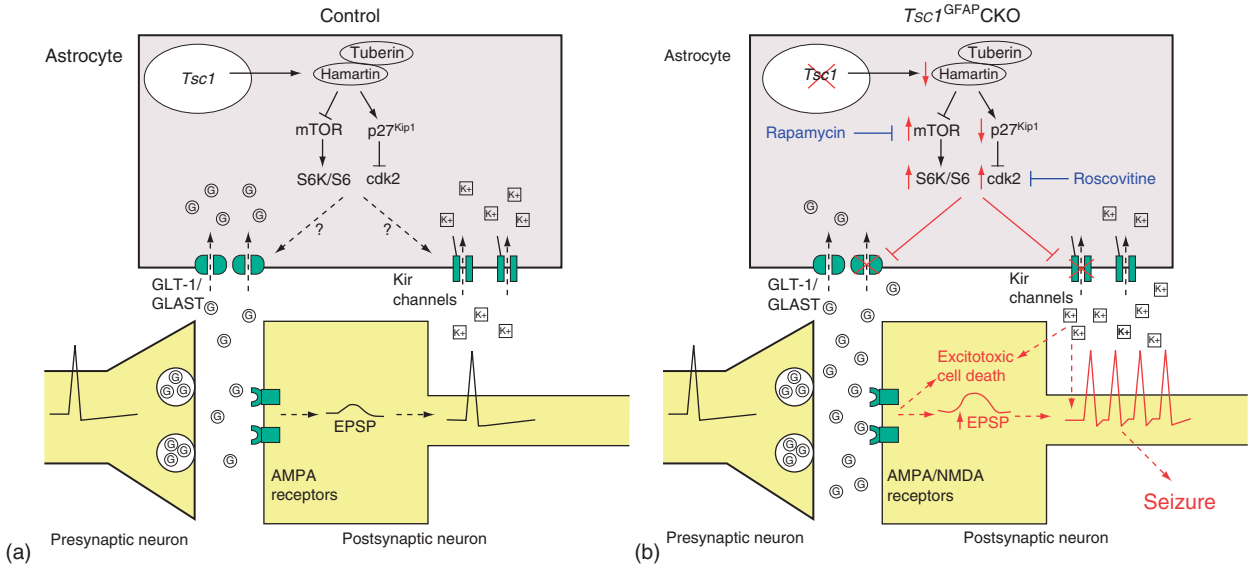


Figure 2 Putative mechanisms of epileptogenesis in *Tsc1*^{GFAP}CKO mice. (a) In control mice, astrocytes closely regulate extracellular glutamate and potassium homeostasis and prevent excessive excitation of neurons by these substances. Glutamate released by neurons during synaptic transmission is taken up by specific astrocyte glutamate transporters, GLT-1 and GLAST. Extracellular potassium that accumulates during neuronal activity is absorbed by astrocytes through potassium channels, such as inward-rectifying potassium (Kir) channels. The role of the *Tsc* genes in regulating these transporters and channels under normal conditions is unknown, but there is some evidence that hamartin and tuberin may have modulatory effects via mTOR/S6K or p27/cdk2 pathways. (b) In *Tsc1*^{GFAP}CKO mice, the expression and function of astrocyte glutamate transporters and Kir channels are reduced, leading to excessive accumulation of extracellular glutamate and potassium. The abnormal glutamate and potassium homeostasis may cause excessive excitation of neurons, as well as predispose to excitotoxic cell death, both of which may promote epileptogenesis and seizures. Preliminary evidence indicates that inactivation of the *Tsc1* gene in *Tsc1*^{GFAP}CKO mice may cause down-regulation of astrocyte glutamate transporters and potassium channels via hyperactivation of the mTOR/S6K and cdk2 pathways. The drugs, rapamycin and roscovitine, have potential therapeutic benefit in TSC by inhibiting the mTOR and cdk2 pathways, respectively. G, glutamate, $K+$, potassium ions.

glutamate transporters, was dramatically decreased in *Tsc1*^{GFAP}CKO mice, both in whole brains and in cultured astrocytes. GLT-1 expression was primarily reduced in the forebrain in *Tsc1*^{GFAP}CKO mice, whereas GLAST expression was decreased most markedly in cerebellum, its primary site of expression, and to a lesser degree in the neocortex. Whole-cell recordings from astrocytes in hippocampal slices and in culture from *Tsc1*^{GFAP}CKO mice also showed a functional decrease in glutamate transporter currents. Thus, *Tsc1*^{GFAP}CKO mice display impaired expression and function of astrocyte glutamate transporters, which could predispose to excessive extracellular glutamate levels. In support of this idea, recent in vivo microdialysis studies demonstrated elevated extracellular glutamate levels in *Tsc1*^{GFAP}CKO mice. Furthermore, the *Tsc1*^{GFAP}CKO mice also exhibit abnormal neuronal death in the neocortex and hippocampus, which likely results from elevated glutamate levels. Importantly, these abnormalities in glutamate homeostasis were observed in mice less than 1 month of age, which precedes seizure onset. Thus, defective glial regulation of extracellular glutamate could represent a primary mechanism promoting epileptogenesis in *Tsc1*^{GFAP}CKO mice.

In addition to regulation of glutamate homeostasis, a related hypothesis for a glial-mediated mechanism of epileptogenesis involves control of extracellular potassium by astrocytes. Astrocytes contain specific potassium channels and pumps that prevent the excessive accumulation of potassium in the extracellular space. Abnormal potassium handling by astrocytes could lead to neuronal hyperexcitability and seizures. In astrocytes, inward-rectifying potassium (Kir) channels are particularly efficient at removing extracellular potassium. Similar to the reduction of glutamate transporters, we found that Kir currents were significantly reduced in both cultured astrocytes and hippocampal slices from *Tsc1*^{GFAP}CKO mice. In addition, expression of the Kir channel protein subunits Kir2.1 and Kir6.1, but not Kir4.1, was decreased in *Tsc1*^{GFAP}CKO astrocytes. This impaired Kir channel function likely predisposes to increased neuronal excitability, since exposing hippocampal slices to a high potassium-containing solution induced epileptiform discharges more readily in tissue from *Tsc1*^{GFAP}CKO mice than in slices from control mice.

While abnormalities in astrocytic neurotransmitter transporters and ion channels may contribute to

epileptogenesis in *Tsc1*^{GFAP}CKO mice, the molecular pathways by which *Tsc1* gene inactivation leads to alterations in these transporters and channels is unknown. Previous studies from numerous laboratories have demonstrated that the *TSC* gene products, tuberin and hamartin, are involved in regulating intracellular signaling pathways that control cell growth and proliferation by activating the mammalian target of rapamycin (mTOR)/S6 kinase (S6K) pathway. Furthermore, *Tsc*-deficient cells have decreased expression of the cyclin-dependent protein kinase (CDK) inhibitor, p27^{kip1}. We have confirmed that similar abnormalities occur in these signaling pathways in *Tsc1*^{GFAP}CKO mice. In cultured astrocytes derived from *Tsc1*^{GFAP}CKO mice, the expression of p27^{kip1} is significantly decreased compared with the expression in astrocytes from control littermates. In addition, *Tsc1*^{GFAP} CKO astrocytes demonstrate constitutive hyperactivation of the mTOR/S6K pathway, as reflected by an increase in phospho-S6K expression.

The relationship of these intracellular signaling pathway alterations to the abnormalities observed in glutamate and potassium homeostasis and neuronal excitability is only beginning to be elucidated. In recent work, we found that the decreased Kir currents in *Tsc1*^{GFAP}CKO astrocytes were reversed by roscovitine, a CDK2 inhibitor, and by retinoic acid, which increases p27^{kip1} levels, suggesting that the decreased p27^{kip1} expression in *Tsc1*^{GFAP}CKO astrocytes may mediate the effects on potassium channels. Recent studies indicate that rapamycin, an inhibitor of the mTOR pathway, can block the hyperactivation of the S6K/S6 pathway and the decreased glutamate transporter expression observed in *Tsc1*^{GFAP}CKO mice in vivo, as well as prevent the development of seizures. These results demonstrate the importance of the mTOR pathway in mediating the neurological phenotype in *Tsc1*^{GFAP}CKO mice, and have exciting potential therapeutic implications for neurological symptoms in human TSC.

Future Goals

As summarized in **Figure 2**, the work accomplished to date strongly implicates a number of mechanisms in astrocytes that may cause or contribute to epileptogenesis in *Tsc1*^{GFAP}CKO mice, as well as identifies potential novel therapeutic targets for treating epilepsy in TSC. In particular, defects in astrocyte regulation of glutamate and potassium homeostasis represent logical mechanisms for promoting neuronal excitability, neurotoxicity, and seizures in these mice. However, more work is required to definitively establish whether these constitute primary, causative mechanisms of epileptogenesis or simply represent secondary effects. The use of detailed pharmacological or genetic techniques to reverse the observed cellular and molecular abnormalities may provide stronger evidence for a primary etiologic role of these defects in the neurological phenotypes of *Tsc1*^{GFAP}CKO mice. A number of other mechanisms involving astrocytes or glial–neuronal interactions might also be critical for epileptogenesis but are yet to be explored in this model. Among these still unexplored issues are the role of different glial cell subtypes, gliotransmitters, calcium waves in astrocytes, spatial buffering properties of glia networks via gap junctions, and glial–vascular coupling affecting blood–brain barrier function.

The various cellular and molecular abnormalities identified in the *Tsc1*^{GFAP}CKO mice have tremendous potential for identifying novel, rational therapeutic strategies for treating the neurological manifestations of TSC. As mentioned earlier, recent data in the *Tsc1*^{GFAP}CKO mice suggest that inhibitors of the mTOR pathway might represent promising treatments for seizures in TSC. Clinical trials of rapamycin have already been initiated for kidney tumors, as well as astrocytomas in TSC, but the effect of rapamycin on epilepsy in humans has not yet been explored. In addition, drugs already in clinical development for other diseases that modulate the CDK-p27^{kip1} pathway, such as roscovitine, have not been considered as therapies for TSC. Finally, relevant to the abnormalities in glutamate transporters in the *Tsc1*^{GFAP}CKO mice, a comprehensive screen of over a thousand FDA-approved drugs has identified several drugs that specifically increase the expression of the astrocyte glutamate transporter, GLT-1. Among these drugs, the commonly-used antibiotic, ceftriaxone, showed a dramatic effect in elevating GLT-1 expression, and prolonging the survival of a mouse model of amyotrophic lateral sclerosis. Future studies in the *Tsc1*^{GFAP}CKO mice may provide evidence for the efficacy of other mTOR inhibitors, roscovitine and ceftriaxone for treating TSC-related epilepsy, and would support their testing in clinical trials for epilepsy in TSC patients.

Acknowledgements

This work was supported by the National Institutes of Health (K02NS045583 and R01NS056872, MW), Department of Defense (DAMD-17-03-1-0073, DHG) and the Tuberous Sclerosis Alliance (MW).

See also: **Glia/Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters; Peritumoral Epilepsy; Properties of Glia in Epileptic Brain; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Plasticity**: Seizure-Related Changes in Dendritic Spine Structure.

Further Reading

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Glial Modulation of Excitability via Glutamate and GABA Transporters

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Introduction

Fine tuning of glutamatergic excitatory and GABAergic inhibitory neurotransmission is a prerequisite for optimal, normal functioning of the central nervous system (CNS). Termination of the transmission processes mediated by glutamate and GABA is controlled by a number of high-affinity transporters for these amino acids, transporters that are expressed in both neurons and astrocytes. The latter cell type has a major role in this fine-tuning process. Any disturbance in glial transporter function will lead to an imbalance in the equilibrium between excitation and inhibition, leading to seizure activity and epileptic episodes.

Disinhibition, resulting from altered GABAergic neurotransmission, has been associated with a number of neurological disorders, including epilepsy, migraine headaches, chronic pain, anxiety, and sleep disorders. Abnormal excitatory neurotransmission, resulting from excessive synaptic glutamate levels, has been associated with a number of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, Huntington's disease, Parkinson's disease, and neuronal damage associated with cerebral ischemia. In addition to a role in these and other neurodegenerative disorders, alterations in glutamate transporters also contribute to the development of seizures and epilepsy. Thus, efforts to regulate the function and trafficking of GABA and glutamate transporters will likely have important implications for the treatment of a number of these neurological disorders and diseases. Ongoing efforts to gain a greater understanding of the factors that control their cellular expression, function, and regulation at the cellular and network level are critical, and will play important roles in the development of a new class of therapeutics for a number of devastating neurological conditions. Indeed, a number of antiepileptic drugs exert their action by modulating these transport processes.

Background

Characteristics and Nomenclature of Glutamate and GABA Transporters

Cloning strategies have led to the identification of five high-affinity glutamate transporters, the operation of which depends on an intact membrane potential as well as a transmembrane sodium gradient. The prevalent cellular localization of the individual transporters is summarized

agents that target these transporters, and the willingness of investigators, drug companies, and funding agencies to invest in the clinical trials that will ultimately determine the utility of this approach for the treatment of epilepsy and other neurological disorders.

See also: **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Peritumoral Epilepsy; Properties of Glia in Epileptic Brain; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Transporters**: Glutamate Transporters in Epilepsy.

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Peritumoral Epilepsy

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Introduction

Each year ~100 000 patients are newly diagnosed with tumors in the central nervous system (CNS) in the United States. According to data from the Central Brain Tumor Registry of the United States (CBTRUS), ~18 500 of these are considered primary brain tumors, i.e., arising initially in the brain; interestingly, the vast majority are of glial origin. These glial-derived tumors are collectively called gliomas and include oligodendrogliomas, astrocytomas, and glioblastomas. These tumors share a number of genetic alterations with other cancers, but also differ in some important aspects. These differences include, for example, their inability to spread via hematogenous routes and their physical containment within the bony cavity of the brain. Biological adaptations that allow gliomas to develop with these constraints may, in part, contribute to the high likelihood with which these cancers produce seizures in patients. Although most brain tumors, including metastatic tumors, can produce seizures, the causal relationship between tumor biology and peritumoral seizures is best understood in glial-derived tumors, which are the principle topic of this article.

Background

Gliomas and Epilepsy

Gliomas arise de novo within the CNS. Although they are generally believed to derive from glial cells or their progenitor cells, their etiology is still in question. Some gliomas are considered benign and can be treated, but the majority of gliomas form aggressive and highly malignant tumors that are largely unresponsive to treatment. Surgical resection is typically unsuccessful as tumor cells tend to invade the normal brain parenchyema diffusely and create satellite tumors. Upon resection of the main tumor, regrowth typically occurs within just a few months. Unfortunately, chemotherapy provides only limited benefit to patients, in part because the blood–brain barrier makes effective delivery difficult and in part also because gliomas tend to be quite resistant to commonly-used chemotherapeutic drugs. Consequently, the outlook for patients with malignant gliomas remains dismal, with average patient survival of less than one year even with all currently available treatments.

In 30–50% of patients, the diagnosis of a primary brain tumor is preceded by epileptic seizures. This percentage

is even greater in low-grade astrocytomas, where close to 75% of patients present with seizures. Interestingly, it is the otherwise benign, low-grade pilocytic astrocytomas that are particularly prone to cause peritumoral seizures, and quite frequently the seizures necessitate surgery more than the growth of the tissue per se. This situation is particularly the case in children, where astrocytomas constitute the leading solid cancer yet often carrying a relatively good prognosis. In many patients, the seizures generated by the tumor are largely refractory to normal antiepileptic medication, most likely because the biology of the underlying seizures is different from other forms of epilepsy.

Although the co-appearance of seizures and brain tumors has been recognized for some time, little effort has been expended to understand the biological interdependence of these phenomena. Indeed, it had been a common assumption that the growing tumor compresses the surrounding brain tissue and that seizures result primarily from mechanical effects on neighboring tissue. This notion has been challenged by several recent studies that examined the effect of gliomas on the peritumoral brain more directly. These studies provide a more logical framework to explain peritumoral seizures, and offer new opportunities to treat the disease and the associated seizures.

Seizures and Glutamate

As extensively discussed in other articles in this encyclopedia and in the published literature, seizures result from an imbalance of excitatory and inhibitory neurotransmission in brain. It is commonly accepted that an increase in excitatory neurotransmission is sufficient to drive seizure initiation, and experimental application of glutamate or other excitatory amino-acid receptor agonists can produce seizures. Glutamate is the principle excitatory neurotransmitter in the brain and its binding to postsynaptic neuronal glutamate receptors mediates fast signal transmission. Its excitatory activity is typically opposed by GABAergic inhibition. However, in various disease conditions this excitation–inhibition balance fails. Most notably, under conditions of energy failure, glutamate release can increase substantially, thereby causing a sustained activation of glutamate receptors. Importantly, under such conditions, the otherwise controlled and modest increases in intracellular [Ca^{2+}] through NMDA receptors become more sustained and massive, thus overwhelming intracellular Ca^{2+}-regulatory processes, ultimately leading to Ca^{2+}-mediated cell death. This process, termed 'excitotoxicity' many years ago, is now believed to be a final common death pathway in numerous diseases ranging from acute trauma and ischemic stroke to progressive neurological conditions such as amyotropic lateral sclerosis (ALS) or Alzheimer's disease.

Because of the vulnerability of the brain to excessive glutamate, several Na^{+}-dependent transport systems for glutamate have evolved that maintain extracellular glutamate at very low micromolar levels. These are primarily expressed on nonmalignant astrocytes. The neuroprotective role that these astrocytic transporters play are evident from studies in knockout mice, showing neurodegeneration and lethal epilepsy in animals that have lost functional astrocytic glutamate transporters. Interestingly, these 'neuroprotective' astrocytic glutamate transporters are not at all functional in astrocytic tumors, which therefore cannot participate in glutamate homeostasis. Much to the converse, these tumor cells actually release glutamate into the peritumoral space, contributing to excitotoxic death of peritumoral neurons, probably allowing the tumor to expand into the vacated space (**Figure 1**).

Methods

Glutamate and cystine transport uptake and assays: To study the transport of amino acids such as glutamate and cystine, we used routine radiotracer studies in which cells are pretreated with a defined ratio or concentration of radiolabeled amino acids (i.e., ^{35}S-cystine and ^{3}H-glutamate or ^{3}H-aspartate), which are also transported by the excitatory amino-acid transporter (EAAT) family of glutamate transporters. The rate of release or uptake can then be determined after defined time periods. Experiments performed in the presence or absence of extracellular Na^{+} allow us to isolate the Na^{+}-dependent component of glutamate release. Furthermore, sister cultures can be treated with defined inhibitors such as S-4CPG or sulfasalazine. We also used a commercial bioluminescence assay kit to measure glutamate release from gliomas, and two fluorescent assay kits to determine glutathione (GSH) production in cell culture; these approaches allow us to quantitatively determine GSH levels (over several days) in the presence and absence of system x_c^- inhibitors.

To examine the effects of system x_c^- inhibitors on tumor growth in vivo, 0.5-mil human glioma cells were concentrated and stereotactically injected into the cranium of anesthetized, severe immune-compromised mice. Following a 30-day period of growth, animals were sacrificed and tumor volumes determined by quantitative histological examination, allowing a direct comparison of treated to untreated animals.

To better understand the mechanism of glioma invasion and the potential contribution of system x_c^--mediated glutamate release, we used Transwell migration assays in which cells are challenged to transverse a barrier membrane with 8-μm pores. The bottom of the filters are coated with the chemoattractant vitronectin.

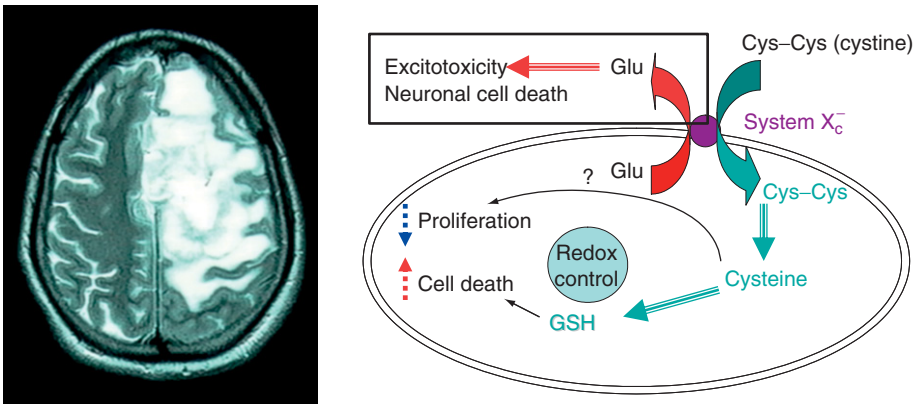


Figure 1 Gliomas destroy normal brain as they grow: Glial-derived tumors are limited in their physical growth space by the cranium. To grow, they must destroy normal brain tissue to vacate room for their own growth. They do so by releasing glutamate, which occurs as an obligatory byproduct of cystine uptake through system x_c^-. The latter serves as precursor for the synthesis of the antioxidant glutathione (GSH). System x_c^- is the primary transport system for the import of cystine into glioma cells, and hence tumor growth is dependent on the continued supply of cystine via this pathway. The released glutamate triggers peritumoral seizures and causes excitotoxic neuronal cell death in peritumoral regions. The illustrated brain is a MRI scan from a patient who suffered a high-grade malignant glioma occupying a large part of the brain.

To study Ca^{2+} signaling in migratory cells, we used standard Fura-2 fluorescence imaging, which allows the investigator to monitor spontaneous or induced changes in intracellular calcium after loading cells with Fura-2 AM dye.

Recent Results

Glutamate Release from Tumor Cells

As illustrated in Figure 2(a), even in culture, glioma cells release up to 500 μM of glutamate over a 12-h period. In contrast, nonmalignant astrocytes remove glutamate from the medium rather than secrete it. Astrocytes use the GLT-1 glutamate transporter for the uptake of glutamate, which, as further discussed below, is not expressed in gliomas (Figure 2(b)). When hippocampal neurons are placed in the vicinity of glioma cells, neurons undergo NMDA receptor-mediated excitotoxic cell death (Figure 2(c)). Moreover, when rat C6 glioma cells are implanted into the brains of host animals, tumors only grow when the implanted C6 cells are capable of releasing glutamate – but not in cell lines deficient of glutamate release. Hence, glioma growth in situ and in vivo appears to require the release of glutamate from the tumor. In all likelihood, the released glutamate is used to kill peritumoral brain tissue in order to vacate room for the tumor expansion. Of note, gliomas are the only cancers that grow in a confined space and hence are physically limited in their ability to grow. The release of glutamate and its excitotoxic action on surrounding brain tissue represent a biological adaptation that may be uniquely suited to the needs of these CNS tumors.

Of note, several studies have now independently demonstrated that glutamate is elevated in the vicinity of tumors in both animal models and in patients suffering from glioblastoma or oligodendrogliomas. This principal finding that glioma tumors release glutamate in vivo is firmly supported by experimental data. These findings also suggest that glutamate released from gliomas would overactivate peritumoral neurons through a continued release of glutamate. This glutamate-mediated excitation, of course, explains the initiation and localization of seizures to peritumoral regions of the brain. However, one would expect that a need to vacate brain tissue through an excitotoxic, possibly seizure-initiating, pathway would be most prominent in very advanced stages of the disease. To the contrary, it is the lower grade tumors that are more likely to present with seizures.

Glutamate Transporters in Gliomas

A natural next step in examining the glioma glutamate release was a search for the release pathway, as this pathway could be therapeutically important. An obvious candidate was one of the five members of the EAAT family, two of which – EAAT1 and EAAT2 – are highly expressed in nonmalignant astrocytes. These transporters typically take up glutamate using the inwardly-directed transmembrane Na^+-gradient, but have been shown to reverse direction under conditions of energy failure and have been suggested to contribute to glutamate release and excitotoxicity in ischemia. Quite surprisingly, however, functional EAAT transporters are absent in gliomas (Figure 2(b)); as a consequence, Na^+-dependent glutamate transport is negligible. This result illustrated in Figure 3, which shows Western blots from patient tissue sections probed for the principal glial glutamate transporter, EAAT2 – prominent in nonmalignant brain but absent in all gliomas (Figure 3(a)). In these cancers,

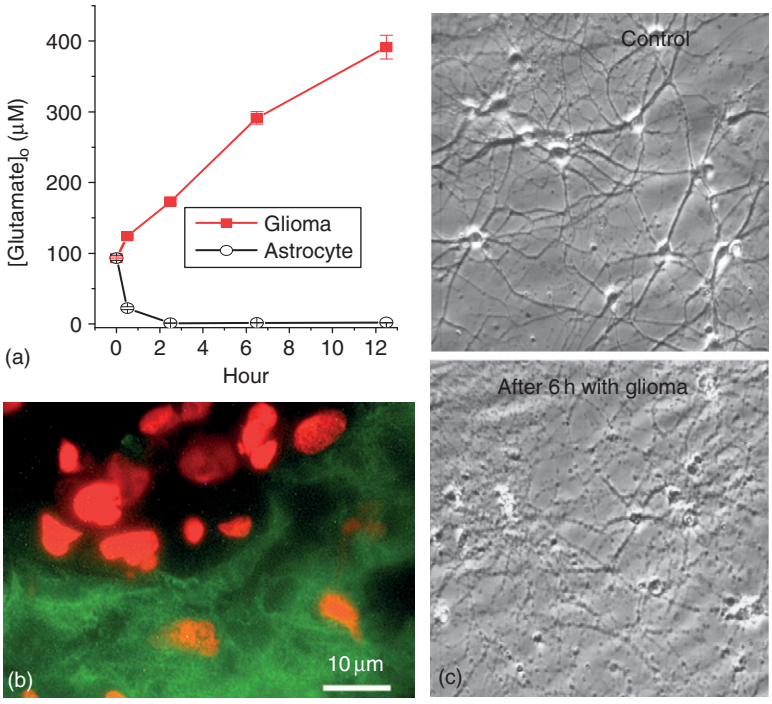


Figure 2 Gliomas release Glutamate. (a) Glioma cells release glutamate as determined by sampling medium in a 70-cc flask measuring bioluminescence as a function of time. By contrast, nonmalignant astrocytes remove glutamate when challenged with 100 mM at the start. (b) Gliomas in vivo do not express the GLT-1 glutamate transporter (green), while the surrounding astrocytes do as shown by immunocytochemistry. The GLT-1 staining demarcates the tumor boundaries. Red staining is propridium iodine to label cell nuclei. (c) Time-lapse video microscopy shows pronounced neuronal cell death when hippocampal neurons are placed in the vicinity of glioma cells yet without touching them (sandwich culture). Reproduced from Ye ZC, Rothstein JD, and Sontheimer H (1999) Compromised glutamate transport in human glioma cells: Reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine–glutamate exchange. *Journal of Neuroscience* 19: 10767–10777.

glutamate release was dependent on the presence of extracellular cystine and was competitively inhibited by extracellular glutamate. These characteristics are signature features of another transport system for glutamate called 'system x_c^-.' This abundant but relatively poorly investigated electroneutral amino-acid transporter transports cystine into the cell in exchange for glutamate release. System x_c^- is a heterodimeric protein complex consisting of a catalytic light chain (xCT) that confers substrate specificity and a regulatory heavy chain (4F2hc). The latter is a cell-surface glycoprotein (previously known as CD98) that is essential for membrane localization of the transporter; only the heteromultimeric protein complex functions as an amino-acid transporter. The light chain that confers the transport of cystine has recently been cloned and shown to belong to the 12-transmembrane domain amino-acid transporter proteins. The catalytic subunit can exist in two splice variants (hxCTa and hxCTb), both of which are up-regulated following oxidative stress. As shown in **Figure 3(b and c)**, both regulatory and catalytic subunits of this transporter are highly expressed in human glioma cell lines and glioma biopsies from patients.

In most cell types, system x_c^- serves as one of multiple pathways through which cells satisfy their demand for intracellular cysteine (the reduced form of cystine), which is required for the biosynthesis of many proteins and the intracellular reducing agent GSH (discussed in more detail below). System x_c^- accounts for the major route of cystine uptake into immature cortical neurons but not mature neurons or astrocytes, and appears to play a role in cystine transport in early oligodendrocyte precursor cells. Neurons and glial cells employ at least two other pathways to meet their cysteine needs, namely the γ-glutamyl cycle and the EAAT family of Na^+-dependent transporters that can also transport cysteine. It has recently been demonstrated that over 85% of the neuronal demand for cysteine in cultured neurons is mediated by the Na^+-dependent uptake of cysteine via the EAAT-2 and EAAT-3 transporters, with little contribution of system x_c^- in these neurons. Gliomas, in contrast, rely almost exclusively on system x_c^- activity for this process.

System x_c Function in Gliomas

The main function of the cystine–glutamate exchanger (system x_c^-) is to supply cells with the essential amino acid cystine, which is an important biosynthetic precursor molecule. Once taken up, cystine is readily reduced to cysteine, one of the principle building blocks for the ubiquitous tri-peptide GSH (L-γ-glutamyl-L-cysteinylglycine), which

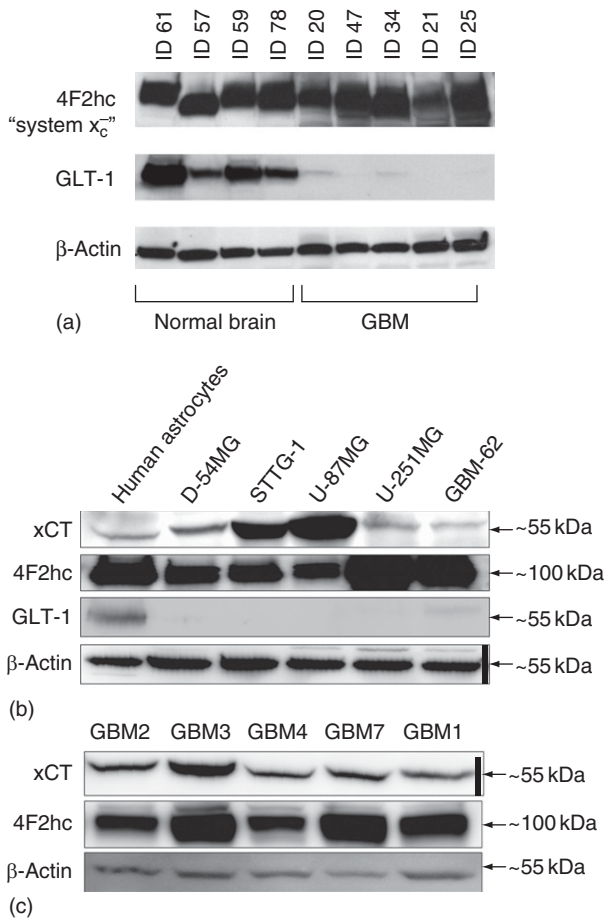


Figure 3 Glutamate transporters in gliomas. (a) The GLT-1 or EAAT2 Na^+-dependent glutamate transporter is highly expressed in normal brain but absent in all glioma biopsies examined by Western blot expression. Five representative glioma biopsies and four nonmalignant brain biopsies are shown. (b) Five glioma cell lines and five acute glioma biopsies (c) highly express the catalytic (xCT) and regulatory (4F2hc) subunit of the system x_c^- cystine–glutamate exchanger. (a): Reproduced from Chung WJ, Lyons SA, Nelson GM, *et al*. (2005) Inhibition of cystine uptake disrupts the growth of primary brain tumors. *Journal of Neuroscience* 25: 7101–7110. (b): Reproduced from Lyons SA, Chung WJ, Weaver AK, *et al*. (2007) Autocine glutamate signaling promotes glioma cell invasion. *Cancer Research* 67: 9463–9471.

is formed from glutamate, cysteine, and glycine. GSH is present in almost all tissues and serves as an intracellular thiol-disulphide redox system. It scavenges peroxides and prevents oxidation of –SH groups by reactive oxygen species (ROS).

GSH has been called the guardian of the cells' redox status, as its primary role is to defend cells against endogenously produced ROS. These ROS include hydroxyl radicals ($^{\cdot}OH$), superoxide anion (O_2^-), nitric oxide (NO), and hydrogen peroxide (H_2O_2). Some of these ROS are inevitable and constant by-products of oxidative metabolism and mitochondrial function. ROS species are all short lived and highly reactive. They can cause lipid peroxidation as well as oxidation of DNA and proteins, and hence can cause significant cell damage if uncontrolled. Under normal conditions, this damage is prevented by the activity of GSH peroxidase, an enzyme that creates a disulphur bond between two GSH molecules to form oxidized GSH (GSSG). ROS production is notably enhanced in rapidly growing cancer cells, including gliomas, and it is thought that these cancer cells use GSH to scavenge ROS. To meet the need for continuous GSH biosynthesis, gliomas require significant cellular uptake of cystine or cysteine, the latter being rate limiting for the production of GSH.

The resistance of certain cancers, including gliomas, to chemo- and radiation therapy has also been linked to the cells' GSH status. First, members of the GSH-S-transferase (GST) superfamily of enzymes catalyze the conjugation of GSH with numerous compounds often referred to as xenobiotics. Such xenobiotics include chemotherapeutic drugs and environmental toxins. These GSH conjugates are converted to mercapturic acids for excretion. A second role for GSH in this context pertains to the inactivation of ROS species produced specifically by ionizing radiation. Hydroxyl free radicals ($^{\cdot}OH$) are a prominent product of ionizing radiation and have been shown to be responsible to a large degree for the DNA degradation and protein peroxidation observed in cells exposed to radiation. Again, GSH serves to protect cells from hydroxyl radicals under such conditions; indeed, cells deficient in GSH show enhanced sensitivity to attack by such ROS. Hence, the vulnerability of gliomas and other cancers to both chemo- and radiation therapy can be enhanced by depriving cells of GSH, which, in gliomas, appears possible through the inhibition of system x_c^- function (as further discussed below).

It should be noted that system x_c^--mediated glutamate release has recently also been discussed as a possible contributor to extracellular glutamate in normal brain function. However, in situ hybridization and PCR studies suggest that, in the normal brain, system x_c^- is primarily expressed in the meninges and periventricular organs, with only very low expression levels in neurons and glia throughout the brain. Nevertheless, owing to its regulation by oxidative stress, system x_c^- expression may become a factor under certain disease conditions. Indeed, CNS inflammation, virally-induced encephalopathy, and periventricular leukomalacia may invoke the release of glutamate through system x_c^-. In addition, the very small basal levels of nonsynaptic glutamate may be regulated by system x_c^-. In most normal brain cells – including nonmalignant glial cells – any system x_c^--mediated glutamate that is released will be immediately taken back into the cells via the Na^+-dependent EAAT family of transporters. However, one of the major differences between gliomas and nonmalignant brain cells is the loss of functional EAAT glutamate transporters from the membrane. Combined with up-regulation of system x_c^-, this loss leads to a situation where cystine uptake causes the net

release of significant amounts of glutamate (as demonstrated in vitro and in vivo). In other words, glutamate release from gliomas appears to be an obligatory byproduct of cystine uptake, as illustrated in the schema in **Figure 1**. Hence the resulting over-activation and seizure activity of peritumoral neurons appears to also be a byproduct of a unique biology of these tumors. This condition would predict that inhibition of glutamate release from gliomas via system x_c^- would be highly beneficial. On the one hand, it should limit GSH synthesis by gliomas, and on the other hand it should stifle glutamate excitotoxicity and seizures. Both results would be expected to curtail the growth of these cancers.

Preclinical Studies Using Pharmacological Inhibition of System x_c to Inhibit Glutamate Release

The activity of system x_c^- can be inhibited by a number of related drugs that also affect metabotropic glutamate receptors. Of these, S-4-carboxyphenylglycine (S-4-CPG) has proven to have the greatest affinity, and in culture models, causes almost complete inhibition at ~100 μM. Their secondary action on mGLUR receptors, however, limits their use in animal studies. This difficulty was overcome with the discovery that sulfasalazine, a drug that is clinically used to treat Crohn's disease, also effectively inhibits system x_c^- activity. As shown in **Figure 4**, sulfasalazine shows a dose-dependent inhibition of cystine uptake and causes a near complete depletion of cellular GSH within 24 h. As a consequence, cells are compromised in their redox status and show growth arrest. Indeed, over prolonged incubation periods, cells undergo caspase-mediated apoptosis. When given to tumor-bearing animals by intraperitoneal injection, sulfasalazine was able to reduce tumor growth by 60–80% (**Figure 4**). To what extent these effects were due to cystine and or GSH depletion – versus a reduction of glutamate release and hence, reduced excitotoxicity – remains to be shown. However, these findings suggest that sulfasalazine may be a useful clinical drug to suppress system x_c^- function in patients with gliomas, a drug that may provide benefits with regards to direct tumor containment and may be of equal utility in inhibiting the generation of peritumoral

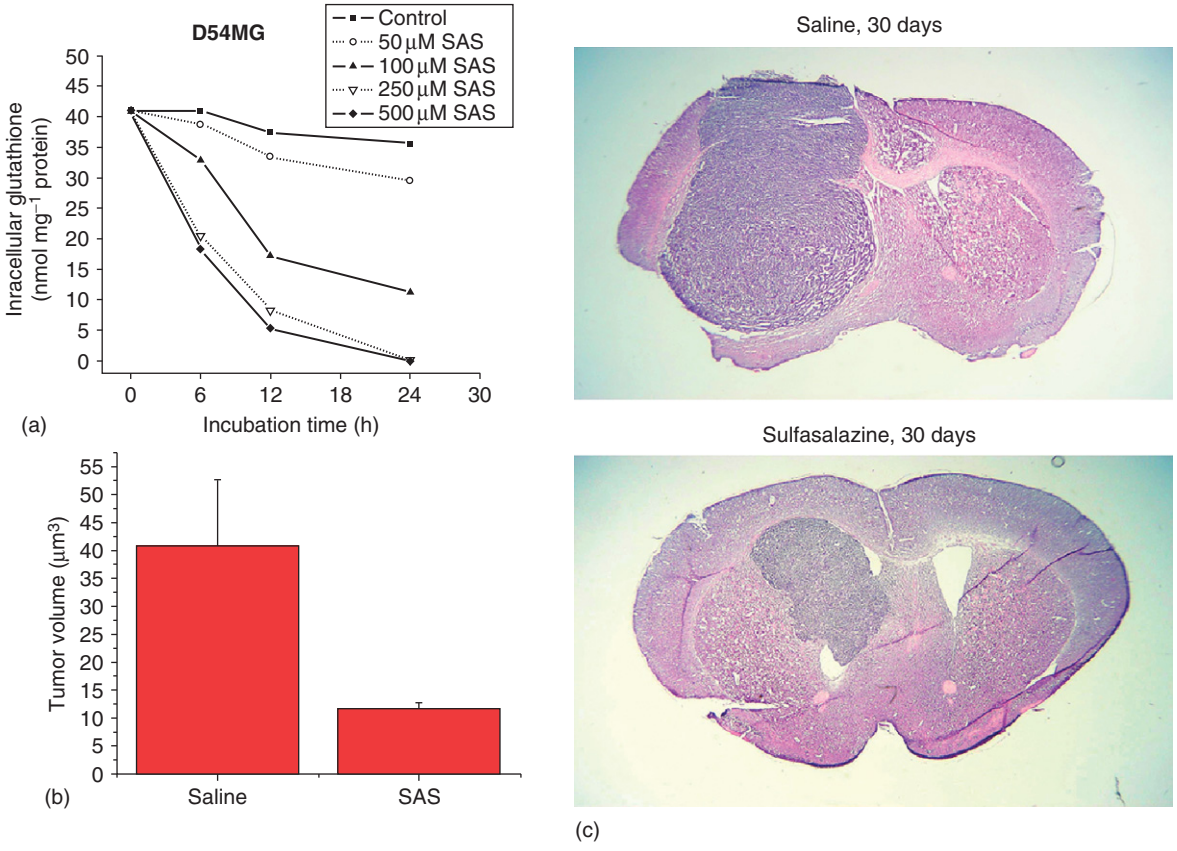


Figure 4 Inhibition of system x_c^- causes glutathione (GSH) depletion and reduces tumor size in vivo. (a) Sulfasalazine, which inhibits system x_c^-, causes a dose- and time-dependent reduction in GSH levels. (b) Intraperitoneal injection, twice daily, of 8 mg kg^{-1} sulfasalazine causes a 75% reduction in tumor volume in tumor-bearing animals. (c) A representative comparison of control saline-injected with sulfasalazine-treated animals at 30 days of treatment is shown in these brain cross sections. Reproduced from Chung WJ, Lyons SA, Nelson GM, *et al*. (2005) Inhibition of cystine uptake disrupts the growth of primary brain tumors. *Journal of Neuroscience* 25: 7101–7110.

seizures. Importantly, sulfasalazine can be safely given to children and may control seizures associated with low-grade gliomas that do not require surgical resection. Its clinical use as adjuvant treatment also deserves further consideration. The suppression of GSH observed in cell culture and animal studies suggests that gliomas would become more susceptible to radiation damage. Hence, sulfasalazine could be given in conjunction with exogenous beam radiation, which is the 'standard of care' treatment for patients with gliomas.

Glutamate and Migration or Dispersal of Glioma Cells

As we conducted animal studies with sulfasalazine, and as we examined the release and action of glutamate on glioma cells further, we made the unexpected observation that glutamate serves an important secondary role in the biology of gliomas – namely, aiding the invasive migration and dispersal of glioma cells. Similar to granule cells, glioma cells show oscillatory changes in intracellular Ca^{2+} as they migrate. In neurons, these oscillations are primarily triggered by glutamate activation of NMDA receptors. Glioma cells do not express NMDA receptors, but instead express Ca^{2+}-permeable AMPA receptors – i.e., receptors that lack the Glu-R2 subunit. Their activation appears to mediate intracellular Ca^{2+} fluctuations that are necessary for glioma-cell migration. This dependence of cell migration on glutamate and Ca^{2+} signaling is reminiscent of migrating cerebellar granule cells. However, the source of glutamate in this context turned out to be system x_c^-, releasing glutamate either onto the same or neighboring cells, hence acting as an autocrine or paracrine signal. Inhibition of either the Ca^{2+}-permeable AMPA receptor or system x_c^- caused the inhibition of cell migration and disrupted dispersal of tumors. In animal studies, tumors remained primarily as solitary growth centers rather than multiple dispersed tumors.

Summary and Challenges for the Future

Although the earlier-described data suggest a mechanism for seizure initiation, further animal studies are needed to determine that glutamate release from tumors are indeed causal of seizures. Therefore, tumor-bearing mice should be examined by imaging tumor volumes along with seizure monitoring (using EEG or related methodologies). It would be important to determine whether tumor size or location correlates with the severity of seizures. Furthermore, the therapeutic benefit of sulfasalazine should be investigated in these animal models. These experiments are in progress.

Taken together, these data suggest that glioma cells have co-opted the neurotransmitter glutamate as a means to kill peritumoral tissue to vacate room for their growth, as well as using the transmitter as a signal to aid the dispersal of tumor cells into normal brain. The collateral damage inflicted by the released glutamate is the over-excitation of neurons in the vicinity of the tumor, and this excitation may present as peritumoral seizures in many patients. The extent to which this glutamate release causes seizures depends on many factors. Tumor location appears to be a very important variable, as seizures are much more frequent in supratentorial tumors than infratentorial and also more prominent in temporal and parietal cortical locations than in occipital areas. Since glutamate is intrinsically linked to GSH production and hence redox status of the cell, it may not be surprising that those cancers with higher aerobic metabolically activity, and hence greatest GSH needs, would release the most glutamate. This factor indeed explains the increased likelihood for lower grade tumors, which have the highest metabolic activity, to present with seizures. The availability of a well-described FDA-approved drug may readily allow 'off-label' use to treat peritumoral seizures, and more extensive clinical evaluation of sulfasalazine as adjuvant treatment in all gliomas appears warranted.

Acknowledgements

The authors are grateful for continued financial support from NIH through the following grants: RO1-NS31234, RO1-NS36692, RO1-NS052634, and P50CA97247.

See also: **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Glial Modulation of Excitability via Glutamate and GABA Transporters; Properties of Glia in Epileptic Brain; Role of Astrocyte Dysfunction in Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment.

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Properties of Glia in Epileptic Brain

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Introduction

The neuron, and networks of neurons, has received the bulk of attention in research on the basic mechanisms of epilepsy. That focus seems reasonable given that epilepsy is a disorder in the electrical discharge of neurons, and the symptomatic manifestations associated with epilepsy – seizures – are driven by the activity of neurons. However, it has been observed that neuroglia, in particular astrocytes, populate a variety of seizure-related pathological foci in the brain. The best evidence for the role of astrocytes in epileptic foci comes from studies on hippocampal seizure foci in temporal lobe epilepsy (TLE). Hippocampi showing hippocampal sclerosis, characterized by patterned neuron loss and an accumulation of astrocytes, were reported over 100 years ago. Many investigators have argued that the sclerotic hippocampus is the cause of seizures in patients with mesial TLE with hippocampal sclerosis. Given the relative paucity of neurons in such seizure foci, however, it has been speculated that the glia at these foci may play an important role in seizure generation and maintenance. Of the various types of glia in the brain, the astrocytes appear to be the predominant cell type at hippocampal seizure foci, along with a population of microglia. Investigations of the properties of astrocytes in particular, and microglia to a lesser degree, have become active areas of recent research in epilepsy.

Background

Unique Properties of Astrocytes

The contribution that astrocytes may make to seizure production and epilepsy arises from some unique properties of astrocytes. Astrocytes express similar sets of ion channels and receptors as do neurons, but with varying relative strength of expression. In particular, their membrane potassium (K^+) channel density exceeds that of Na^+ channels, with a preferential expression of the inwardly rectifying $K_{ir}4.1$ channel. The $K_{ir}4.1$ channels are found predominantly on astrocytic end feet surrounding capillaries and synapses, where they are colocalized with the water channel Aquaporin 4. These two types of channels together help maintain K^+ and water homeostasis, thus reducing the possibility of excessive neuronal excitation in the normal brain. Two astrocyte-specific glutamate transporters – EAAT1 (GLAST) and EAAT2 (GLT-1) – play a significant role in the removal of glutamate released into the extracellular space by active neurons, and these transporters also help control neuronal excitability. Finally, astrocytes can also release neuroactive substances, including neurotransmitters; such release is dependent on Ca^{2+} release from intracellular stores, which in turn is mediated through the activation of a variety of receptors on astrocytes.

Astrocytes also make a unique contribution to brain metabolism, mediated by a set of enzymes not found in neurons. In particular, astrocytes possess the enzyme pyruvate carboxylase, which can de novo carboxylate pyruvate to oxaloacetate, used to replenish carbon skeletons lost from the tricarboxylic acid (TCA) cycle. Astrocytes also uniquely possess glutamine synthetase, which catalyzes the conversion of glutamate (released by neurons and taken up by astrocytes) into glutamine, which is returned back to neurons in a process described as the glutamate–glutamine cycle. Under conditions of high extracellular glutamate, the TCA cycle product α-ketoglutarate may leave the TCA cycle as malate, to be converted in the cytosol to

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pyruvate, catalyzed by the cytosolic malic enzyme (which is restricted to astrocytes). Glia, and in particular astrocytes, have a high capacity to produce lactate in the presence of normal oxygen levels. The presence of the lactate dehydrogenase 5 (LDH5) isoform in astrocytes favors conversion of pyruvate to lactate, which is released and used by neurons as an energy source. Changes in any of these unique properties of astrocytes may contribute to the development of epilepsy through a variety of mechanisms.

Methods

Our laboratory has used a variety of methods to study astrocyte properties at hippocampal seizure foci. A primary method has been the use of immunohistochemistry to localize and compare the expression of a variety of astrocytic proteins – such as metabolic enzymes, neurotransmitter receptors, and membrane transporters – in human hippocampi surgically removed for the control of medically intractable TLE. This approach has been supplemented by enzyme activity assays and in vitro slice electrophysiology (some of which has been done in laboratories other than our own but is relevant to this review). Most recently, we have adopted DNA microarray technology to study gene expression patterns in hippocampal seizure foci.

Results

Properties of Astrocytes in a Hippocampal Seizure Focus

Resting astrocytes in the brain are spatially distributed in such a manner that the terminal ends of their processes do not invade into the space occupied by adjacent astrocytes. This phemonenon is sometimes described as 'tiling.' This rather even distribution pattern of astrocytes changes in a seizure focus (i.e., the sclerotic hippocampus), where the astrocytes become 'reactive'; they show aberrant extension and intermingling of their processes, with an increase in expression of a microfilament protein glial fibrillary acidic protein (GFAP). Along with this 'reactive gliosis,' several other properties of the astrocytes also change.

Membrane ion channels

Several lines of investigation have demonstrated alterations in astrocyte membrane ion channels. Most patch clamp studies on astrocytes from a sclerotic seizure focus show an increase in Na^+ channels on their membranes and a more depolarized resting membrane potential (although in one study, no change was found). Astrocytes also have reduced K_{ir} channel function. Immunostaining for the L-type Ca^{2+} channels demonstrates an upregulation in sclerotic hippocampi, suggesting enhanced Ca^{2+} uptake.

Neurotransmitter receptors

Glutamate receptor expression on astrocytes is also altered. Immunoelectronmicroscopic examination of sclerotic hippocampi has revealed the expression of mGluR2/3, mGluR4, and mGluR8 in reactive astrocytes. In like manner, mGluR3, mGluR5, and mGluR8 are reported to be upregulated in the hippocampus in experimental animal models of TLE. Activation of these receptors leads to an increase in intracellular Ca^{2+} and Ca^{2+} wave propagation, leading to astrocytic glutamate release. An increase in calcium transients and calcium wave propagation can be demonstrated in primary astrocyte cultures from human seizure foci. An elevated flip-to-flop mRNA ratio of GluR1 (AMPA receptor) in reactive astrocytes from hippocampal seizure foci has also been reported, suggesting an increased responsiveness of astrocytes to glutamate. Indeed, it has been shown that astrocytes from hippocampal seizure foci can be depolarized to generate action potentials.

Transporter molecules

A downregulation of astrocytic glutamate transporters EAAT1 and EAAT2 has been hypothesized to explain the well-documented observation of increased levels of extracellular glutamate in hippocampal seizure foci. Consistent with this hypothesis, some authors have reported a downregulation of these glutamate transporters in sclerotic hippocampi; however, others have been unable to confirm such an observation. A careful review of these discrepant data leads to the conclusion that astrocytic glutamate transporter differences in sclerotic versus nonsclerotic hippocampi are an inadequate explanation for high extracellular glutamate levels observed by in vivo dialysis in seizure foci.

γ-Amino butyric acid (GABA) is an inhibitory neurotransmitter that contributes to the balance between inhibition and excitation in the normal brain. In vivo microdialysis studies in human hippocampal seizure foci have demonstrated reduced levels of extracellular GABA in the epileptogenic seizure focus during the ictal state. The expression of the GABA transporter GAT3 on astrocytes in the epileptogenic hippocampus appears to be increased, which may contribute to excess removal of GABA and thus explain the earlier observation.

Aquaporin 4 is a water transporter molecule that is found on astrocytes. Under normal conditions, the distribution of AQP4 molecules on astrocytes shows a polarity, with a high density of the transporter on the astrocytic perivascular end feet. However, in astrocytes at the sclerotic hippocampal seizure focus, the density of AQP4 is reduced along the perivascular membrane domain, with no change in density on the astrocytic membrane facing the neuropil.

Astrocyte-specific enzymes

The expression of the enzyme glutamine synthetase is significantly downregulated in astrocytes in hippocampal

seizure foci. Such a reduction in this enzyme is postulated to diminish the efficient removal of extracellular glutamate, and thus its accumulation – perhaps resulting in overstimulation of neurons and subsequent seizures. To date, there are no published studies on the astrocyte-specific enzymes pyruvate carboxylase, cytosolic malic enzyme, or LDH5 in hippocampal seizure foci.

Astrocytes and inflammatory responses

Astrocytes are known to contribute to the inflammatory response of the central nervous system. In vivo and in vitro studies have established that astrocytes can produce a range of immunologically relevant molecules, including class II major histocompatibility complex antigens, many cytokines and chemokines, interleukins (IL-1, IL-6, and IL-10), interferons (INF-α and β), tumor necrosis factor (TNF) α, and transforming growth factor (TGF). A strong association of a polymorphism in the *IL-1*β gene has been found in patients with hippocampal sclerosis (as compared to patients without sclerosis and to nonepileptic controls), and intrahippocampal injection of IL-1β in rats is reported to worsen seizures. IL-1β has been shown to regulate the phenotype of astrocytes. A biallelic polymorphism in the promoter region of the *IL-1*β gene favors high production of IL-1β in patients with hippocampal sclerosis. IL-1β can act on astrocytic IL receptors and induce, via the transcription factor NFkB, the expression of a variety of molecules such as chemokines (CCL2, CCL3, CCL4), the ezrin–radixin–meosin complex of proteins, cyclooxygenase-2, and others. Immunohistochemical analysis of sclerotic hippocampi from TLE patients has revealed increased expression of the NFkB-p65 subunit in astrocytes. Differential gene expression analysis of hippocampi from TLE patients shows that several genes regulated through the NFkB pathway are upregulated in the sclerotic hippocampi. Some of these – such as S100B, ezrin, radixin, and meosin; the chemokines CCL2, CCL3, CCL4, and CX3CL1, and the chemokine receptor CXCR4 – may be associated with astrocytes or endothelial cells at the astrocyte–endothelium interface, but this possibility needs verification.

Association with blood–brain barrier

Astrocytes in normal brain have a close association with the microvasculature. Astrocytic end feet wrap around the blood–brain barrier, formed by the tight coupling of endothelial cells, and play a role in the movement of water and other molecules between the blood and the brain parenchyma. The proliferation of the microvasculature in the sclerotic hippocampal seizure focus was reported over 100 years ago. More recently, it has been suggested that the blood–brain barrier may be altered – among other possibilities, it may become leaky – during seizures. Astrocytes in the sclerotic hippocampi show several changes in the expression of molecules on their end feet associated with the microvasculature. In addition to changes in AQP4 distribution (see earlier sections), plectin 1, erythropoietin receptor (EPO-r), and the multiple drug resistance protein P-glycoprotein (PgP) expression are increased.

Contribution of Astrocyte with Altered Properties to Seizure-Inducing Mechanisms

Astrocytes at epileptic foci, because of the altered properties they possess, may contribute to seizure-inducing mechanisms in a number of ways. In all of the physiological mechanisms in which astrocytes are involved, their impact on seizure generation is assumed to occur through influencing the excitability of surviving neurons – at the epileptic brain focus, or more probably on neurons in the penumbra of the epileptic focus (since sclerotic hippocampal foci have a paucity of neurons).

The prominent reduction in the astrocytic enzyme glutamine synthetase has been postulated to lead to inadequate clearance of glutamate from the extracellular space because of reduced catabolism of glutamate to glutamine in astrocytes, resulting in an accumulation of extracellular glutamate. While it has been argued by some that the reduction in astrocytic glutamine synthetase is secondary to inadequate uptake of glutamate by the astrocyte (because of reduced astrocytic glutamate transporters), this possibility appears unlikely in as much as reduction in astrocyte glutamate transporters is equivocal and perhaps minimal at best. Further, experimental blocking of glutamine synthetase function in the hippocampus (e.g., by competitive inhibition of glutamine synthetase with methionine sulfoximine (MSO)) also results in seizures and increased extracellular glutamate. Concomitant with reduced glutamate catabolism, there may also be glutamate release from astrocytes, contributing to the enhanced extracellular glutamate pool. For example, enhanced metabotropic glutamate receptors on astrocytes may be activated to induce intracellular Ca^{2+} release and glutamate release by astrocytes. While enhanced levels of intracellular Ca^{2+} release in astrocytes at human sclerotic seizure foci have been documented, there is no experimental evidence yet that this effect is due to metabotropic receptor stimulation or results in glutamate release. Additionally, while the unique metabolic pathways in astrocytes could result in their synthesizing additional glutamate for release, such a functional mechanism in reactive astrocytes has yet to be demonstrated.

Another mechanism by which astrocyte abnormalities at a seizure focus may influence seizure activity is via altered K^+ clearance. The downregulation of inwardly rectifying K^+ channels on astrocytes may be a cause of this impairment, and could be compounded by defective water homeostasis. The buffering of K^+ by means of inwardly rectifying K^+ channels on astrocytes depends

on a parallel flux of water through the plasma membrane of these cells. The loss of AQP4 from the perivascular membrane will impair such water movement, and lead to increased extracellular concentrations of K^+, which can depolarize neurons in adjacent regions (such as the subiculum in the case of hippocampal foci).

The immunological responsiveness of astrocytes at seizure foci may provide clues as to how a variety of initial precipitating factors trigger TLE and its associated pathology. Early childhood febrile seizures, which predispose adults to TLE, are associated with increased IL-1β levels in CNS and blood plasma. IL-1β can act on interleukin receptors on astrocytes, and via the transcription factor NFκB induce a variety of molecules, including the chemokines, cytokines, and ERM proteins that are upregulated in the sclerotic hippocampus. Infections of the brain – such as meningitis, human herpes virus 6 (HHV6), and herpes simplex virus (HSV1) – that are shown to be associated with TLE may also be the cause of the inflammatory responses of glia. The effect of these infectious agents on astrocytes may be via astrocytic toll-like receptors (TLR) or may be via activation of inflammatory factors like interleukins, both of which can trigger signal transduction pathways leading to the activation of the transcription factor NFκB; through activation of NFκB, other molecular pathways may be induced that cause intracellular Ca^{2+} release and subsequent glutamate release, as well as chemokine and cytokine mechanism that can modulate the seizure focus microvasculature. The presence of such mechanisms requires experimental confirmation.

The modification of the membrane properties of astrocytes – increased sodium channel densities, a more depolarized membrane potential, and the potential to generate action potentials if sufficiently depolarized – confers on them neuron-like properties. This phenomenon and its potential contribution to seizure generation have received little attention to date. Recently, investigators have reported that there is a population of glial cells in the normal rat brain that express the proteoglycan NG2 (also called CSPG2), and that these astrocytes can generate action potentials when depolarized. This observation takes on increased significance, as in the sclerotic hippocampus, where astrocytes with a predisposition to generate action potentials are found, there is a significant increase in expression of CSPG2 (as demonstrated in recent microarray and immunohistochemical studies). Could astrocytes in a seizure focus serve as a source of electrical excitation in the brain?

Microglia in the Hippocampal Seizure Focus

Relatively little is known about the role of microglia in brain seizure foci. The few available studies clearly point to their role in inflammatory responses. Increased expression of complement system proteins C1q, C3c, C3d, and the C5–C9 proteins of the membrane attack protein complex, is observed in cells of the microglial/macrophage lineage – especially in sclerotic hippocampi in regions of neuronal loss (such as the hilar area and CA fields). Direct infusion of the proteins of the membrane attack pathway into the hippocampus in rats induces behavioral and electrographic seizures, suggesting their potential role in modulating epileptogenicity. Additionally microglia in sclerotic hippocampal seizure foci show increased expression of HLA-DR (a Class II major histocompatibility antigen), IL-1b, IL-1 receptor Type 1, the low-affinity p75 neurotrophin receptor, and the chemokine receptor CXCR4. This known activation pattern of microglia is, in all likelihood, only but a snapshot of the role that microglia may play in seizure foci. How and why this activation occurs, and its potential for seizure induction, remain unclear.

Challenges for the Future

The study of the properties of astrocytes at a seizure focus, such as the sclerotic hippocampus in patients with TLE, has thrust glia into the forefront as significant contributors to the pathophysiology of focal epilepsies. The intriguing altered properties of astrocytes at a seizure focus suggest their participation in one or more of several mechanisms, leading to a number of questions that will undoubtedly guide future research. For example: (1) Does their function result in the production of pathologically high levels of glutamate, resulting in the excitation of neurons surrounding the focus to generate seizures? (2) Do they function as the agents mediating an inflammatory response to various initial seizure precipitation factors (fevers, infectious agents, etc.), which in turn directly or indirectly lead to seizure generation? If so, what are these mechanisms? (3) Do astrocytes constitute an electrically excitable substrate, in and of themselves, that can generate seizures?

See also: **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Glial Modulation of Excitability via Glutamate and GABA Transporters; Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Peritumoral Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain; **Non-Synaptic Mechanisms**: Role of Aquaporins in Non-Synaptic Mechanisms of Epilepsy; **Pyramidal Cells**: Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain; **Transporters**: Glutamate Transporters in Epilepsy.

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Role of Astrocyte Dysfunction in Epilepsy

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Introduction

Epilepsy comprises a group of brain disorders characterized by the periodic and unpredictable occurrence of seizures. Even with optimal current antiepileptic drug (AED) therapy, ~30% of patients have poor seizure control and become medically refractory. Because many AEDs act as general CNS depressants and must be taken chronically for seizure suppression, they often have marked inhibitory effects on cognition.

Thus, more specific AEDs that target cellular and molecular abnormalities responsible for epilepsy but not globally affect cerebral function need to be developed. In this regard, recent developments in understanding glial (especially astrocytic) changes in epilepsy can potentially provide novel therapeutic targets. For simplicity, in this article we refer to different types of cells with astroglial properties as 'astrocytes.'

Background and Results

Glial Morphological Changes in Temporal Lobe Epilepsy

Alterations in astrocytic properties have been best described in the specific case of human temporal lobe epilepsy. The most common pathology found in patients with medically-intractable temporal lobe epilepsy is hippocampal sclerosis, more generally termed mesial temporal sclerosis. Mesial temporal sclerosis is characterized by neuronal cell loss in specific hippocampal areas, gliosis, microvascular proliferation, and synaptic reorganization. One striking hallmark of sclerotic hippocampus is that while there is a specific pattern of neuronal loss, there is also 'reactive gliosis' with hypertrophic glial cells exhibiting prominent Glial Fibrillary Acidic Protein (GFAP) staining and long, thick processes. Most of the changes in astrocytic channels and transporters described later have been discovered in sclerotic hippocampi from temporal lobe epilepsy patients. However, the cellular and molecular processes leading to astrocytic changes during epileptogenesis are not yet understood.

Glial Glutamate Receptors and Transporters in Temporal Lobe Epilepsy

Dysfunction of glutamate transport and synthesis

Glutamate transporters are expressed by several CNS cell types, but astrocytes are primarily responsible for glutamate uptake. The astroglial transporter GLT-1 is responsible for the clearance of bulk extracellular glutamate in the CNS, and increased extracellular levels of glutamate have been found in epileptogenic foci. GLT-1 knockout in mice caused spontaneous seizures and hippocampal pathology resembling alterations in temporal lobe epilepsy patients with mesial temporal sclerosis. Several human studies have supported the hypothesis that reduced or dysfunctional glial glutamate transporters in the hippocampus may trigger spontaneous seizures in patients with mesial temporal sclerosis (**Table 1**), yet the underlying mechanisms are unclear.

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Table 1 Involvement of astroglial membrane channels, transporters, and receptors in specific epilepsy syndromes

Epilepsy syndrome	*Astroglial molecule*	*Effect*	*Species*	*Methods*
Temporal lobe epilepsy	GLT-1 GLAST	No change No change	Human	IHC, WB, ISH
Temporal lobe epilepsy	GLT-1 GLAST	↓ No change	Human	IHC
Temporal lobe epilepsy	GLT-1 GLAST	↓ ↓	Human	IHC, ISH
Temporal lobe epilepsy	GLT-1 (Glutamine synthetase)	No change (↓)	Human	IHC, WB, enzyme activity
Temporal lobe epilepsy	GluR1 ('flip' variant)	↑	Human	PC, pharmacology (CTZ, PEPA), single-cell rtPCR, RA
Temporal lobe epilepsy	mGluR2/3 mGluR5 mGluR8	↑ ↑ ↑	Human	IHC
Temporal lobe epilepsy	Kir channel	↓	Human	PC
Temporal lobe epilepsy	Kir channel	↓ ↓	Human Rat (pilocarpine)	ISM, Ba^{2+}
Temporal lobe epilepsy	Kir channel	↓	Human	PC, Ba^{2+}, single-cell rtPCR
Temporal lobe epilepsy	AQP4	↑ overall ↓ perivascular	Human	IHC, rtPCR, gene chip, EM
Focal cortical dysplasia	mGluR2/3 mGluR5	↑ ↑	Human	IHC
Tuberous sclerosis	GLAST GLT-1	↓ ↓	*Tsc1*^{GFAP}CKO mouse	WB, PC
Tuberous sclerosis	Kir channel	↓	*Tsc1*^{GFAP}CKO mouse	PC, WB, Ba^{2+}, mRNA analysis
Tumor-associated epilepsy	GluR2	↓ Q/R editing	Human glioma	rtPCR, sequencing
Tumor-associated epilepsy	GLT-1 GLAST	↓ Mislocalized	Human glioma	IHC
Tumor-associated epilepsy	Kir channel	↓ Mislocalized	Human glioma	PC WB, IHC
Posttraumatic epilepsy	Kir and Kv channels	↓	Rat (fluid-percussion injury)	PC, ISM
Posttraumatic epilepsy	GLT-1 GLAST	↓ No change	Rat (ferrous chloride)	WB

CTZ, cyclothiazide; EM, electron microscopy; IHC, immunohistochemistry; ISH, in situ hybridization; ISM, ion-sensitive microelectrodes; PC, patch clamp; PEPA, 4-[2-(phenylsulfonylamino)ethylthio]-2,6-difluoro-phenoxyacetamide; RA, restriction analysis; rtPCR, reverse transcriptase polymerase chain reaction; WB, Western blot. Modified from Binder DK and Steinhäuser C (2006) Functional changes in astroglial cells in epilepsy. *Glia* 54: 358–368.

Alternatively, alterations in glutamate metabolism may be important. de Lanerolle's group has found reduced glutamine synthetase, an enzyme in astrocytes that converts glutamate into glutamine, in sclerotic rather than in non-sclerotic hippocampus of temporal lobe epilepsy patients (Table 1). Downregulation of glutamine synthetase would slow down glutamate–glutamine cycling and accumulation of the transmitter in astrocytes and in the extracellular space. This condition would provide a metabolic mechanism for astrocyte-dependent hyperexcitability.

Alterations of ionotropic glutamate receptors

A few studies have addressed the potential involvement of ionotropic glutamate receptors in seizure generation. Astrocytes abundantly express receptors of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype composed of the subunits GluR1 to GluR4. Combined functional and single-cell transcript analyses revealed that enhanced expression of GluR1 flip variants accounts for the prolonged receptor responses observed in hippocampal astrocytes of epilepsy patients with mesial temporal sclerosis (Table 1). Prolonged opening of receptor will promote influx of Ca^{2+} and Na^{+} ions. Steinhäuser's group has shown that enhanced intracellular Na^{+} blocks inwardly rectifying astroglial K^{+} (Kir) channels, further strengthening depolarization and reducing the K^{+} buffering capacity of astrocytes, and thus contributing to hyperexcitability.

Metabotropic glutamate receptors and astroglial Ca^{2+} signalling in epilepsy

mGluR3 and mGluR5 are the predominant metabotropic glutamate receptor subtypes expressed by glial cells. Activation of these receptors affects cAMP accumulation and leads to an increase in intracellular Ca^{2+}. Group II

mGluRs (mGluR 2, 3) have been shown to be coupled to cAMP levels in cultured astrocytes. mGluR-triggered rise in Ca^{2+} may cause oscillations and initiate Ca^{2+} wave propagation within the astrocyte network, activate Ca^{2+}-dependent ion channels, and induce glutamate release from astrocytes. In experimental epilepsy, reactive astrocytes of the hippocampus persistently upregulate mGluR3, mGluR5, and mGluR8 protein. Electron microscopic and immunohistochemical inspection of hippocampal tissue from temporal lobe epilepsy patients revealed expression of mGluR2/3, mGluR4, mGluR5, and mGluR8 in reactive astrocytes, suggesting an involvement of these receptors in gliosis. Upregulation of astroglial mGluR2/3 and mGluR5 was also observed in epileptic specimens from patients with focal cortical dysplasia (**Table 1**). However, the functional role of glial mGluR upregulation in epilepsy is not yet clear.

Astrocytic Glutamate Release in Epilepsy

Astrocytes are capable of releasing glutamate through a Ca^{2+}-dependent process, which could be involved in seizure generation. In chemically-induced, acute epilepsy models, it was recently reported that astrocytes contribute to the generation of synchronized epileptiform activity. In these studies, epileptiform discharges were provoked through the application of 4-aminopyridine, $GABA_A$ receptor antagonists, or bath solutions containing low concentrations of divalent cations. It appeared that astrocytic increase in $[Ca^{2+}]_i$ is sufficient to stimulate release of glutamate from glial cells, which was critical for the generation of paroxysmal depolarization shifts (PDSs), a hallmark of epileptiform activity. In addition, in vivo imaging showed that some antiepileptic drugs suppressed astrocytic Ca^{2+}-signalling. An important caveat to these studies is that human epilepsy is associated with significant morphological alterations that are absent in acute models that have been studied.

Astrocyte Potassium and Water Channels

Since both extracellular K^+ concentration and osmolarity have been shown to markedly modulate neural excitability, it is plausible that changes in astrocytic K^+ or water channels could contribute to hyperexcitability in epilepsy. Indeed, recent studies have found changes in astroglial Kir channels and AQP4 water channels in temporal lobe epilepsy specimens.

K^+ channels

During neuronal hyperactivity, extracellular $[K^+]$ may increase from ~3 mM to a ceiling of 10–12 mM; and K^+ released by active neurons is thought to be primarily taken up by glial cells. Any impairment of glial K^+ uptake would be expected to be proconvulsant. In the hippocampus, millimolar and even submillimolar increases in extracellular K^+ concentration enhance epileptiform activity. High-K^+ also reliably induces epileptiform activity in hippocampal slices from human patients with intractable temporal lobe epilepsy and hippocampal sclerosis.

A primary mechanism for K^+ reuptake is thought to be via inwardly rectifying glial K^+ channels (Kir channels). Glial Kir channels may contribute to K^+ reuptake and spatial K^+ buffering, which has been most clearly demonstrated in the retina. Although multiple subfamilies of Kir channels exist (Kir1–Kir7) differing in tissue distribution and functional properties, the expression of Kir4.1 in brain astrocytes has been investigated most thoroughly. Pharmacological or genetic inactivation of Kir4.1 leads to impairment of extracellular K^+ regulation. However, members of the strongly rectifying Kir2 family may also contribute to astroglial K^+ buffering.

Downregulation of astroglial Kir channels has been found in the injured or diseased CNS. Kir currents are reduced following injury-induced reactive gliosis *in vitro*, entorhinal cortex lesion, freeze lesion-induced cortical dysplasia, and traumatic and ischemic brain injury. In addition, several studies have indicated downregulation of Kir currents in specimens from patients with temporal lobe epilepsy. Using ion-sensitive microelectrodes, U. Heinemann's group compared glial Ba^{2+}-sensitive K^+ uptake in the CA1 region of hippocampal slices obtained from patients with or without mesial temporal sclerosis. Ba^{2+}, a blocker of Kir channels, augmented stimulus-evoked K^+ elevation in non-sclerotic but not in sclerotic specimens, suggesting an impairment in K^+ buffering in sclerotic tissue. Direct evidence for downregulation of Kir currents in the sclerotic CA1 region of hippocampus came from a comparative patch-clamp study in which a reduction in astroglial Kir currents was observed in sclerotic hippocampi compared with that in non-sclerotic hippocampi (**Table 1**). These data indicate that dysfunction of astroglial Kir channels could underlie impaired K^+ buffering and contribute to hyperexcitability in epileptic tissue. When and how this dysfunction develops during epileptogenesis is not yet clear.

Water channels

Alterations in astroglial water regulation could also highly affect excitability. Brain tissue excitability is extremely sensitive to osmolarity and the size of the extracellular space (ECS). Decreasing ECS volume produces hyperexcitability and enhanced epileptiform activity; conversely, increasing ECS volume with hyperosmolar medium attenuates epileptiform activity. These experimental data parallel extensive clinical experience indicating that hypo-osmolar states such as hyponatremia lower seizure

threshold while hyperosmolar states elevate seizure threshold.

The aquaporins (AQPs) are a family of membrane proteins that function as 'water channels' in many cell types and tissues in which fluid transport is crucial. Aquaporin-4 (AQP4) is expressed ubiquitously by glial cells, especially at specialized membrane domains including astroglial endfeet in contact with blood vessels and astrocyte membranes that ensheath glutamatergic synapses. Activity-induced radial water fluxes in neocortex have been suggested to occur as a result of water movement via aquaporin channels in response to physiological activity. Mice deficient in AQP4 have shown marked decrease in accumulation of brain water (cerebral edema) following water intoxication and focal cerebral ischemia and impaired clearance of brain water in models of vasogenic edema, suggesting a functional role for AQP4 in brain water transport. Similarly, mice deficient in dystrophin or α-syntrophin, in which there is mislocalization of the AQP4 protein, also show attenuated cerebral edema.

Alteration in the expression and subcellular localization of AQP4 has been described in sclerotic hippocampi obtained from patients with mesial temporal sclerosis. One study using immunohistochemistry, rt-PCR, and gene chip analysis reported an overall increase in AQP4 expression in sclerotic hippocampi. However, using quantitative immunogold electron microscopy, the same group found that there was mislocalization of AQP4 in the human epileptic hippocampus, with reduction in perivascular membrane expression (**Table 1**). The authors hypothesized that the loss of perivascular AQP4 perturbs water flux, impairs K^+ buffering, and results in an increased propensity for seizures.

Several lines of evidence support the hypothesis that AQP4 and Kir4.1 may act in concert in K^+ and H_2O regulation. First, reuptake of K^+ into glial cells could be AQP4-dependent, as water influx coupled to K^+ influx is thought to underlie activity-induced glial cell swelling. Second, studies in the retina have demonstrated subcellular co-localization of AQP4 and Kir4.1 via both electron microscopic and co-immunoprecipitation analyses. Third, Kir4.1$^{-/-}$ mice, like AQP4$^{-/-}$ mice, have impaired retinal and cochlear physiology presumably due to altered K^+ metabolism. Fourth, AQP4$^{-/-}$ mice have remarkably slowed K^+ reuptake in models of seizure and spreading depression in vivo associated with a near-threefold increase in seizure duration. Fifth, afferent stimulation of hippocampal slices from α-syntrophin-deficient mice demonstrates a deficit in extracellular K^+ clearance. These data are consistent with the idea that AQP4 and Kir4.1 participate in clearance of K^+ following neural activity. However, further studies are required to clarify the expression and functional interaction of AQP4 and Kir4.1 in the brain and their changes during epileptogenesis.

Astrocyte Dysfunction Involved in other Epilepsy Syndromes

Tuberous sclerosis

Tuberous sclerosis (TS) is a multisystem genetic disorder resulting from autosomal dominant mutations of either *TSC1* or *TSC2* genes. The *TSC1* gene encodes the protein hamartin and *TSC2* encodes tuberin, which are thought to be regulators of cell signalling and growth. Epilepsy occurs in 80–90% of cases of TS, frequently involves multiple seizure types and is often medically refractory. Cortical tubers represent the pathologic substrate of TS, and microscopically consist of a specific type of dysplastic lesion with astrocytosis and abnormal giant cells. Although this suggests that astrocytes are involved in the pathologic lesion, in itself this is not the evidence for a causative role of astrocytes in TS epileptogenesis. However, recent evidence using astrocyte-specific *TSC1* conditional knockout mice has provided insight into a potential role of astrocytes in the etiology of TS. These mice, which have conditional inactivation of the *TSC1* gene in GFAP-expressing cells (*Tsc1*^{GFAP}CKO mice), develop severe spontaneous seizures by 2 months of age and die prematurely. Intriguingly, the time point of onset of spontaneous seizures in these mice is concordant with increased astroglial proliferation. Furthermore, two functions of astrocytes – glutamate and K^+ reuptake – are impaired in these mice. These mice display reduced expression of the astrocyte glutamate transporters GLT1 and GLAST. In addition, recent evidence indicates that astrocytes from *Tsc1*^{GFAP}CKO mice exhibit reduced Kir channel activity, and hippocampal slices from these mice demonstrated increased sensitivity to K^+-induced epileptiform activity (**Table 1**). Together, these studies demonstrate that in this model, changes in glial properties may be a direct cause of epileptogenesis.

Tumor-associated epilepsy

Tumor-associated epilepsy is an important clinical problem, seen in approximately one-third of cases. Surgical removal of tumors usually results in seizure control, but many tumors cannot safely be resected, and tumor-associated seizures are often resistant to anticonvulsant therapy. Classic epilepsy-associated brain tumors include astrocytoma, oligodendroglioma, ganglioglioma, dysembryoplastic neuroepithelial tumor, and pleomorphic xanthoastrocytoma. Microdialysis studies of gliomas have revealed reduced glutamate in the tumor compared to peri-tumoral tissue. A 'glutamate hypothesis' of tumor-associated epilepsy has been advanced which suggests that tumors excite surrounding tissue by glutamate overstimulation. Two lines of evidence are relevant to this hypothesis. First, the glutamate receptor subunit GluR2 has been found to be underedited at the Q/R site in gliomas, which would

increase AMPA receptor Ca^{2+} permeability and potentially result in increased glutamate release by glioma cells (Table 1). Second, glioma cells release larger than normal amounts of glutamate *in vitro*. The release of glutamate from glioma cells is accompanied by a marked deficit in Na^+-dependent glutamate uptake, reduced expression of astrocytic glutamate transporters, and upregulation of cystine-glutamate exchange (Table 1). Hence, glioma cell glutamate release at the margins of the tumor may initiate seizures in peritumoral neurons. A distinct potential mechanism underlying tumor-associated epilepsy is altered K^+ homeostasis. In support of this hypothesis, both reduced Kir currents and mislocalization of Kir4.1 channels have been found in malignant astrocytes (Table 1).

Posttraumatic epilepsy

Posttraumatic epilepsy refers to a recurrent seizure disorder whose cause is believed to be traumatic brain injury. It is a common and important form of epilepsy, and develops in a variable proportion of traumatic brain injury survivors depending on the severity of the injury and the time after injury. Anticonvulsant prophylaxis is ineffective at preventing the occurrence of late seizures. Weight-drop and fluid-percussion injury animal models of posttraumatic epilepsy have demonstrated characteristic structural and functional changes in the hippocampus, such as death of dentate hilar neurons and mossy fiber sprouting. Recently, studies have also implicated altered astrocyte function in posttraumatic epilepsy models. Recordings from glial cells in hippocampal slices 2 days after fluid-percussion injury demonstrated reduction in transient outward and inward K^+ currents (Table 1), and antidromic stimulation of CA3 led to abnormal extracellular K^+ accumulation in posttraumatic slices compared to controls. This was accompanied by the appearance of electrical afterdischarges in CA3. Thus, this study suggests impaired K^+ homeostasis in posttraumatic hippocampal glia. Another study demonstrated reduction in expression of the astrocyte glutamate transporter GLT1 in a posttraumatic epilepsy model induced by intracortical ferrous chloride injection (Table 1), suggesting impaired glutamate transport. Further studies of the role of glial cells in posttraumatic epilepsy appear warranted now that reliable posttraumatic epilepsy animal models have been developed.

Perspectives and Future Directions

Astrocytes undergo cellular and molecular changes in epilepsy, including alteration in glutamate transporters and receptors as well as Kir channels and water channels. So far, most of these changes have been demonstrated in sclerotic hippocampi from patients with temporal lobe epilepsy or animal models resembling this particular human condition. However, the various functions of astrocytes in modulation of synaptic transmission and glutamate, K^+ and H_2O regulation suggest that astrocyte dysfunction could also be part of the pathophysiology of other forms of epilepsy.

One important recent development is the recognition of structural and functional heterogeneity of cells with astroglial properties. It is clear that a subset of hippocampal astroglial cells ('classical' astrocytes or GluT cells) expresses glutamate transporters and not ionotropic glutamate receptors and another (NG2 glia or GluR cells) expresses ionotropic glutamate receptors but not glutamate transporters. However, the lineage relationship of NG2 glia/GluR cells and the relative roles of *bona fide* astrocytes versus NG2 glia/GluR cells in epilepsy still remain unclear. In addition, the functional roles of ionotropic glutamate receptors, Kir and AQP4 channels in these subsets of glial cells in the hippocampus are not yet understood. Hippocampal NG2 glia/GluR cells lack gap junctional coupling but receive direct synaptic input from GABAergic and glutamatergic neurons. Gap junctions may also regulate excitability, although available data are inconsistent regarding the impact of altered connexin expression on epileptogenesis. The availability of mice with genetically uncoupled astrocytes will allow examination of this question, by separating the effects produced by alterations of neuronal versus glial gap junctions. It will be important in future studies to examine the cellular and molecular properties of subsets of hippocampal glial cells in human epileptic tissue and unravel the course of their functional alterations during epileptogenesis in appropriate animal models.

Another recent focus in astrocyte biology that may become important for epilepsy research is the 'gliovascular junction.' Microvascular proliferation in the sclerotic hippocampus was noted as early as 1899, but the role of the vasculature and the blood-brain barrier in epilepsy is not yet clear. The intimate relationship between astroglial endfeet ensheathing blood vessels, the targeted expression of AQP4 and Kir4.1 on astroglial endfeet, and the role of astrocytes in blood-brain barrier permeability and control of microcirculation have only recently been appreciated. Local pathological alterations in the gliovascular junction could perturb blood flow, K^+ and H_2O regulation and constitute an important mechanism in the generation of hyperexcitability. Indeed, a recent study suggests that transient opening of the blood-brain barrier is actually sufficient for focal epileptogenesis. The cellular and molecular roles of the gliovascular junction in metabolic homeostasis and changes during epileptogenesis are only beginning to be explored.

In conclusion, the exact changes taking place in astroglial functioning during epilepsy are still poorly understood. The term 'reactive gliosis' is too descriptive and should be replaced by careful morphological, biochemical, and electrophysiological studies of identified glial cell subtypes in human tissue and animal models. In addition to changes in preexisting glial cell populations, newly-generated glial cells with distinct properties may migrate into the hippocampus and contribute to enhanced seizure susceptibility. The available data likely represent only the 'tip of the iceberg' in terms of the functional role of astroglial cells in epilepsy. In view of the many physiologic functions of astrocytes that have been elucidated within the past decade, it can be expected that the next few years of research will yield evidence of similar important roles for glial cells in pathophysiology. Further study of astrocyte alterations in epilepsy should lead to the identification of novel molecular targets that might open new avenues for the development of alternative antiepileptic therapies.

Acknowledgements

D.K.B. is supported by a American Epilepsy Society/Milken Family Foundation Early Career Physician Scientist Award. C.S. is supported by grants from the Deutsche Forschungsgemeinschaft (SFB/TR3; SPP1172). We apologize to all those whose work could not be discussed because of space constraints.

See also: **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Glial Modulation of Excitability via Glutamate and GABA Transporters; Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Peritumoral Epilepsy; Properties of Glia in Epileptic Brain; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Neurotrophic Factors**: Role of BDNF in Animal Models of Epilepsy; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Role of Aquaporins in Non-Synaptic Mechanisms of Epilepsy; **Transporters**: Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures.

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Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment

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Tumor-Induced Epilepsy

Introduction

Tumors were known to cause seizures as early as the eighteenth century (and likely earlier still), as evidenced by the standard text on epilepsy at that time – Samuel Auguste Tissot's 'Traité de l'épilepsie' of 1770. How tumors bring about seizures, however, has been a matter of continuing speculation, certainly well into the 1970s and indeed even today. Several theories about underlying mechanisms have been put forward, including (a) increased intracranial pressure per se, (b) deafferentiation, (c) loss of gap junctional coupling (particularly in highly malignant cells, which would as a consequence reduce spatial buffering of extracellular potassium, (d) increased glial cell excitability and glial acquisition of neuronal properties (including the ability to fire action potentials), and (e) glia-mediated shifts in synaptic properties of neurons.

Background

The earlier-mentioned theories provide several viable hypotheses. However, they can all be challenged owing to several reasons, and do not afford as yet a comprehensive explanation for the appearance of epileptic discharges with brain tumors. Although all classes of brain tumors (including metastases, neurinomas, and meningeomas) undoubtedly lead to an increase in brain pressure, the link between pressure and neuronal excitability is at present based only on clinical observation. The hypothesis has the advantage of 'explaining' tumors that do not grow within the CNS itself (such as meningeomas) but still appear to produce seizures, but the support is only correlative. Further research is needed to determine whether, e.g., mechanosensitive channels of the TRP-family could indeed be involved in pressure-induced hyperexcitability. Further, there is no direct correlation between the extent of edema/tumor expansion and the likelihood of seizure appearance.

The deafferentiation hypothesis was raised in the late 1950s, and was based mainly on the observation of increased electrographic activity in surgically isolated brain areas in vivo. Recent studies have supported the view that that deafferentation is associated with increased excitability, as reflected in enhanced recurrent connectivity in isolated neocortical areas (as demonstrated by mapping the number of sites at which optical-uncaging of glutamate is able to evoke EPSCs, and by measuring the corresponding EPSC amplitudes), and in impaired chloride extrusion in the neurons in these isolated cortical slabs (shown by measuring chloride equilibrium potential in perforated-patch recordings and assessing its recovery after chloride loading induced by muscimol-activation of GABA-receptors). However, the extent of deafferentiation induced by tumors has not been investigated yet, so the hypothesis is currently viewed as the one applying to brain injury rather than to brain tumors.

The loss of gap junctional coupling does occur in malignant gliomas, as demonstrated in migration/fluorescence studies in vitro of clonal glioma cell lines (correlating migration rate to coupling ratios, using dye diffusion). And loss of astrocytic gap junctional coupling can indeed support increased network excitability, as shown by recent connexin knockout studies. However, loss of gap junctional coupling does not result in spontaneous seizures, and more importantly does not explain why low-grade astrocytomas (presumably still expressing higher numbers of gap junctions) are much more likely to be associated with seizure activity than high-grade gliomas (or why non-glial tumors, such as meningiomas, produce seizures). Electrophysiological recordings from glial cells in slices taken from oligodendroglioma/gliobalstoma patients, as well as from glial in primary glial cultures obtained from these tissues, reveal glia with neuronal properties – i.e., these cells exhibit inward sodium and outward potassium currents sufficient to generate action potentials when the cells are depolarized to threshold. It is thus intriguing to speculate that the glia cells themselves partake in, or even initiate seizure activity. However, a follow-up study combining MEG/EEG techniques for source localization and electrophysiological investigations of sodium currents in glial cells revealed that seizure initiation in gliomas usually occurs in the tumor border zone – not in the tumor mass with highest glial cell density. More importantly, there was no correlation between sodium current expression in glia and focus localization. Hence, glial cells do not seem to be directly involved in seizure generation.

There is a growing body of evidence, however, showing that glial cells shape and determine a host of neuronal functions by providing transmitters (or their precursors) and substrates (such as lactate), by influencing the pH and

ionic micromilieu (for example, by spatial buffering of K^+), by uptake and release of neurotransmitters, etc. To review of these physiological actions is beyond the scope of this article. However, it is certainly conceivable that, at least in glial tumors, there are alterations of these glial functions that contribute to epileptogenesis. For example, it has been shown that in peritumoral areas of gliomas, there are shifts in NMDA and GABA-receptor densities; however, these changes are neither very specific nor consistent, since both up- and downregulations can be observed, not only in tumors but also in various other brain lesions. The same holds true for actual transmitter levels, as determined with in vivo microdialysis in patients. Paradoxically, most of such studies show a trend toward downregulation of glutamate levels within tumors (or in the peritumoral surround), and an upregulation of GABA content – both changes being difficult to reconcile with increased excitabiltiy. There is, however, conflicting evidence, for moderately increased glutamate levels in animal models with glioma implants. Bioluminescence and spectroscopic analyses of experimentally-induced tumor tissue in cats reveal alkalinization in the tumor border zone. Although such a pH shift is often associated with increased excitability, to date there is no evidence that it causes seizures. Taken together, the data summarized earlier do not satisfactorily explain epileptogenesis occurring with gliomas (the neoplasms on which attention has been most intensively focused) or with brain tumors in general; further experiments are necessary to unravel the underlying mechanisms.

Methods

For the investigation of tumor-induced epileptogenesis, several approaches have been taken. These approaches can be roughly grouped into three categories: (1) studies on patients or tissue/cells of human origin (usually glioma, ganglioglioma, astrocytoma, glioblastoma); (2) studies on animal glioma models; and (3) studies in surrogate models, not involving tumors but focusing on perhaps related conditions (e.g., traumatic lesions). The studies on human tissue involve the largest number of approaches, including clinical correlations of symptoms (seizures) to tumor mass (e.g., to assess the role of intracranial pressure in epileptogenesis), immunohistochemical analyses of receptor density or gap junction expression (e.g., to address indirectly such issues as synaptic or spatial buffering dysregulation), and measurements of transmitter levels; the latter studies may employ either in vivo analysis using microdialysis, or in vitro measurements (e.g., in tissue slices from tumor resections), using a 'functional' (and in the case of microdialysis, dynamic) approach to assessment of synaptic release. Further, several in vitro studies have used human brain tissue infiltrated with tumor cells (glioma, astrocytoma, glioblastoma, oligodendroglioma), employing either whole slices, slice-derived glial tissue cultures, or membrane/cell fractions (i.e., synaptosomes). Most of these studies have concentrated on the electrophysiological properties, primarily of glial cells and in a few instances of neurons (see below). Lastly, human glioma-derived cells lines have been investigated to test transmitter release/uptake in vitro, and have shown that glutamate uptake (in these cell lines) is decreased.

Experimental models of glioma, at least in the context of epilepsy-related research, have been used much more rarely. These models involve, in essence, transplantation of different glioma cell lines in rat (e.g., C6, RG2, or G5-glioma cell lines) or cat (glioma clone F98) brain (usually neocortex or striatum). In some studies, irradiation has been used to improve glioma cell growth rate.

Recent Results

A series of recent papers have addressed the hypothesis of increased transmitter levels in tumor tissue (initially put forward in the 1980s) by determining the correlation between glutamate release and malignancy/invasiveness of glioma tissue on the one hand, and glutamate release and the initiation of epileptic activity on the other (**Figure 1(a)**). Using subclones of the C6-glioma cell line, differentiated for their expression of excitatory amino acid carrier 1 (EAAC1), it became evident that the clones devoid of EEAC1 actively released glutamate and produced larger tumors when implanted in rat striatum, suggesting invasiveness to be linked to the ability to release glutamate (**Figure 1(b)**). Intriguingly, this invasiveness could be significantly reduced by treatment with NMDA-antagonists, suggesting that glutamate-receptor activation, presumably on neurons, made brain tissue permissive for tumor growth – possibly by inducing apoptosis/excitotoxic cell death in surrounding healthy nervous tissue, thus surrendering space to the neoplasm. In a follow-up study on acute models of epileptiform activity in vitro, investigators showed that astrocytes can indeed function as an extrasynaptic source of glutamate sufficiently large to trigger epileptiform discharges. Taken together, these findings suggest that glutamate released from gliomas could indeed act both as an excitotoxic and epileptogenic agent. Animal data on increased glutamate levels within and (more importantly) surrounding experimental gliomas in rats (RG2 cell line) appear to support this notion. However, clinical observations have challenged this interpretation, since microdialysis studies in glioma patients rather show a decrease in glutamate levels.

Very few studies actually focus on the consequences of tumor invasion for excitability of surrounding neuronal tissue, for example by assessing direct electrophysiological correlates such as repetitive or burst firing. In analysis of spontaneous activity and synaptically-induced firing of neurons in brain slices from patients with tumors (glioma,

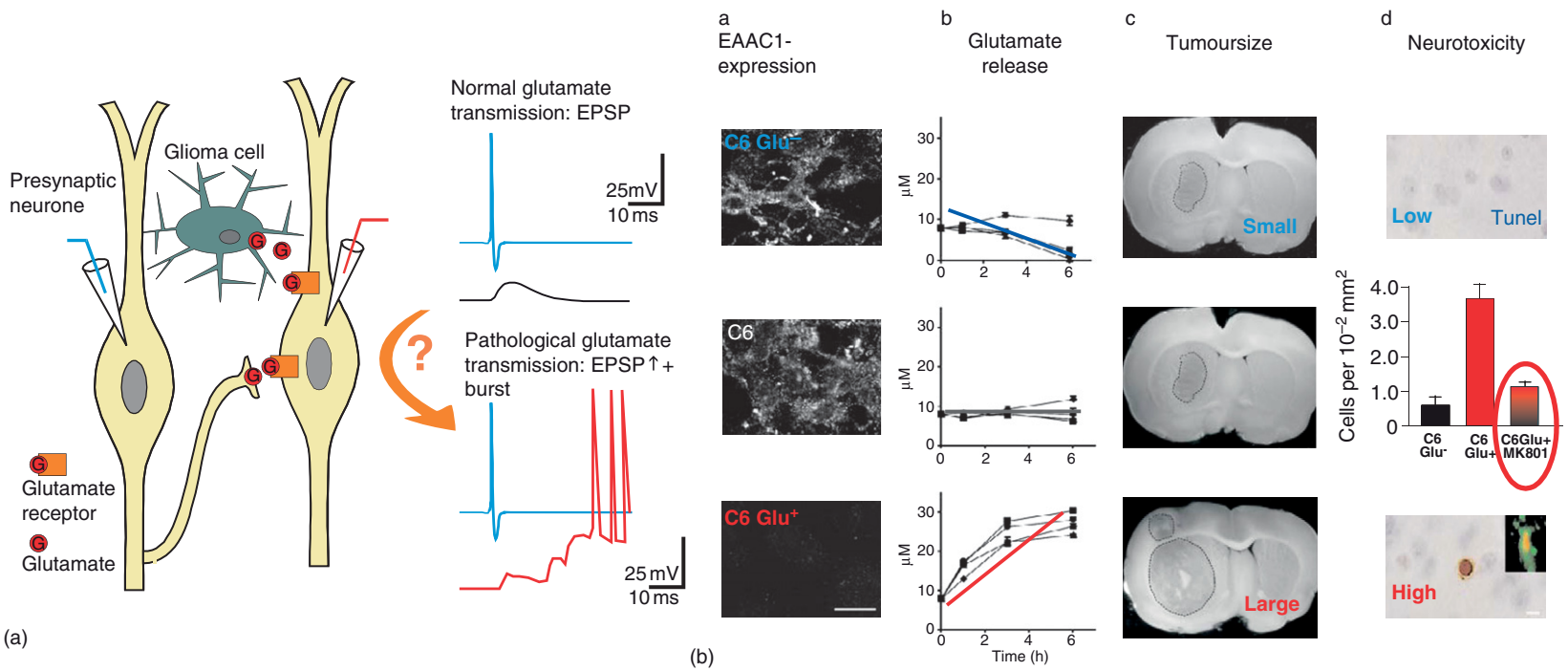


Figure 1 Processes leading to neuronal hyperexcitability in glioma models. A: Cartoon of glutamate release hypothesis: Normal synaptic transmission occurs at synaptic contacts between pre- and postsynaptic neuron. In case of excitatory contacts, the most common transmitter is glutamate (G) acting on glutamate receptors. In gliomas, astrocytes also release glutamate as a consequence of reduced expression of excitatory amino acid carrier 1 (EAAC1). This release may lead excitotoxic neuronal death and synaptic activation. As a – hitherto unconfirmed-consequence, glioma-mediated astrocytic glutamate release might also lead to prolonged/increased postsynaptic receptor-mediated potentials, converting normal EPSPs to pathologically increased responses and bursting. B: Rat glioma cell lines (C6) can be subcloned to yield cell lines with high (C6 Glu^-), normal (C6) and low (C6 Glu^+) EAAC1 expression (EEAC1 immunoreactivity of cell cultures in a), resulting in cell cultures showing decreasing, constant or increasing glutamate levels, respectively, corresponding to slow uptake, balanced uptake/release, and net release (plots of glutamate concentration over time in cell suspension in b). Implants of the corresponding cells into striate body result in gliomas significantly differing in size (histological sections of coronal slices of tumor-implanted rat brains in c) and, importantly, associated with low or high levels of neuronal degeneration (as evidenced by TUNEL staining) in Glu^- and Glu^+ groups, respectively (TUNEL-histochemistry in d). This neuronal degeneration could be significantly reduced (to levels of the Glu-group) if NMDA-receptor blocker MK-801 was administered to the animas (bar chart in d showing TUNEL positive neurons per mm^2). Adapted from Takano *et al*. (2001), *Nature Medicine* 7(9): 1010–1015, copyright 2001, with permission from Macmillan Publishers Ltd.

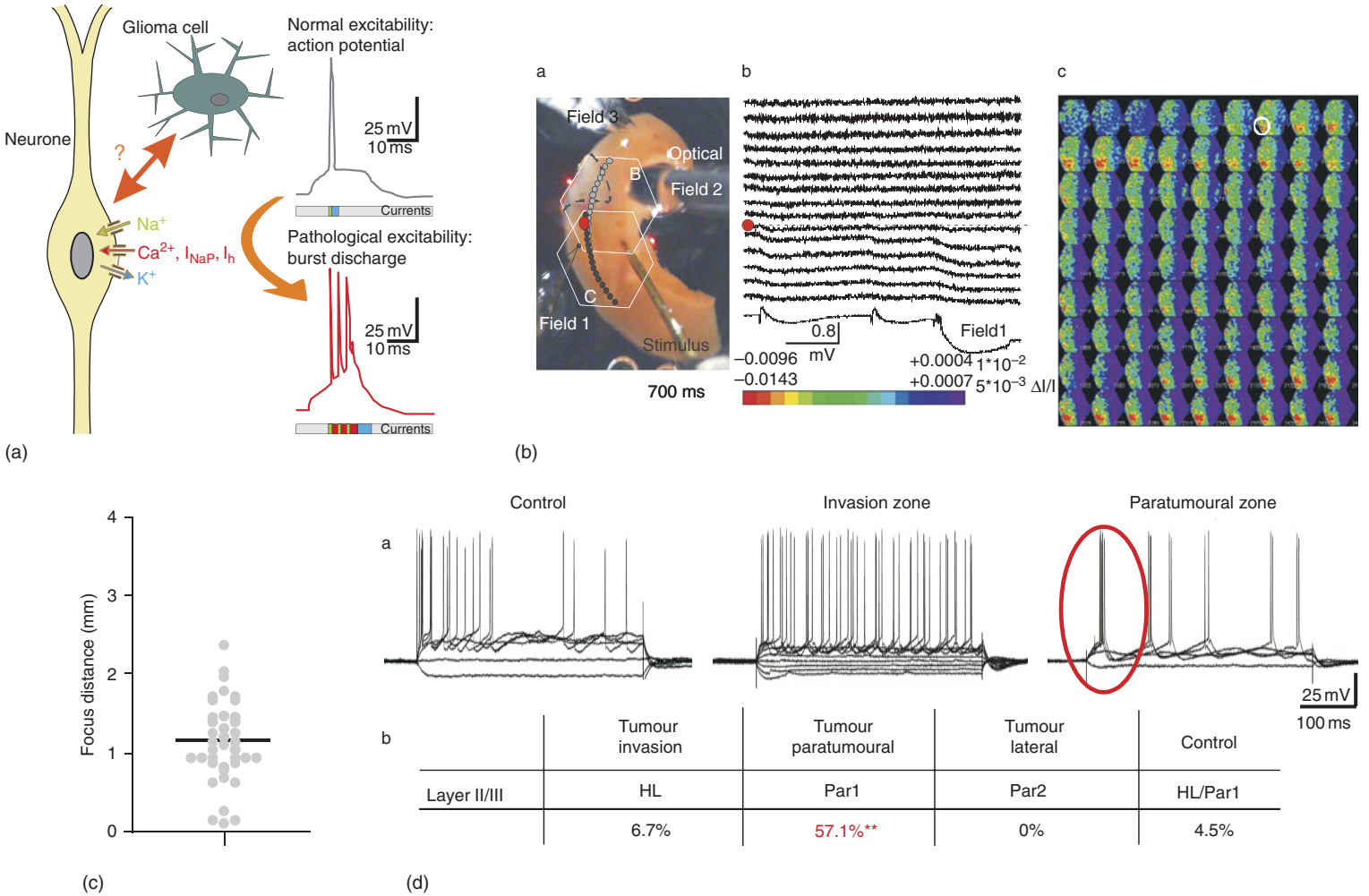


Figure 2 Processes leading to neuronal hyperexcitability in glioma models. A: Cartoon of hyperexcitabiltiy hypothesis: Glioma cells induce (by hitherto unknown interaction with neurons; arrow and question mark) an altered expression of transmembraneous ion channels. This converts action potentials into burst-discharge behaviour, i.e., normal excitability is shifted to pathological one, as a consequence, neurons fire repetitively. The contribution of different ion cannels to the membrane potential changes is indicated as colour-coded bars symbolising the periods during which the respective currents are active. Putative currents responsible for the initiation and plateau phase of bursts (red) are calcium, persistent sodium or hyperpolarization-activated unspecific cation currents (Ca^{2+}, I_{NaP}, I_h). Fast sodium (Na^+) and repolarising potassium currents (K^+) are coded in green and blue, respectively. B: Identification of foci of epileptiform activity in glioma model. a. Photograph of cortical brain slice with invasive glioma induced 2 weeks earlier by C6-glioma-cell injection. The tumor border is marked by the dotted line. Hexagrams indicate optical recording fields (opt; B, C); field 1 and field 2 denote recording positions of conventional microelectrodes for field potential recording. (b) Traces of photodiode signals (deflection downwards = depolarization; recording position B of Ba) and conventional field potential recording (field 1) of initiation of ictal discharge induced by perfusion of 0-Mg^{2+} solution. The red dot marks the trace with the initial signal, indicating the focus. C: False-color coding of a series of 81 consecutive optical recordings taken at intervals

astocytoma, oligodendroglioma), investigators showed that some neurons in the superficial cortical layers – 0.5–2 cm away from the lesion rim – exhibit burst firing. Although the specificity of this finding is difficult to gauge (there was no appropriate control tissue), this discharge pattern is atypical of supragranular-layer neurons (e.g., as seen in temporal lobe epilepsy specimens). This observation suggests that tumors may actually change intrinsic neuronal properties – especially considering these changes were seen comparatively far away from the tumor border, and might be much more pronounced in cells closer to the lesion. A recent investigation explored this phenomenon in more detail (**Figure 2**). In rat glioma-implantation experiments, spontaneous epileptiform activity induced by epileptogenic conditions ($0Mg^{2+}$) were observed to arise in the border zone of the lesion, 1–2 mm from its rim (**Figure 2(b,c)**) – a finding reminiscent of EMG/EEG data in patients who show epileptic foci in close proximity to, but not within, the glioma. It is in this border zone where disproportionately many bursting neurons were observed in supragranular layers of cortex. This increased density of burst-generating cells might be viewed as pivotal for the generation of the epileptic activity seen in ECoG recordings from these animals (**Figure 2(a,d)**). In summary, the observations of increased glial glutamate release and changes of intrinsic excitability of peritumoral neurons are likely to represent important facets of tumor-related (glioma-induced) epileptogenesis.

Future Goals

Future investigations of tumor-induced epileptogenesis should pursue the specific leads described earlier, and more generally look for common factors associated with epilepsy-inducing tumors in the clinical setting. Such common factors may constitute likely candidate mechanisms of epileptogenesis, and can be exploited (e.g., in the development appropriate animal models) for mechanistic studies. Among specific questions to be investigated, the issue of loss of gap junctional coupling in gliomas still needs to be addressed in studies that actually establish a correlation to K^+ spatial buffering defects or alterations in resting K^+ levels. Further, the pH changes seen in vitro must be systematically confirmed in vivo, and linked to excitability changes of neurons (and glia). In view of the neuron-like properties of glial cells from a variety of tumors, it will also be interesting to clarify (in vitro or in vivo) whether there is any pathological communication between these cells and surrounding neurons, be it via gap junctions or (as suggested by some recent findings) via transmitters released from glioma cells. That is, studies should examine whether activation of glia indeed leads to neuronal or EEG spiking. In this context, further systematic combined microdialysis/electrophysiological experiments are needed to reconcile the opposing findings on glutamate levels in human brain and animal models, and to correlate these changes with network excitability changes. Both tumor type and the degree of disease progression may be important factors here. Regarding the observation of bursting neurons in the surround of implanted gliomas, experiments on human tissue (looking at cells closer to the tumor border than previously investigated) are necessary to confirm this finding in the clinical setting. Additionally, the processes underlying these changes need to be unravelled. Possible candidate mechanisms include alterations of transmembranous currents associated with bursting behaviour (e.g., I_m, I_h, persistent I_{Na}, and low-threshold calcium).

More generally, one of the main criticisms one can raise against the experimental findings to date is that not only gliomas (and especially the lower-grade malignant type rather than very invasive ones), but above all also other tumors such as meningeomas or metastases, will readily (and perhaps even more efficiently than gliomas or glioblastomas) induce seizures. Hence, new animal models have to be established in which to investigate these tumor types. One single denominator common to all brain neoplasms is an increase in brain pressure. Therefore, a brain-pressure model in which the investigator can mimic slow increases of intracranial pressure may be useful for investigating network and neuronal excitability changes.

Tumor Treatment-Induced Excitabiltiy Changes

Not only brain tumors, but also their treatment, may affect neuronal excitability. This important point is evident as one reads case reports on the epileptogenic (and also antiepileptic) potential of cytostatic drugs. Radiotherapy, at least transiently, appears to support epilepsy management in tumor patients. And although tumour surgery is, in most cases, beneficial for patients with respect to seizure

of 5 ms each. Red denotes depolarization, blue normopolarization. White circle identifies the focus in the recording; the position corresponds to the red dot in optical recording position B (of Ba). C: Scatter plot of all epileptic foci identified by optical methods as a function of their distance from the tumor rim. The bar denotes the median value at around 1.2 mm. Da: Discharge patterns of neurons in control preparations, in the immediate invasion zone of glioma-invaded tissue, and from the paratumoral zone, i.e., 1–2 mm from the tumor rim. Only in the zone in which the foci are located, more than half of the neurons show burst firing. Db: Table giving the percentages of neurons displaying burst firing in upper cortical layers II and III in the immediate tumor invasion zone (hindlimb area; HL), in the paratumoral zone corresponding to parietal area 1 (Par1), lateral to the tumor (parietal area 2, Par2) and in control tissue without tumor in HL and Par1 regions. Adapted and reprinted from Köhling *et al.*, Epileptiform activity preferentially arises outside tumor invasion zone in glioma xenotransplants. *Neurobiology of Disease* 22: 64–75, 2006, copyright 2006, with permission from Elsevier.

activity, a pro-epileptic outcome has occasionally been observed. The mechanisms underlying these mostly clinical observations are only now beginning to emerge.

Recent Developments

Chemotherapy. Only few drugs used in the treatment of brain tumors, be they primary or metastatic, have been investigated for their effects on neuronal/network excitability. Vincristine, a toxin inhibiting microtubule arrangement, has been hypothesised to disturb intramembranous receptor anchoring. Indeed, IP3-induced upregulation of GABAergic currents can be blocked by vincristine, suggesting a pro-epileptic mechanism. A follow-up investigation on vincristine's effects on postsynaptic density composition, however, failed to establish a link to GABA-receptor clustering. Methotrexate, a folate antagonist, was hypothesised to interfere with transmitter uptake mechanisms, and in vitro studies in brain slices have confirmed this notion. The details of its interaction with transporters remains to be elucidated. Cyclosporine A (CsA) a drug used experimentally in gliomas (and more commonly in leukemia treatment) as a protein phosphatase 2B (calcineurin) inhibitor, may have a number of epilepsy-relevant effects on intracellular signalling cascades, including: a possible inhibition of potassium currents (demonstrated in annelids), a disruption of K_v 2.1 clustering, a block of cholinergically-induced downregulation of voltage-gated calcium current (and as a result, rendering cells more vulnerable to excitotoxicity), and a block of KCC2-activation in neurons (likely resulting in depolarizing GABA-actions). The cytostatic agent Temozolomide was recently reported to have anti-epileptic potential in the clinical setting (treatment of glioblastoma), but experimental data which might explain this effect are lacking.

Radiotherapy. Ionizing radiation has also been reported to dampen excitability. Recent (and not so recent) publications suggest this effect is dose- and time-dependent. Low dose radiation appears to be associated with an increase in excitability; similarly, high dose treatment appears to be associated with an early increase in excitability due to increases in membrane resistance, but is then followed by decreases in resistance. Further, radiation appears to downregulate presynaptic release machinery (SNAP25), and to interfere with presynaptic depolarization – resulting in a reduction of transmitter release. A note of caution is appropriate here: Most of the studies conducted to date in this field have been made in the context of defense/warfare issues, and not with the aim to clarify actions of therapeutic radiation. Future studies should be tailored to radiation schemes used in the clinical setting.

Surgery. Neurosurgery, in essence, produces 'controlled trauma' in the brain. Rare clinical reports on seizures initiated in tissue remaining at the rim of the resection zone suggest that tissue defects/deafferentiation may be a common mechanism for both traumatic epilepsy and these rare instances of surgery-induced hyperexcitability. Recent studies have revived the initial experiments of the 1950s on so-called undercutting lesions, whereby the grey matter of the cortex is undercut to sever it from ascending afferents. These studies show that both chloride-extrusion mechanisms after chloride loading (prolonged inhibitory GABAergic activation) are slowed in neurons from tissue treated in this manner, and that the density and amplitude of recurrent excitatory innervation is higher. Both changes would result in increased excitability, as GABA-ergic potentials are more likely to become depolarizing, and overall glutamatergic input is increased. Clinical studies are needed to reveal to what extent such processes actually occur as a result of surgery, and what impact they may have on seizure activity in patients.

Future Challenges

The questions in the field of treatment-induced changes of neuronal excitability in the context of brain tumor therapy are far from resolved. Regarding chemotherapeutic agents, basic research into their actions on the neuronal level must be extended. Radiation studies should be focused on experimental designs relevant to clinical treatment, and should also include in vivo animal experiments to clarify whether seizure susceptibility is reliably altered by radiation protocols. Lastly, clinical studies are needed to assess the extent of surgery-induced excitability changes associated with surgical tumor resections.

See also: **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; Glial Modulation of Excitability via Glutamate and GABA Transporters; Peritumoral Epilepsy; **Non-Synaptic Mechanisms**: Role of Aquaporins in Non-Synaptic Mechanisms of Epilepsy.

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GLUTAMATE

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Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability
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Introduction

Among the most complex neurological disorders, the epilepsies have been traditionally associated with hyperexcitability resulting from an impaired balance between excitation and inhibition in neuronal circuits. Temporal lobe epilepsy (TLE) is the most common adult form of treatment-resistant epilepsy. Patients suffering from TLE present seizures involving the temporal cortex and limbic structures such as the hippocampus and the entorhinal cortex. In the hippocampus, TLE is associated with a complex functional and structural reorganization involving principal cells and different subtypes of inhibitory interneurons. The mechanisms of TLE have been extensively studied in experimental animal models such as the kainic acid (KA) model, which reproduces well the main features of human TLE. Using this model, we searched for the factors that might contribute to imbalance between excitation and inhibition in the CA1 hippocampus. We found that, apart from axonal sprouting and synaptic reorganization in CA1 pyramidal cells, cell type-specific loss and synaptic remodeling occur in CA1 inhibitory circuits, thus contributing to local network hyperexcitability. Specifically, cellular loss affects a subpopulation of parvalbumin- and somatostatin-positive GABAergic interneurons in stratum oriens/alveus (O/A). This cell type-specific vulnerability is not due to the differential sensitivity of different subtypes of inhibitory interneurons to the excitotoxic action of KA because interneurons within stratum oriens/alveus (vulnerable population) and lacunosum moleculare (LM; resistant population) demonstrate similar membrane depolarizations and associated somatic Ca^{2+} elevations in response to acute KA application. However, interneuron type-specific vulnerability may result from the activation of other glutamate receptor mechanisms – e.g., the group I metabotropic glutamate receptors (mGluRs), which are differentially expressed in distinct subpopulations of hippocampal interneurons. Alternatively, this vulnerability may reflect the cell type-specific synaptic remodeling induced at interneuron excitatory synapses by epileptiform activity, which may lead to the interneurons' overexcitation and death. Indeed, specific cell loss is significantly reduced by treatment with a

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group I mGluR antagonist. Moreover, we found that both subtypes of the group I mGluRs (mGluR1a and mGluR5) contribute to epileptiform discharges in O/A interneurons but not in LM cells. Additionally, we observed specific alterations in the l-methyl-D-aspartate receptor-mediated currents at excitatory synapses onto O/A interneurons of KA-treated animals. The mechanisms of this cell type-specific synaptic remodeling are unknown, but may involve mGluR1a/5, which play an important role in synaptic plasticity. Thus, we attempted to characterize the cellular and molecular mechanisms of mGluR1a/5 signaling in O/A interneurons. We found that mGluR1a and mGluR5 are localized to distinct dendritic microdomains, linked to specific intracellular Ca^{2+}-signaling cascades producing Ca^{2+} rises with distinct temporal properties, and play specific functional roles in O/A interneurons. In the following paragraphs, we will discuss the view that mGluR1a/5 subtype-specific mechanisms are involved in the induction of synaptic plasticity and contribute to epileptiform activity, suggesting that their activation during seizures may induce long-lasting modifications at excitatory synapses onto O/A interneurons. The findings in support of this hypothesis may be particularly important, since they identify cell type-specific targets that are activated in epilepsy, and provide a basis for the development of target-selective therapy.

Background

mGluR1/5 Signaling and Epileptiform Activity

Group I metabotropic glutamate receptors (mGluR1 and 5) represent a family of $G_{q/11}$-protein coupled receptors. In the hippocampus, mGluR1a demonstrates cell type-specific distribution, while mGluR5 is present in the majority of neurons. Subcellularly, mGluR1/5 is located near excitatory synapses, at peri- and extrasynaptic sites. Accordingly, high-intensity presynaptic activity, leading to the glutamate 'spillover,' is required to activate these receptors. mGluR1/5 activation is classically linked to the phospholipase C (PLC)/inositol 1,3,5-triphosphate (IP_3)/Ca^{2+} release pathway. However, mGluR1/5 may also be organized with members of Homer scaffolding proteins into multiprotein complexes, suggesting additional coupling to different transduction pathways. Indeed, there is now considerable evidence demonstrating G-protein-independent signaling via group I mGluRs. For instance, in CA3 pyramidal neurons, mGluR1/5 is linked to the activation of Src-family nonreceptor tyrosine kinase. Furthermore, mGluR1/5 has been demonstrated to activate the extracellular signal-regulated kinase (ERK1/2)/mitogen-activated protein kinase (MAPK) cascade in a number of preparations. While mGluR1 has been shown to physically interact with a member of the canonical subfamily of transient receptor potential (TRP) channels (TRPC1), mGluR5 is linked to the NMDA receptors by a chain of anchoring proteins, including PSD-95, Shank, and Homer. Furthermore, mGluR1/5-L-type Ca^{2+}-channel interaction has been reported and represents a potential mechanism for the regulation of the Ca^{2+}-channel function by synaptic activity. Thus, group I mGluRs may be involved in multiple cellular functions, from neuronal excitability and different forms of synaptic plasticity to neuronal differentiation and cell-death mechanisms.

Importantly, these receptors are involved in both induction and maintenance of epileptiform activity. Indeed, in hippocampal CA3 pyramidal cells, transient activation of mGluR5 alone is capable of inducing epileptiform discharges that last for hours, and involve protein synthesis-dependent mechanisms. Furthermore, concurrent mGluR1/5 participation in epileptiform discharges significantly increases the duration of epileptic events, leading to the initiation of ictal discharges and facilitating seizure propagation. Interestingly, while mGluR5 has been shown to play a principal role in the induction phase of epileptiform activity, mGluR1 has been implicated as a key player in the maintenance of ictal-like discharges. These receptor-specific roles may be explained by particular signaling microdomains formed by each receptor, as well as by unique receptor-interacting partners. Indeed, generation of prolonged epileptiform discharges in CA3 pyramidal neurons involves specific TRPC channels. Given that these channels may physically interact with mGluR1, their activation may represent the mGluR1-specific mechanism of seizure prolongation. These data point to important roles for both mGluR subtypes in the initiation and spread of epileptiform activity. Furthermore, cell type-specific functions of mGluR1/5 in the hippocampus, and in particular in different types of inhibitory interneurons, may determine the behavior of a particular cell type during epileptiform activity. While such cell type-specific mechanisms may be critically important for our understanding of cellular basis of network reorganization, very little is currently known about mGluR1/5 cell-specific roles throughout epileptogenesis.

Methods

To study receptor-specific signaling mechanisms, as well as the roles of distinct receptor subtypes in interneuron function in normal and pathological conditions, we combine whole-cell patch-clamp electrophysiology, cellular imaging, and immunohistochemistry in rat hippocampal slices from normal and epileptic animals. We use a KA model of TLE in rats, as well as a model of acute epileptiform activity induced in hippocampal slices by application of the $GABA_A$ receptor antagonist bicuculline and the K^+-channel blocker 4-aminopiridine. Patch-clamp recordings in voltage- and current-clamp

mode are performed from distinct interneuron types, visually identified using the infrared and differential interference contrast microscopy. To study specific Ca^{2+}-signaling mechanisms activated by different receptor subtypes, we use two-photon Ca^{2+} imaging in combination with whole-cell patch-clamp recordings in acute hippocampal slices. We also perform morphological and neurochemical post hoc identification of recorded interneurons, using intracellular labeling with biocytin or fluorescent dyes and immunohistochemistry for specific Ca^{2+}-binding proteins and neuropeptides.

Recent Results

mGluR1/5 Signaling Mechanisms in O/A Interneurons

Group I mGluRs (specifically mGluR1a) are abundantly expressed in O/A interneurons. We first determined the mechanisms of these receptors' activation under physiological conditions. Using a combination of two-photon Ca^{2+} imaging and whole-cell recordings, we found that mGluR1a/5 can be activated by intense repetitive synaptic stimulation, consistent with their perisynaptic location. The receptors' activation in interneuron dendrites was associated with a relatively slow Ca^{2+} transient (CaT; time-to-peak, ~1 s; decay time, ~10 s) and an inward current sensitive to both the mGluR1a antagonist LY367385 and the mGluR5 antagonist MPEP. In addition, theta-burst synaptic stimulation coupled with postsynaptic depolarization also provided efficient activation of mGluR1a/5, pointing to a regulation of receptor function by membrane depolarization. Together, these data indicate that both mGluR subtypes contribute to excitatory transmission under conditions of high excitation.

We next attempted to examine specific signaling mechanisms linked to mGluR1a versus mGluR5. Using two-photon Ca^{2+} imaging and micropressure applications of the group I mGluR agonist (*S*)-3,5-DHPG to interneuron dendrites, we were able to discriminate between Ca^{2+} signaling microdomains produced by local activation of mGluR1a or mGluR5, and subsequently focus on receptor subtype-specific signaling mechanisms. The results of these experiments showed that mGluR1a activation produces a relatively fast-rising Ca^{2+} signal (time-to-peak, 1–2 s) resulting from Ca^{2+} entry via TRP channels, with a minor contribution of intracellular Ca^{2+} release. Using Western blot analysis and immunocytochemistry, we also found mGluR1a-dependent activation of ERK via Src tyrosine kinase in O/A interneurons. Moreover, mGluR1a-TRP Ca^{2+} signal in interneuron dendrites was dependent on Src and ERK activation, pointing to an important role of Src/ERK cascade in the regulation of the TRP-channel function and mGluR1a Ca^{2+} signaling. Although it is unclear which particular type of TRP channels may be coupled with mGluR1a in O/A interneurons, several recent findings in our laboratory point to a role of TRPC1/5. First, mGluR1a/5 CaTs are associated with outwardly rectifying membrane currents, similar to those produced by the activation of heteromeric TRPC1/5 channels in heterologous expression systems. Second, TRPC1 and TRPC5 show overlapping distribution in the hippocampus and are detected in O/A interneurons. In addition, mGluR1a-TRPC1 interaction has been reported in cerebellar Purkinje neurons; this interaction resulted in the generation of inward current similar to that induced by mGluR1a activation in O/A interneurons. Finally, our data on the TRP channel regulation by ERK point to a specific ERK–TRP interaction. Indeed, TRPC1 has a few potential ERK phosphorylation sites on its intracellular N-terminal. Interestingly, recent data indicate that both ERK and TRP channels may be involved in the generation of prolonged epileptiform discharges in CA3 pyramidal cells. Thus, ERK–TRP-channel interaction may represent a novel cell type-specific mechanism for the generation of epileptiform activity.

In contrast to mGluR1a Ca^{2+} signaling, mGluR5 activation in O/A interneuron dendrites produced slowly rising, plateau-like CaTs (time-to-peak, ~6 s) or Ca^{2+} oscillations (peak power at 0.37 Hz), which resulted solely from intracellular Ca^{2+} release via ryanodine-sensitive stores. These Ca^{2+} signals occurred independently of Src but were negatively modulated by ERK. Thus, while mGluR1a is linked to Src, ERK, and TRP channels (as well as intracellular Ca^{2+} stores), mGluR5 is coupled exclusively to internal stores. Together, these data show that mGluR1a/5 in O/A interneuron dendrites form distinct Ca^{2+}-signaling microdomains with specific properties that may serve specific signaling functions (**Figure 1**).

mGluR1/5 Contribution to Synaptic Plasticity

What can be the functional roles of these receptor subtype-specific dendritic Ca^{2+} microdomains in O/A interneurons? First, postsynaptic Ca^{2+} elevations control the induction of different forms of synaptic plasticity in central neurons. Excitatory synapses of O/A interneurons demonstrate a Hebbian form of long-term potentiation (LTP) induced by pairing synaptic theta-burst stimulation with postsynaptic depolarization. The induction of this form of LTP is critically controlled by mGluR1a and postsynaptic Ca^{2+} elevation. Indeed, LTP is blocked by the mGluR1a antagonist LY367385 or intracellular BAPTA, and is impaired in mGluR1a knockout mice. Moreover, specific mGluR1a Ca^{2+} signaling via TRP channels, regulated by Src/ERK cascade and store-dependent mechanism, appears to be directly involved in LTP induction: blocking TRP channels alone, or blocking Src/ERK together with intracellular Ca^{2+} stores, prevents LTP induction at interneuron excitatory synapses. In contrast, mGluR5 does not

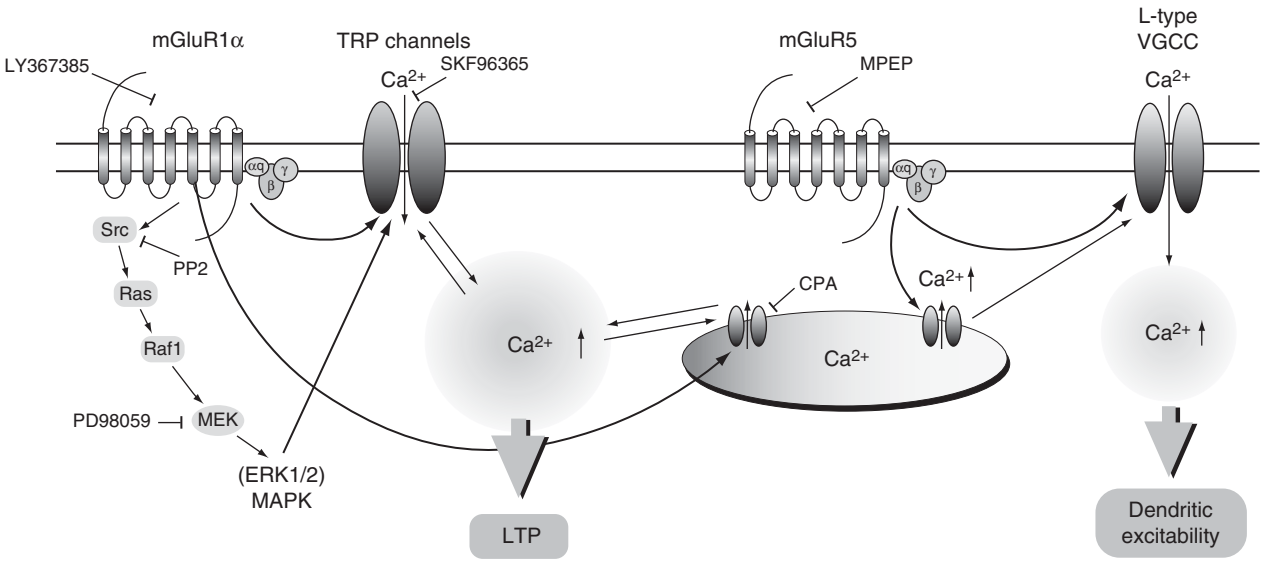


Figure 1 Schematic representation of mGluR1a- versus mGluR5-mediated Ca^{2+}-signaling pathways and their physiological roles in O/A inhibitory interneurons. mGluR1a activation initiates two parallel Ca^{2+}-signaling pathways: Src/ERK-dependent Ca^{2+} entry via TRP channels and intracellular Ca^{2+} release from internal stores. Ca^{2+} signal arising from the interaction between these two pathways control the induction of LTP at interneuron excitatory synapses. mGluR5 activation produces intracellular Ca^{2+} release but may be directly linked to the L-type VSCC. mGluR5-induced long-term potentiation of Ca^{2+} signal via L-type VSCCs represents an important mechanism for the modulation of dendritic excitability, which may play a role in the regulation of synaptic plasticity and other cellular functions. Reproduced from Topolnik L, *et al*. (2006) mGluR1/5 subunit-specific Ca2+ signalling and long-term potentiation in rat hippocampal oriens/alveus interneurons. *Journal of Physiology (London)* 575: 115–131, with permission from Wiley-Blackwell.

contribute to the induction of this form of synaptic plasticity in O/A interneurons. In principal cells, mGluR5 activation is involved in the potentiation of NMDA receptor function via G-protein- and PKC-dependent pathway. Given the essential role of NMDA receptors for a wide range of CNS functions (from neuronal development to synaptic plasticity), their regulation represents an important adapting mechanism to activity-dependent changes. However, it remains to be determined whether an mGluR5–NMDA receptor interaction operates in inhibitory neurons.

Second, local Ca^{2+} signaling may play an important role in the modulation of ion-channel function and dendritic excitability. Accordingly, by tuning the function of specific ion channels, it can provide an alternative mechanism for the regulation of synaptic plasticity. Indeed, our recent data point to the mGluR5-induced regulation of the L-type Ca^{2+}-channel function. It should be noted that multiple Ca^{2+} sources contribute to Ca^{2+} signaling in dendrites of O/A interneurons, including different types of voltage-sensitive Ca^{2+} channels (VSCCs). For instance, R/T-, L-, and P/Q-types of VSCCs contribute to CaTs evoked by bursts of action potentials (APs). Interestingly, local activation of mGluR5 produces long-lasting potentiation of APs-CaTs. This potentiation is not affected by the P/Q- or R/T-types of VSCC blockers, but prevented by the intracellular store inhibitor ryanodine or L-type VSCC blocker nifedipine. Moreover, APs-CaT potentiation is associated with an increased contribution of L-type VSCCs. What can be the functional significance of this mGluR5-induced L-type VSCC potentiation in O/A interneurons? Our data show that L-type VSCCs are activated during pairing protocols, which induce a Hebbian LTP at interneuron excitatory synapses. Furthermore, blocking these channels prevents LTP induction. This observation suggests that L-type VSCCs contribute to a build-up of Ca^{2+} signal necessary for LTP induction. Accordingly, modulation of these channels may represent a mechanism for modulation of synaptic plasticity. From this point of view, mGluR5-induced potentiation of L-type VSCCs may act as a priming stimulus, decreasing the Ca^{2+} threshold necessary for LTP induction. Indeed, our data show that in O/A interneuron dendrites, mGluR1a/5 activation is associated with distinct Ca^{2+}-signaling microdomains that play specific physiological roles. While mGluR1a is primarily involved in the induction of synaptic plasticity, mGluR5 acts as a regulator of specific ion-channel function and, by this means, regulates synaptic plasticity.

mGluR1a/5 Involvement in Seizure Activity In Vitro

How do these mechanisms function under pathological conditions – e.g., during epileptiform activity? Do mGluR1a/5 subtype-specific Ca^{2+} signals demonstrate similar properties and involve the same mechanisms as under normal conditions? Are there still plastic changes induced by the mGluR1a/5 activation? And what can be their role throughout epileptogenesis? To begin

answering these questions, we first determined whether mGluR1a/5 are activated during acute epileptiform activity, and examined how mGluR1a/5 may participate in epileptiform discharges. In disinhibited slices (in the presence of bicuculline and 4-aminopiridine), a brief train of electrical stimulation induced prominent long-lasting epileptiform discharges in O/A interneurons. Interestingly, this type of activity was cell type-specific, since the same stimulation paradigm applied to LM cells elicited epileptiform activity of significantly lower magnitude and duration. Given that O/A and LM interneurons differ in their mGluR1/5 content, it was particularly interesting to examine the different mGluR contribution to cell type-specific epileptiform activity. Our data showed that both mGluR1a and mGluR5 contributed to epileptiform activity in O/A interneurons but not in LM cells. Moreover, while mGluR5 played a principal role in the generation of current underlying epileptiform discharge, mGluR1a contributed specifically to a slow, long-lasting component of the discharge. These data are in agreement with previous findings in CA3 principal neurons, where mGluR5 was shown to be critically involved in the induction of epileptiform activity, while mGluR1a contributed to its prolongation and generation of ictal-like discharges. The mechanisms underlying the different roles of mGluR5 versus mGluR1a in the generation of epileptiform activity in O/A interneurons are presently unclear, but suggest receptor subtype-specific consequences of activation of mGluR1a/5 during seizures. Moreover, our data show that interneuron-type specific distribution of mGluR1a/5 may underlie the differential involvement of different types of inhibitory interneurons in epileptiform activity. Furthermore, the receptor-subtype specific roles of distinct mGluRs suggest that differential activity-dependent plastic changes might occur in particular cell types during epileptiform activity. Such activity-dependent changes may underlie the functional modification at interneuron excitatory synapses or the induction of cellular death mechanisms in specific populations of hippocampal inhibitory neurons.

Epileptiform Activity and Synaptic Remodeling in O/A Interneurons

Following epileptiform discharges, hippocampal circuitry undergoes extensive synaptic reorganization. Given that long-lasting changes in the molecular composition of synapses are typically associated with the incorporation of newly synthesized proteins, seizure-induced synaptic reorganization may be associated with activity-dependent gene expression and new protein synthesis. Indeed, at least several classes of transcription factors, including c-*jun* and c-*fos*, are induced by pathological (seizure) activity. It should be noted that the same factors play an important role in hippocampal synaptic plasticity, suggesting that the same pathways may control activity-dependent plastic changes in normal and epileptic hippocampus. Both mGluR1a and mGluR5 induce specific plastic modifications in O/A interneurons in normal hippocampus. Both receptor subtypes are also activated during epileptiform activity and may trigger Ca^{2+}-sensitive gene transcription. Thus, mGluR1a/5-specific Ca^{2+} mechanisms may be involved in synaptic remodeling in O/A interneurons. Indeed, in parallel with interneuron loss, we have recently uncovered changes at excitatory synapses of KA-resistant O/A interneurons. Single-fiber excitatory postsynaptic currents in O/A interneurons showed slower kinetics in KA-treated animals. Interestingly, only NMDA receptor-mediated current appeared to be changed, suggesting a specific alteration in NMDAR composition at excitatory synapses of O/A interneurons as a mechanism contributing to hippocampal remodeling. The cellular or molecular mechanisms of this plasticity are currently unknown but may involve activation of mGluR1a/5, since mGluR5 has been demonstrated to play an important role in the regulation of NMDAR function. However, its role in synaptic reorganization in O/A interneurons in normal and pathological conditions remains unknown, and elucidating this role will be an interesting direction for future studies.

Future Directions

Our findings demonstrate that, in interneurons, distinct group I mGluRs are linked to specific Ca^{2+}-signaling pathways, control different types of synaptic plasticity, and contribute to epileptiform activity in a cell type- and synapse-specific manner. Given this important role of mGluR1a/5 in epileptiform activity and regulation of interneuron excitation, it would be interesting in future studies to focus on the signaling mechanisms activated by mGluR1a/5 in different interneurons during epileptic activity, and the consequences of differential activation for synaptic function. Specifically, the role of Src/ERK cascade, TRP and L-type calcium channels, and protein synthesis should be explored in this context, as well as the involvement of mGluR1a/5 in modification of NMDAR expression and function. Uncovering the roles of mGluR1a/5 in epileptiform activity and seizure-induced synaptic remodeling would improve our understanding of the mechanisms underlying the functional reorganization of inhibitory circuits in epilepsy.

See also: **Glutamate**: Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability; Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on

Seizure Expression and Implications for Epileptogenesis; **Interneurons**: Properties of Interneurons and their Relation to Epilepsy; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures.

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Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain

F E Jensen, Harvard Medical School, Boston, MA, USA

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Introduction

Glutamate receptors are the most abundant type of excitatory neurotransmitter receptors in the mammalian forebrain. Glutamate receptors are responsible for the excitatory drive in neuronal networks, and are uniquely involved in activating downstream signaling cascades required for synaptic plasticity, learning, and memory. Overactivation of glutamate receptors occurs during a seizure, and in vivo microdialysis studies reveal increased extracellular glutamate during status epilepticus. Glutamate receptor antagonists are potently anticonvulsant in in vitro and in vivo models of epilepsy and seizures. Importantly, during early development, excitatory synaptic density is highest compared to any other part of the lifespan, while functional inhibitory neurotransmission is still relatively underdeveloped compared to later in life.

Background

Clinically, seizure incidence is highest in the neonatal period and during infancy. Neonatal seizures are commonly refractory to conventional AEDs, suggesting that their mechanisms are uniquely different from seizure mechanisms in the adult. In addition, seizures in early postnatal development result in an increased risk of later life epilepsy, as well as cognitive impairment, including autism. Animal studies mimic this low threshold for seizures in infants; in rodents, this heightened seizure-susceptibility is observed between 1 and 2 weeks of age. Compared to the adult, the immature animal shows a lower threshold for seizures induced by a variety of insults, including chemoconvulsants, increased temperature, trauma, and hypoxia or hypoxia/ischemia. The developmental regulation of a number of factors that

Seizure Expression and Implications for Epileptogenesis; **Interneurons**: Properties of Interneurons and their Relation to Epilepsy; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures.

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Introduction

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control neuronal excitability likely contributes to the increased seizure susceptibility of the immature brain. The balance between excitation and inhibition is tipped to create more excitable neuronal networks, as excitability is necessary for synaptogenesis and synaptic plasticity, both of which are heightened during this critical period of early development.

The enhanced excitability of the immature brain is multifactorial, but two major features that likely contribute to this condition are: (1) GABAergic inhibition is relatively underdeveloped compared to the adult brain and (2) glutamate receptors, the major class of excitatory neurotransmitter in the brain, are robustly expressed in the immature brain (see Silverstein and Jensen in Further Reading section). There are several subtypes of excitatory glutamate receptors found on neurons in forebrain structures (neocortical, hippocampal, and limbic structures) known to be activated in epilepsy models. Glutamate receptors can be divided into two categories, ionotropic and metabotropic. Considerable information is available regarding the potential involvement of ionotropic receptors in epilepsy; the contribution of metabotropic receptors is less well understood. For the purposes of this review, we focus on ionotropic glutamate receptors.

The ionotropic glutamate receptors include the *N*-methyl-D-aspartate receptor (NMDAR), the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPAR), and the kainate receptor. NMDARs and AMPARs are primarily located postsynaptically on principal cells and interneurons, while kainate receptors have been observed to be both pre- and postsynaptic, depending on the cell type. In the adult brain, NMDARs are permeable not only to Na^+ and K^+ but also to Ca^{2+}, while AMPA and kainate receptors – on principal neurons – are permeable only to Na^+ and K^+ (interneurons possess Ca^{2+} permeable AMPARs). NMDARs require membrane depolarization, with consequent releases Mg^{2+} channel blockade, to become activated. The subsequent Ca^{2+} influx through NMDAR channels has been linked to the activation of many Ca^{2+}-dependent signaling cascades, involved in posttranslational protein modification, the induction of protein synthesis, and the induction of gene transcription, that have been implicated in normal activity-dependent synaptic plasticity. All iontropic receptors are heteromeric and composed of different subunits. The function of ionotropic receptors is dictated by their subunit composition. Importantly, glutamate receptor subunit expression is developmentally regulated. The obligate subunit of NMDARs is the NR1 subunit, and receptors are made up of combinations of the NR1 subunit with NR2 or NR3 subunits. Rodent studies reveal that NR2A is expressed at higher levels in the adult brain, whereas NR2B is present in the first 2 postnatal weeks. Human studies reveal a similar pattern of early NR2B expression in the infant brain. When NR2B is expressed, current decay times are prolonged, increasing excitability. In addition, the sensitivity of the NMDAR to Mg^{2+} blockade is decreased when NR2D and NR3A are expressed, and these are expressed predominantly in early postnatal development in rats and humans.

Similar to the NMDAR, the AMPAR is also developmentally regulated in a manner that increases postsynaptic excitability and signaling in early development coincident with the critical period (**Figure 1**). AMPARs can contain GluR1, GluR2, GluR3, and GluR4 subunits. In the adult brain, many receptors appear to be GluR1 and GluR2/3 containing. GluR1 isoforms are differentially expressed over development. The 'flip' isoform of GluR1 is associated with less glutamate desensitization than when the 'flop' isoform is expressed. During early development, the expression of the 'flip' isoform is high, increasing synaptic excitability. In addition, in early development (i.e., the first 2 weeks in the rodent and the first 1–2 years in the human), principal neurons show a relative lack of GluR2 in forebrain structures such as hippocampus and cortex. The presence of GluR2 renders the AMPARs impermeable to Ca^{2+}; hence, the absence of this subunit in the immature neurons renders them more Ca^{2+}-permeable. In addition, the GluR2 mRNA undergoes an editing step in the adult brain, substituting a Q for R in its sequence. When the mRNA is edited, a functional GluR2 protein is translated; however, when the editing step is omitted, the unedited form of GluR2 is nonfunctional, and equivalent to the GluR2-deficient condition.

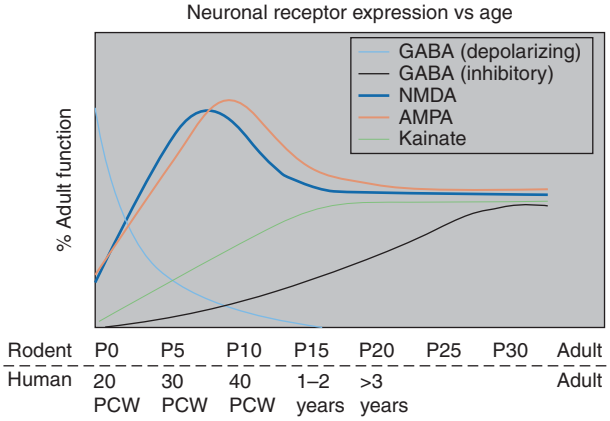


Figure 1 Schematic depiction of maturational changes in glutamate and GABA receptor expression and function in the developing brain. Developmental pattern for rat (top X axis) and human (bottom x axis) are shown. GABA receptors are depolarizing early in the first postnatal week in the rat and up to and including the neonatal period in the human, while functional inhibition is gradually reached over development (6). Prior to full maturation of GABA-mediated inhibition, the NMDA and AMPA subtypes of glutamate receptors peak between the first and second postnatal weeks in the rat and in the neonatal period in the human. Kainate receptor binding is initially low and gradually rises to adult levels by the fourth postnatal week (12). Reproduced from Silverstein FS and Jensen FE (2007) Neonatal seizures. *Annals of Neurology* 62: 112–120, with permission Wiley-Blackwell.

To date, no GluR2 editing is known to take place during development, although editing can take place in response to injury and seizures (see the following sections).

Kainate receptors are comprised of GluR5, GluR6 and/or KA1 and KA2 subunits. Like GluR2, the GluR5 and GluR6 subunits can undergo an editing step and the unedited subunit protein results in increased Ca^{2+} permeability. Regarding their maturational profile, kainate receptors on principal neurons have been observed to gradually increase in expression with development. However, kainate receptors on GABAergic interneurons are expressed at high levels early in development, when the GluR5-containing kainate receptors are localized at terminals where they can directly inhibit GABA release. Hence, kainate appears to have important modulatory actions on GABAergic transmission in the immature brain.

A number of rodent protein and gene analyses, as well as electrophysiological studies, have revealed a baseline enhanced expression of excitatory glutamate receptors subtypes that promote excitability of the immature brain compared to the adult. Given the enhanced excitability of the immature brain, there are a number of approaches that have been taken to investigate the effects of glutamate receptor expression on seizures during development, to explore both mechanisms leading to long term epilepsy and to elucidate neurocognitive impairment associated with early seizures.

Methods

In our laboratory, we have used a model of neonatal seizures (in the Long Evans rat) that is based on the natural epileptogenic potential of hypoxia (rather than a chemoconvulsant). Hypoxic or hypoxic/ischemic encephalopathy is the most common cause of neonatal seizures clinically, and these seizures can be refractory to conventional AED medication and lead to long-term epilepsy and neurocognitive deficits. Hence, we use hypoxia as our epileptogenic stimulus, and have based the timing of its delivery (i.e., the animal's age) on the developmental profile of glutamate receptor expression in Long Evans rats (**Figure 1**). Global hypoxia induces EEG and behavioral seizure activity optimally at postnatal days 10–12 (P10–12) in our animals. This period of development is characterized by heightened expression of calcium-permeable GluR-2-deficient AMPARs, as well as expression of NR2B and NR3A containing NMDARs. We have also evaluated these developmental profiles in Sprague–Dawley rats, which appear to be developmentally advanced compared to the Long Evans strain. The gestational period in the Sprague–Dawley rat is 23 days, whereas the Long Evans is only 21 days; hence, for postconceptional age, a P10–12 Long Evans rat is equivalent to a P7–8 Sprague–Dawley rat. Indeed, we have shown that with respect to the developmental regulation of AMPAR subunit expression, these relative ages appear to be analogous. In addition, preliminary unpublished studies from our laboratory have shown that the C57/BL6 mouse is more similar to the Sprague–Dawley rat than the Long Evans rat in terms of the cortical and hippocampal neuronal and glial AMPAR expression.

We expose the rats to graded hypoxia, starting with room air and decreasing the oxygen content to 4% over a 15-min period. Too rapid a decline in O_2 results in an isoelectric EEG, severe bradycardia, and apnea, whereas a more gradual decrease appears permissive of only mild bradycardia, sustained respiration, and prominent EEG and behavioral seizures (**Figure 2**). Others and we have also shown that animals that experience these early hypoxic seizures go on to develop long-term increases in susceptibility to seizure-induced neuronal injury and exhibit learning impairment. In addition, recent unpublished studies have shown that these animals have a high incidence of EEG and behavioral spontaneous seizures at P50–80 (**Figure 2**). Hence, this model is well suited to the study of factors responsible for acute seizure susceptibility as well as mechanisms of epileptogenesis and the long-term neurobehavioral deficits. In addition, we have employed this model to conduct therapeutic trials aimed at both acute seizure suppression and antiepileptogenic intervention.

We study this model at several levels. First, whole animal behavioral and EEG studies show that there is a seizure phenotype acutely and chronically. Depth electrodes show activation of cortical and hippocampal areas acutely. We can also evaluate behavioral and learning deficits: these animals show deficits in water maze as well as open field and handling tests, suggesting limbic and hippocampal dysfunction. Hence, we have both acute and long-term outcomes for therapeutic trials. Second, we can evaluate acute and long-term modification of gene and protein expression. A major advantage of this model is that, like other models of early life seizures, there is no acute neuronal injury. Thus, observed changes in gene and protein expression are less likely related to cell death cascades (which are often confounding features of some adult chemoconvulsant models where there is prominent acute cell death). In addition to sacrificing animals at intervals following seizures to track gene and protein alteration, we have performed both extracellular and whole cell patch clamp studies on hippocampal and cortical slices, removed ex vivo at intervals ranging from immediately after the hypoxia-induced seizure (at P10) to weeks after the acute seizures. These slice studies allow us to characterize changes in glutamate and GABA receptor function in specific neuronal populations, and to correlate these changes with altered protein or gene expression.

In order to determine the clinical relevance of our findings related to developmental glutamate receptor

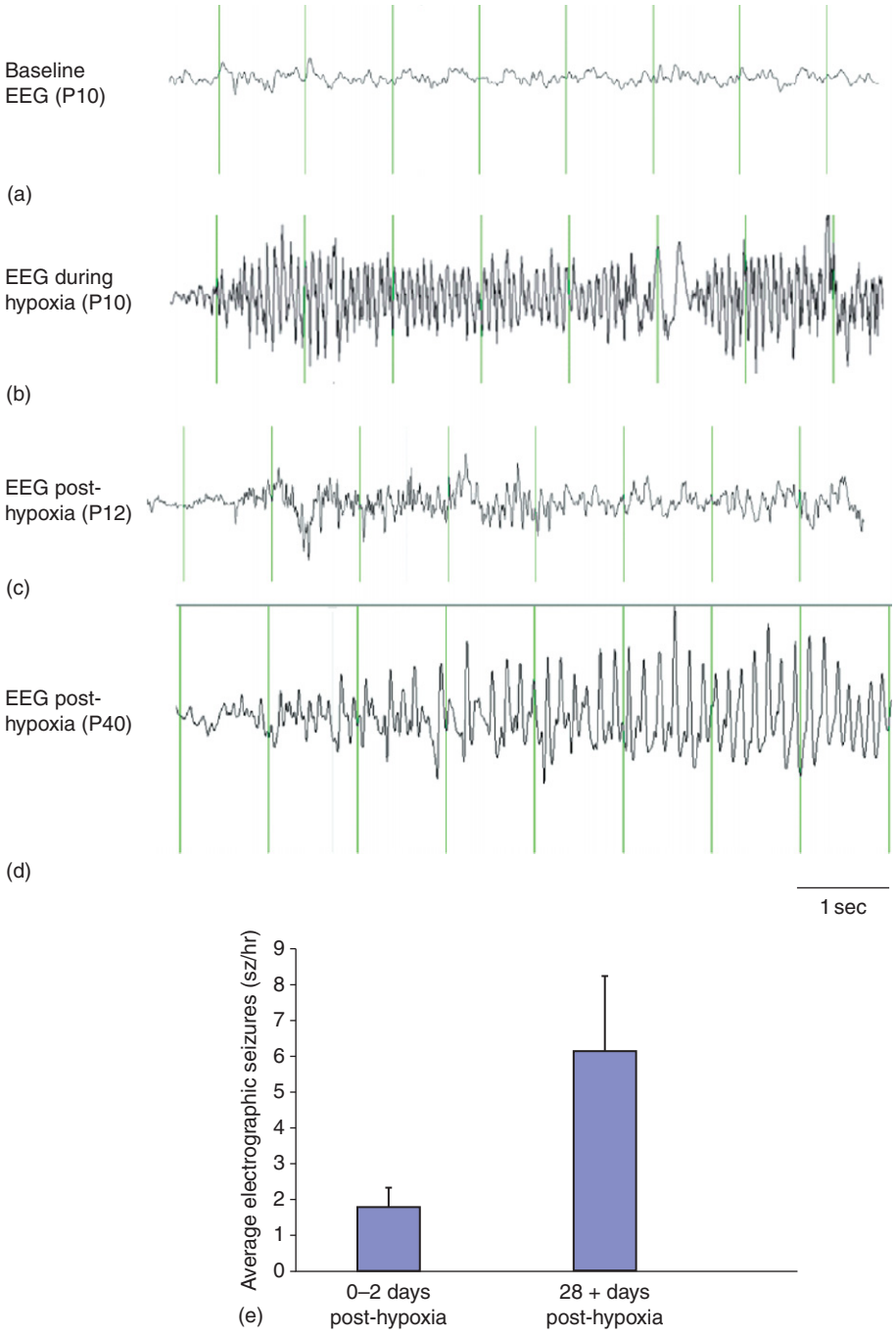


Figure 2 Electrographic seizures recorded from rat pups during and post hypoxia. Electrographic recordings obtained using subdermal wire electrodes in rat pups show abnormal epileptiform activity during graded global hypoxia (b) 2 days following hypoxia induction (c) and at 4 weeks following hypoxia induction (d). Baseline EEG activity in a P10 long Evans rat pup is shown (a). The average seizure frequency recorded using video-EEG recording apparatus is shown in (e).

regulation and alterations due to early life seizures, we have evaluated postmortem samples of normal human brain, at ages ranging from midgestation to adulthood. Double labeling with glutamate receptor subunit-specific antibodies in combination with neuronal and glial markers has allowed us to make comparisons, across ages, between rodent and human cortical samples.

Recent Results

Early studies using this hypoxia model suggested a critical role for AMPARs, in that systemic pretreatment with the AMPAR antagonist NBQX was effective in blocking the acute seizure activity (at P10) as well as the long-term outcome. The NMDAR antagonists MK-801 and the

GABA agonists phenobarbital and lorazepam had minimal effects. Given that clinically there are no biomarkers to identify hypoxic infants at risk for seizures, we next evaluated *post*seizure treatment. We used both NBQX and the clinically available drug, topiramate – which has AMPAR antagonist action; both agents exhibited protective effects against the long-term outcomes when administered over 48 h post seizure. These results suggested that seizure-induced changes in neuronal excitability could be prevented following the initial seizures, and that AMPAR activation appeared to be necessary and sufficient for these changes.

Given that AMPARs appeared to play a role, and that their antagonists showed efficacy in the rodent model treatment trials, we next evaluated whether glutamate receptors were developmentally regulated in the human brain in a manner similar to that observed in the rat. Indeed we showed that cell-specific AMPARs expression was developmentally regulated, with cortical and hippocampal neuronal glutamate subunit expression studies showing the presence of GluR2-deficient AMPARs during the neonatal period and throughout the first year of life. In addition, our unpublished studies reveal that these neurons also express NR2B and NR3A containing NMDARs, which are expressed at much lower levels later in infancy and childhood. Parallel studies of developmental expression in human tissue help validate the experimental observations in the animal model.

Recently, we have been examining how early-life seizures alter hippocampal and cortical networks. Using hippocampal and cortical slices removed at intervals following P10 hypoxia-induced seizures, we have shown (with both extracellular recordings and whole cell patch clamp recordings) that there is a rapid and sustained increase in network excitability. AMPAR currents are rapidly increased in amplitude and frequency, and they become more inwardly rectifying in CA1 pyramidal cells from hippocampal slices removed within hours following seizures. In addition, there is no increase in paired pulse facilitation, suggesting that the change is largely due to a postsynaptic modification. In the setting of an increased/decreased receptor mediated current, paired pulse stimulation can help determine the presynaptic vs. postsynaptic source for the change in synaptic strength; any change in the amplitude of the second of two paired pulses is generally thought to indicate a change in presynaptic transmitter release, while the absence of an altered paired pulse response is more consistent with a postsynaptic modification. Indeed, we recently showed that sites on the AMPAR GluR1 (ser 831 and ser 845) and GluR2 (ser 880) are phosphorylated rapidly following seizures (**Figure 3**). These posttranslational subunit modifications have been observed by a number of laboratories studying models of long-term potentiation, where AMPAR-mediated synaptic efficacy is enhanced. GluR1 phosphorylation at Ser 831 and Ser 845 results in trafficking of the GluR1 subunit to the synaptic membrane, with a functional increase the amplitude and open time of AMPARs. Phosphorylation of GluR2 at Ser 880 results in subunit removal from the membrane. GluR2-deficient synapses are characteristically inwardly rectifying and Ca^{2+}-permeable compared to GluR2-containing synapses. Indeed, we have shown that within hours to days after hypoxic seizures, AMPARs are more inwardly rectifying. In addition, using the cobalt uptake assay as a surrogate for calcium permeability, we have shown significant increases in cobalt staining of pyramidal neurons in CA1 following seizures at P10.

In addition to these changes at the AMPAR, we have recently investigated seizure-induced alterations in NMDARs. NMDARs are NR2B-containing in hippocampal CA1 neurons at P10, and show a characteristic ifenprodil sensitivity. However, whole cell patch clamp recordings in slices removed at 24 h following seizures at P10 show a decrease in ifenprodil sensitivity. In addition, NR2B expression is reduced on western blot analysis of hippocampal homogenates at 24 h post seizure. Analysis of other NMDAR subunit changes, and studies to determine whether or not these rapid changes are posttranslational, are ongoing.

Other studies using this model include an investigation of the mechanism of protection by posttreatment with AMPAR antagonists. We hypothesized, based on our earlier observations, that the rapid alterations in glutamate receptor trafficking might represent early steps in the epileptogenic cascade in this model. To test this hypothesis, we treated rats immediately following hypoxic seizures with AMPAR antagonists, and then either sacrificed the rats for hippocampal slice electrophysiology or for immunoprecipitation and western blot analysis for phosphorylated AMPAR subunits. We found that if AMPAR antagonist posttreatment was initiated immediately following the initial hypoxia-induced seizures, the expected alterations in AMPAR function and subunit phosphorylation were blocked, as was the increased seizure susceptibility. We tested NBQX and topiramate, as well as the new AED candidate drug talampanel, which is an uncompetitive AMPAR antagonist (GYKI 53773). NBQX 30 mg kg^{-1}, topiramate 20 g kg^{-1}, or talampanel 7.5 mg kg^{-1}), when administered immediately following seizures at P10, prevented the seizure-induced alterations in AMPAR function when slices were prepared at 2–3 h after hypoxia in vivo. In addition, hippocampal homogenates from rats sacrificed at 24 h after two doses (immediate and at 12 h post seizure) showed that treatment blocked the posttranslational changes in subunit phosphorylation. These studies demonstrate how this model can be used to examine putative steps in the epileptogenic cascade and determine points for therapeutic intervention.

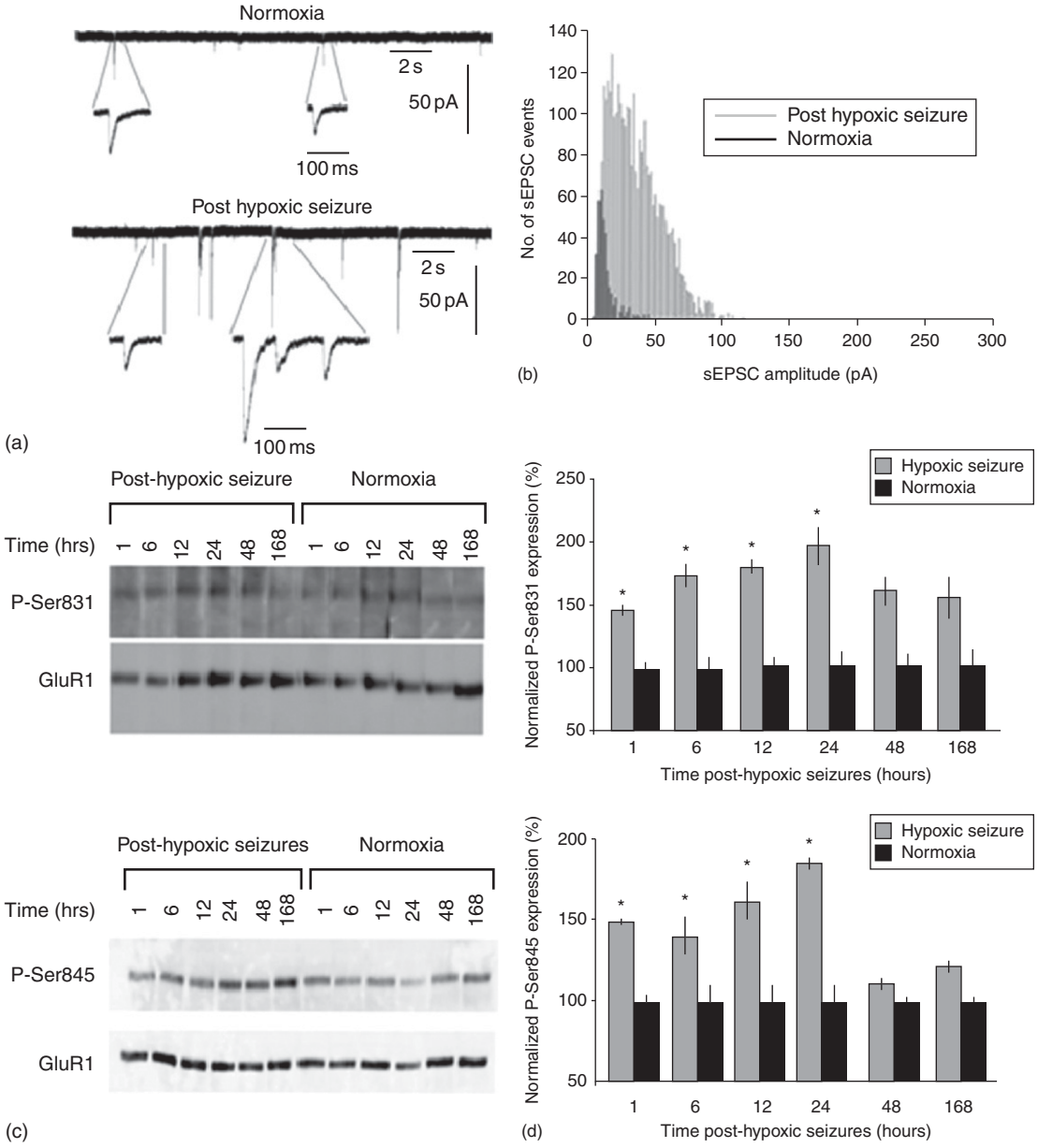


Figure 3 Enhanced AMPAR-mediated sEPSCs in CA1 pyramidal neurons and rapid increase in phosphorylation of GluR1 S831/S845 immediately following hypoxic seizures. (a) Representative recordings of AMPAR-mediated sEPSCs in CA1 neurons from normoxic control hippocampal slices (top) versus slices removed at 1 h after hypoxic seizures in vivo at postnatal day 10 (bottom). Individual sEPSCs are shown in detail at expanded time scale below the continuous traces (whole cell patch-clamp recordings at room temperature). (b) Amplitude histograms (normoxia sEPSC event $n = 368$, post-hypoxic seizure event $n = 3161$) were increased with larger amplitudes in CA1 pyramidal neurons in slices removed after hypoxic seizures (-24.4 ± 2.5 pA; $n = 12$) compared to control slices (-15.4 ± 1.0 pA, $n = 12$, $p < 0.05$). (c) Immunoblots showing significant, increase in phosphorylation of GluR1 S831, and (d) GluR1 S845 at 1, 6, 12, and 24 h post-hypoxia. Densitometric analysis and relative quantitation of phosphorylation of GluR1 subunit revealed the maximal increase at 24 h post-hypoxia for both GluR1 S831, (183%, $n = 3$, $p < 0.05$) and GluR1 S845 (190%; $n = 3$, $p < 0.05$) compared to normoxic controls. Reproduced from Rakhade S, *et al*. (2008) Early alterations of AMPA receptors mediate synaptic potentiation induced by neonatal seizures. *Journal of Neuroscience* 28: 7979–7990, with permission from Journal of Neuroscience.

Future Directions

We have employed a model that has been useful in identifying the roles of glutamate receptors in contributing to baseline seizure susceptibility in the developing brain, and for determining the consequences of glutamate receptor activation in early steps in epileptogenesis. The model has clinical relevance in that it uses a physiological convulsant stimulus, hypoxia, to generate ictal activity. Future studies will continue to probe the timeline of changes in glutamate receptors and their downstream signaling cascades that occur during brain development.

Beyond the early posttranslational changes, there are likely modifications in gene expression and in protein synthesis, and alterations in microarchitecture. Many status epilepticus models show that at any age, seizures appear to trigger the expression of more immature forms of glutamate receptors, even in mature tissue. Thus, insights into therapeutic interventions gained from investigations during early development, when these forms are robustly expressed, may have application to other epilepsy syndromes. In this context, we have begun to examine cortical dysplasias and tuberous sclerosis-associated lesions. Many laboratories have shown that neurons and glia in these 'islands' of cortical maldevelopment appear to have similar glutamate receptor expression patterns compared to those seen in early development.

Importantly, the contribution of the immature forms of glutamate receptors to the enhanced excitability of the immature brain does not occur in isolation. Inhibition is likewise developmentally regulated, with the full complement of functional GABAergic inhibitory synapses developing later than the peak expression for glutamatergic synapses. In addition, GABA receptors that are present contain a higher fraction of the α4 subunit, which encodes relative benzodiazepine insensitivity. Also, concurrent with the immature expression of glutamate receptors, the GABARs are depolarizing by virtue of the higher intracellular Cl^- concentration, as determined by the age-dependent relative expression of membrane Cl^- cotransporters (the importer NKCC1 and the exporter KCC2). During early postnatal development in the rat, KCC2 is expressed at low levels, and Cl^- remains high intracellularly; thus, upon activation of the GABAR, Cl^- flows out of the cell rather than in, resulting in a depolarization of immature neurons. We have recently shown that NKCC1 is upregulated in the second postnatal week in the rat and in the first year of life in the human, while KCC2 is expressed at minimal to low levels. Consequently, NKCC1 blockers (such as bumetanide) appear to improve the efficacy of GABAR agonists in suppressing seizures in the immature brain, including those in the hypoxia model. It is likely that there are a number of major factors contributing to seizures in the immature brain in addition to the unique patterns of expression of excitatory and inhibitory neurotransmitters. Future directions will include the study of interaction between the developmental regulation of glutamate receptors and other systems regulating circuit excitability.

Finally, we are in the process of developing this hypoxia-seizure model in mice. Given our observations suggesting critical roles for particular glutamate receptors and downstream signaling pathways, studies on transgenic mice – with up- or down-regulation of key factors – represent important 'proof-of-principle' experiments. We are presently evaluating the contribution of posttranslational modification of the GluR1 AMPAR subunit, using Ser 831/Ser 845 double phosphomutant mice in which these sites cannot be phosphorylated. These animals show decreased LTP and learning, and our hypothesis is that if the GluR1 site phosphorylation is truly necessary and sufficient to activate later events in epileptogenesis (following P10 hypoxia-induced seizures), these animals should show fewer seizures and less long-term epileptogenesis than wild-type mice. Similarly, we can evaluate the role of other glutamate receptor subunits. For example, using the NR3A knockout mouse, we will determine if the transient increase in NR3A seen in the seizure-susceptible second postnatal week in the rodent (and in the human neonatal brain) is essential for the process of epileptogenesis in the developing brain. Other mice, such as TSC2 mutant mice, where glutamate receptors are abnormally expressed on dysplastic neurons, might be useful in determining how seizure activity in early life affects glutamate receptor expression on dysplastic neurons. Our studies of human cortical biopsy tissue from TSC patients show that the dysplastic neurons and glia in tubers express immature forms of glutamate receptors NMDAR and AMPAR subunits, reminiscent of those expressed on immature neurons, radial glia and astrocytes in fetal and infant forebrain. The TSC2 mutant mouse is a good example of how these mouse models might prove useful in determining the role of specific glutamate receptors in generating epilepsy in rodent developmental models.

The developmental expression of glutamate receptors is likely to play an important role in a variety of early life seizure models. Many investigators have observed the relative hyperexcitability of the immature brain, as well as a relative underdevelopment of GABAergic inhibition. Given that much is being learned about age-specific targets for epilepsy suppression, investigators are now addressing specific mechanisms whereby seizures in early life disrupt network function beyond simply inducing epilepsy. Detailed understanding about the unique signaling cascades mediated by these immature glutamate receptors will be an important advance that could yield novel therapeutic strategies for the prevention of epilepsy and neurocognitive comobidities that arise in patients with early life seizures.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury from Seizures: Influence of Models, Development and Genetics; The Ontogeny of Seizure-Related Programmed Cell Death in the Developing Brain; **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; Epileptogenesis in Childhood; **Transporters**: Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures.

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Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability

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Introduction

In the search for animal models of seizures, treatment with convulsant compounds provides models of differing utility. Both acute and chronic models of seizures have a place in epilepsy research. Where the aim is primarily pharmacological, acutely generated seizures provide a convenient method for the assessment of anticonvulsant activity. In contrast, where the aim is to investigate basic mechanisms that may underlie seizures, the model should mimic epilepsy as much as possible – showing, for example, persistent, progressive seizures and appropriate histological changes. Since the 1970s, kainic acid has been known to produce acute seizure activity that was readily explained by dramatic neuronal depolarization caused by potent activation of glutamate receptors.

Administration of kainate in vivo, either intracerebroventricular (i.c.v.) or intraperitoneal (i.p.), results in seizures and hyperexcitability long after the kainate itself would have dissipated from the affected areas. The seizure induction in the kainate model is also accompanied by hippocampal neuronal loss and glial proliferation as observed in temporal lobe epilepsy. Thus, by displaying appropriate pathology, the model went beyond mere pharmacological mimicry of seizures. These observations served to promote i.c.v. kainate treatment as a model for temporal lobe epileptiform activity.

This early use of kainate tacitly recognized that there would be long-lasting consequences, although at that time there was no clear idea how these could occur. Major progress in defining some ideas and new hypotheses that could be tested resulted from advances in the understanding of glutamate receptor subtypes and the roles played by different subunits. We now realize, for example, that early use of kainate may not have discriminated between AMPA and kainate subtypes of glutamate receptors. Furthermore, the demonstration of metabotropic actions of glutamate receptors has now provided a plausible mechanism for seizure activity, which outlasts the presence of transmitter.

Background

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Background

Through the 1990s, the cloning and characterization of glutamate receptor subunits led to a now conventional

view of glutamate receptors. We now think of three types of tetrameric ionotropic receptors (AMPA receptors, NMDA receptors, and kainate receptors) as well as a 7-transmembrane domain, G protein-coupled metabotropic glutamate receptor. In tandem with these developments, the use of pharmacological tools that distinguished between the receptor categories helped investigators to build a picture of where and how these receptors contribute to neurotransmission.

The tetrameric kainate receptor (KAR) can comprise five different subunits (GluR5, GluR6, GluR7, KA1, and KA2). This diversity of subunit composition obviously allows for functional differences, which may also be correlated with the regional or cellular expression of the KAR. This point is illustrated by the research on postsynaptic and presynaptic actions of KARs.

KAR genes are common throughout the brain, but good evidence for their contribution to synaptic transmission was elusive until the advent of ligands that distinguished between the various glutamate receptors. The glutamatergic epsc was first shown to contain a kainate receptor component in pyramidal cells of the hippocampal CA3 region; synaptic activation of KARs has now been described for several synapses throughout the brain and spinal cord. In most cells, the KAR synaptic current has a peak amplitude that is small compared with that of the rest of the epsc, and has a slower time course.

In addition to their anticipated function in generating the epsc, KARs have also been shown to depress the release of both inhibitory and excitatory transmitters. Depression of glutamate release is thought to be caused by presynaptic KARs that decrease stimulus-evoked calcium influx. Depression of GABA release is caused by a presynaptic GluR5-containing KAR that is linked to G proteins and that requires PKC. It was this action on inhibitory synapses that first showed that kainate receptors on interneurons had a metabotropic function, usefully described by Lerma as 'noncanonical' signaling. The evolution in our understanding of KAR signaling is charted in a number of reviews, which are included in the Further Reading section.

Following the elucidation of ionotropic glutamate receptor subtypes and their respective functions in synaptic physiology, there were a few outstanding paradoxes where KARs failed to contribute to synaptic responses. For example, in CA1 pyramidal cells KARs cause robust inward currents, which are lacking in GluR6-deficient mice, yet these active receptors make no contribution to excitatory synaptic responses. This demonstration of a KAR whose main ionotropic action was unused, and the potential for its mediating metabotropic effects, provided the stimulus for our work on the role of KARs and intrinsic excitability.

One of the most prominent postsynaptic targets of metabotropic transmitter action in the hippocampus is the slow afterhyperpolarization (sAHP) of CA1 and CA3 pyramidal cells. This calcium-dependent potassium current is activated according to the number and frequency of action potentials within a burst, and provides a negative feedback on excitability. In this review, we use the acronym sAHP to refer either to the voltage recording or to the underlying current. The large variety of transmitters (and their second messengers) that inhibit the sAHP includes noradrenaline via cAMP/protein kinase A and muscarinic actions of acetylcholine via Ca^{2+}-calmodulin-dependent protein kinase II. Protein kinase C activation also represents a potent pathway for sAHP inhibition; although initially PKC was presumed to be the second messenger for acetylcholine, this notion had been discredited and the transmitter/receptor for this PKC action is unclear.

We tested whether high-affinity KAR sites might provide a postsynaptic metabotropic function in CA1 pyramidal cells. The likely target of the metabotropic action was postulated to be the slow AHP. This current is susceptible to modulation by a wide range of transmitters that increase excitability (as described earlier) and had been known, years before, to be attenuated in kainate epilepsy models. Here, we describe some key experiments that tested the hypothesis and provided a basis for understanding the transduction mechanism.

Methods

The work described in this article was performed on the slice preparation of a rat hippocampus maintained in vitro. Single cell electrophysiological recordings were used as the functional readout for investigations into kainate receptor actions in hippocampal CA1 pyramidal cells. Well-defined synaptic pathways within the slice are preserved and can be stimulated to release endogenous glutamate. The hippocampal slice is additionally accessible to pharmacological interventions at either the extracellular or the intracellular level. In the latter case, compounds can be introduced into the cell via whole cell recording pipettes, and assessed for their actions in modulating second messenger pathways.

Recent Results

An important first step was to distinguish between metabotropic and ionotropic actions of kainate. **Figure 1(a)** shows that application of 200 nM kainate inhibited the slow AHP by 30–40%. This inhibition had an IC_{50} of ~15 nM and a plateau of 100 nM; these values are comparable to the reported binding affinities of the KAR. However, at these concentrations kainate does not generate inward currents in CA1 pyramidal cells; that is, no detectable ionotropic response is generated.

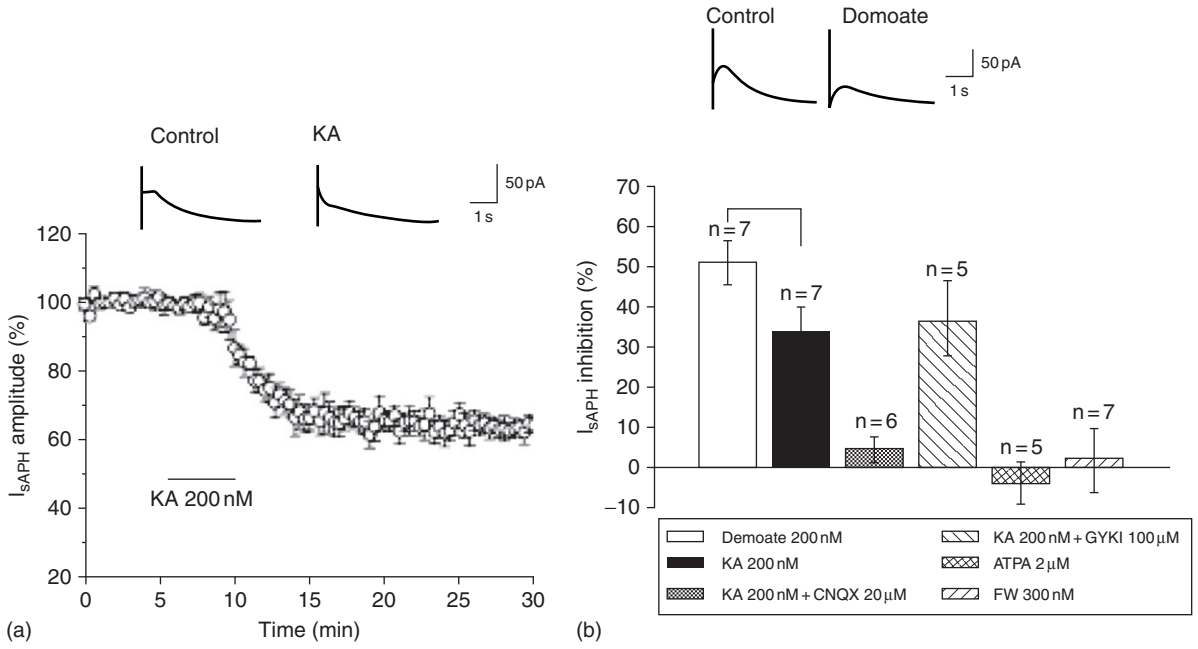


Figure 1 Inhibition of the sAHP current is mediated by kainate receptors. (a) Time course of sAHP inhibition ($n = 7$). Traces show the sAHP current before and after application of 200-nM kainate. (b) Traces show the effect of 200-nM domoate on the sAHP current. The histogram shows average percentage inhibition of sAHP current (mean ± SEM). Domoate and kainate both inhibit the sAHP current; this is blocked by the mixed GluR5/GluR6 antagonist CNQX. The action is not blocked by a GluR5 antagonist (GYKI52466). The GluR5-preferring agonist ATPA and the AMPA receptor agonist fluorowillardine (FW) had no inhibitory effect on the sAHP current.

Following this initial finding, we carried out a series of experiments to test whether exogenous kainate was acting directly on CA1 cells, and then to define the receptor type that might be involved. Significantly, the KAR ligand, domoate – which is more potent as a KAR agonist than kainate itself – caused greater inhibition of the sAHP. This result lent credence to the idea that a KAR was involved in this effect. **Figure 1(b)** shows the activity of different AMPA and KAR ligands on the inhibition of the sAHP. The inhibitory action of kainate on the sAHP was neither blocked by GluR5-preferrring antagonists (GYKI) nor mimicked by GluR5-preferring agonists (ATPA). This pharmacology distinguishes the KAR composition on the CA1 pyramidal cell from that found on interneurons, where GluR5 is a component subunit. A potent AMPA receptor agonist was without effect of the sAHP, even though the nonselective AMPA-kainate receptor antagonist CNQX was an effective blocker of kainate action. These data were supported by later work in other laboratories, which showed that metabotropic regulation of the sAHP by kainate was absent in GluR6-deficient mice and persisted in GluR5 deficiency. In summary, both pharmacological and genetic manipulations suggest that kainate receptors containing GluR6 subunits are essential for modulation of the sAHP by KARs.

It is clear then that kainate application can alter the membrane properties of hippocampal pyramidal cells. A more valuable interpretation can be placed on this information if we know that these KARs can be engaged by synaptically released glutamate. Glutamate is released by the vast majority of the excitatory afferents onto CA1 pyramidal cells. To test if this endogenous transmitter could generate metabotropic activity of KARs, synaptic stimulation was performed in the presence of a cocktail of blockers, which excluded the following from possible contributions to an effect: AMPA receptors, NMDA receptors, $GABA_A$ and $GABA_B$ receptors, metabotropic glutamate receptors, and muscarinic, opioid, and cannabinoid receptors.

Under these conditions, the synaptic current in response to afferent fiber stimulation is abolished and no further ionotropic response is observed even after a short tetanus (**Figure 2(a)**). The sAHP was then subjected to a control recording period of 10 min, after which the glutamate released by the short stimulus train was sufficient to cause inhibition of the sAHP, as illustrated by the pooled data from eight cells in **Figure 2(b)**. This action was blocked by CNQX and so was likely to result from synaptically released glutamate acting on KARs left operational by the blocking cocktail. Following the synaptic release of glutamate, the time course of inhibition of the sAHP was similar to that observed with bath-applied kainate. Since we can safely assume that synaptically released glutamate is taken up quite rapidly in comparison with washout of bath-applied kainate, we postulated that the prolonged time course of the inhibition may reflect the transduction mechanism.

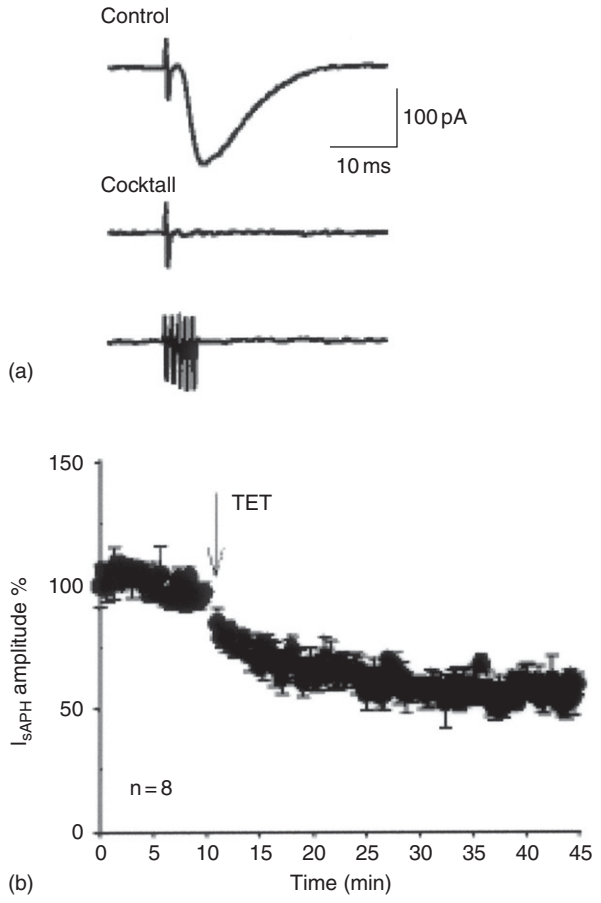


Figure 2 Synaptically released glutamate inhibits the sAHP current. (a) An epsc evoked by stimulation of the Schaffer/commissural afferents to CA1 is blocked by an antagonist mixture (see text for details). No further synaptic response was evoked by a short train of five stimuli. (b) Time course of inhibition of the sAHP current ($n = 8$) following the train of five stimuli to evoke glutamate release. Reproduced from Melyan *et al*. (2004) *Journal of Neuroscience* 24: 4530–4534, with permission from the Society for Neuroscience.

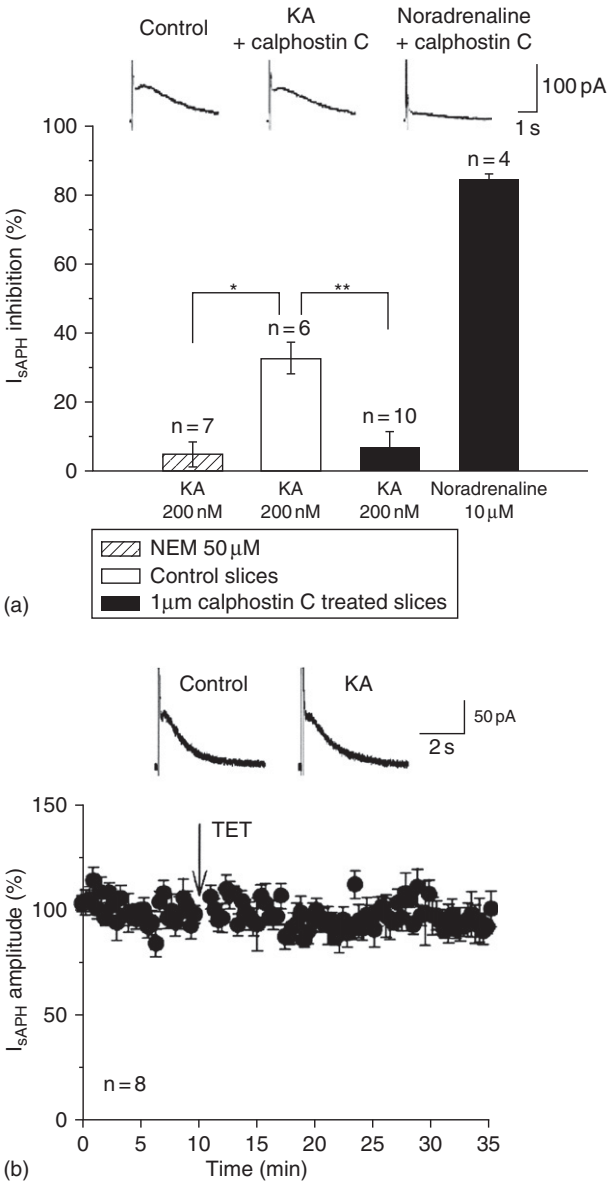


Figure 3 Metabotropic action of kainate receptors on the sAHP current. (a) Sample traces showing that calphostin treatment prevents the action of kainate (KA) while sparing noradrenaline inhibition of sAHP current. The first three bars in the histogram show that inhibition of sAHP current by 200-nM kainate is blocked by the G protein inhibitor NEM or the PKC inhibitor calphostin C. The final bar shows that inhibition by noradrenaline is intact after block of PKC. (b) Time course of sAHP current after tetanic stimulation to release glutamate in calphostin C-treated hippocampal slices. PKC is required for the action of both kainate and synaptically released glutamate. Reproduced from Melyan *et al*. (2004) *Journal of Neuroscience* 24: 4530–4534, with permission from the Society for Neuroscience.

It is axiomatic that a metabotropic receptor action requires a G protein, which acts to couple the receptor with downstream elements of the transduction mechanism. There is no working hypothesis to account for this G protein coupling for a classical ionotropic receptor, and to date it remains somewhat a puzzle – although pharmacological approaches unequivocally point to involvement of G proteins. First, we showed that inhibition of the sAHP by kainate was prevented by NEM (**Figure 3(a)**), which is a known blocker of pertussis toxin-sensitive G proteins. Since NEM is not a very specific inhibitor; we performed follow-up experiments using intracellular exposure to pertussis toxin itself or the $G\alpha_{i/o}$ blocker, NF023. These compounds preferentially depressed kainate action, but β-adrenergic inhibition of the sAHP was unaffected (a control). Thus, three pharmacologically distinct blockers point to the involvement of $G\alpha_{i/o}$ in the metabotropic action of KARs.

Protein kinase C has long been known to inhibit the sAHP. When first observed, this effect was assumed to be linked with muscarinic receptor action. As this and other receptors were invalidated as triggers, PKC-dependent inhibition of the sAHP started to look like an orphan

mechanism. However, in 1998 Rodriguez-Moreno and Lerma reported a role for PKC and presynaptic KARs. This report prompted us to perform similar tests, which demonstrated that inhibition of PKC (using calphostin C) blocked the action of kainate to inhibit sAHP (**Figure 3(a)**). In contrast, the inhibitory action of noradrenaline (a cAMP-dependent mechanism) applied later in the same experiment remained unaffected. Corresponding to these observations, calphostin C also abolished the action of synaptically released glutamate on the sAHP (**Figure 3(b)**).

One general limitation of antagonist experiments is that they may simply demonstrate a constitutive requirement rather than revealing a step in a critical pathway. To provide a fuller picture of the role of PKC, we tested specifically whether PKC activation alone could inhibit the sAHP. Both indolactam V and OAG activate PKC directly, and both caused inhibition of the sAHP (**Figure 4(a)** and **4(b)**), which corroborated earlier data using phorbol esters.

We also noted interesting quantitative differences in their action. The action of OAG was partial and reversible (**Figure 4(b)**); these properties are possibly because OAG is a synthetic analog of the natural ligand (DAG) and is subject to metabolic breakdown. In contrast, indolactamV is a powerful but unnatural activator; accordingly, inhibition ($IC_{50} \sim 110$ nM) was complete at 1 μM. The inactive analog of indolactam was without effect.

Up to this point, these data demonstrated that both kainate and synaptically released glutamate could act on -GluR6-containing KARs in CA1 pyramidal cells to trigger a PKC-mediated inhibition of sAHP. In the study of PKC action, we had been using noradrenergic inhibition of the sAHP as a comparator, to contrast with the transduction mechanism involved in the action of kainate and act as a control. To complete the logic of assessing the presumed independence of the two transduction pathways, we performed the reverse experiment – that is, blocking the protein kinase involved in noradrenergic action (PKA) while examining kainate action. The result overturned our expectations and we were instead surprised to discover that PKA was required for kainate inhibition of the sAHP. Two distinct PKA inhibitors (Rp-cAMPs and H89) prevented the action of kainate; the adenylyl cyclase inhibitor DDA showed that there was also a corresponding requirement for cAMP production. In an attempt to impose an order on these observations, we performed experiments to determine the position of PKA and PKC in the signaling pathway. The action of PKC on the sAHP was unaffected by inhibition of PKA, which led to the proposal that PKC is downstream of PKA.

Despite these new observations about the KAR transduction mechanism, it was still difficult to account for the slow recovery from kainate action. Did this slow recovery represent a unique property of KARs or an unexplored property of the transduction pathway? In CA1 cells, both PKC and β-adrenergic receptors, acting

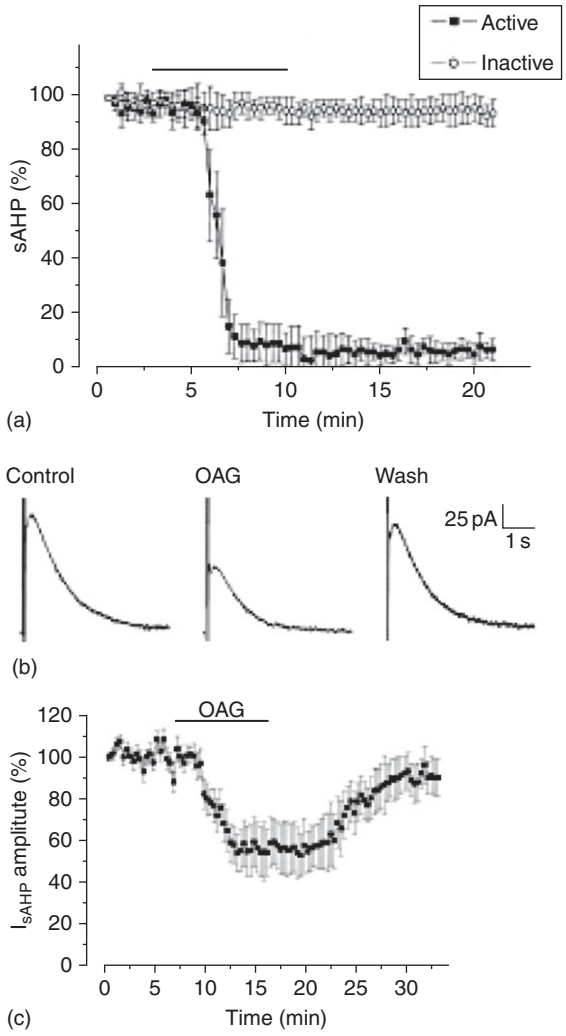


Figure 4 PKC activity is sufficient to inhibit the sAHP current. (a) Time course of the sAHP current in response to extracellular application of active or inactive forms of the PKC activator indolactam V (1 μM, $n = 8$). (b) Extracellular application of the PKC activator diacylglycerol analog OAG (50 μM, $n = 5$) causes reversible inhibition of the sAHP current.

via PKA, are able to engage the MAP kinase cascade. This effector mechanism had not been tested on the sAHP, but had the potential to account for long-lasting actions of transmitters since MAP kinases are known to be involved in late stage long-term potentiation. This action is not, however, inevitable because activation of MAP kinases by β-adrenergic receptors is a product of time and transmitter concentration.

Normally, brief exposure to isoprenaline causes profound inhibition of the sAHP, which recovers fully. Given that β-adrenergic receptor coupling to MAP kinase depends on the duration of receptor activation, we tested extended isoprenaline exposure. The result was a prolonged inhibition of the kind seen with KARs. As a consequence we were encouraged to test the involvement of MAP kinase in kainate inhibition of sAHP.

MAP kinase activity was tested initially with an in vitro assay for phosphorylated MAP kinase. This testing revealed that tonic MAP kinase activity exists in the hippocampus under the conditions of our experiments, and that this MAPK kinase activity is enhanced by 15-min exposure to 200-nM kainate; recovery back to a stable control level occurs within 30 min. The effect of kainate is abolished by the overall loss of phosphorylated MAP kinase in the presence of the MEK inhibitor U0126 (**Figure 5(a)**).

The ability of bath-applied kainate to inhibit the sAHP was blocked either by U0126 or by a structurally distinct inhibitor, PD098059. These data illustrate that the MAP kinase cascade contributes to the metabotropic response of GluR6-containing KARs in the hippocampus. To assess further the physiological readout of the MAP kinase activity observed in the in vitro assay, we measured (in two separate groups of experiments) the sAHP during up- or downregulation of MAP kinase. **Figure 5(b)** illustrates that the slow AHP increases during extracellular application of the MEK inhibitor U0126; conversely, when activated MAP kinase is included within the recording pipette to provide access to the cytoplasm, the slow AHP is suppressed (**Figure 5(c)**). These results provide a demonstration of a bidirectional mechanism in which MAP kinase activity is 'read out' as changes in electrical membrane properties and neuronal excitability.

Future Goals

There is an important missing link in the presumptive metabotropic aspect of KAR function: namely, that there is no obvious molecular mechanism to explain how the glutamate receptor subunits that comprise KARs might allow for G protein interaction. A proper accounting for the observations with G protein inhibitors will be an important test of the metabotropic hypothesis of KAR function.

The finding of a requirement for the MAP kinase cascade represents the most recent part of our work and so raises the most unanswered questions. Obviously, we need to know what happens next – does MAP kinase activation by KARs lead to regulation of gene expression, as proposed in synaptic plasticity? A more general, but important, question is to determine if MAP kinase represents a common hub through which many transmitters act to inhibit the slow AHP. This unifying notion would help to explain why many receptors seem able to target this particular current.

Studies of β-noradrenergic receptors suggest that duration of receptor occupancy can be an important determinant of downstream events – in particular, activation of second messengers that do not become involved when there are more transient changes in transmitter. This characteristic may be an adaptive feature in the case of long-term potentiation. In other circumstances, prolonged exposure to transmitter may be maladaptive. For example, what are the long-term consequences when glutamate receptors are exposed to prolonged glutamate release during seizure activity? The metabotropic action of KARs

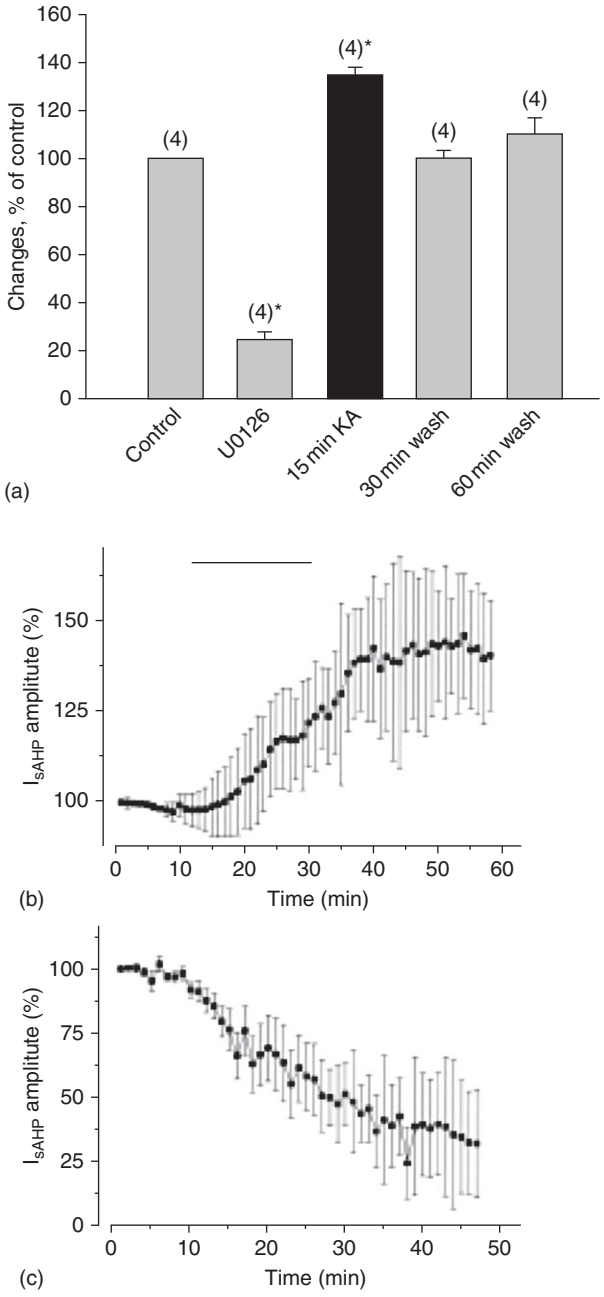


Figure 5 The MAP kinase signaling pathway is involved in suppression of the sAHP current by kainate. (a) Fluorescence intensity measurements of a phosphorylated MAP kinase assay. Kainate enhances the phosphorylated MAP kinase signal; this effect was not observed in the presence of U0126, which greatly reduces levels of phosphorylated MAP kinase. Bars represent mean ± SEM from four experiments (b) Time course of the effect of application of U0126 (bar, 10 μM) on amplitude of the sAHP current ($n = 4$). (c) Effect of intracellular dialysis with activated MAPK on the amplitude of the sAHP current.

would predict a persistent loss of the slow AHP – a phenomenon that has been described after i.c.v. kainate. Even in normal tissue, this loss would enhance excitability because the cell would be more responsive to all excitatory input. Additionally, since the slow AHP functions partly to curtail bursting, loss of this negative feedback could lead to an increased frequency and duration of the paroxysmal depolarization shifts seen in epileptiform activity. Thus, activation of KARs during seizure activity could have long-term consequences for neuronal electrical properties by sensitizing and exacerbating the responses to epileptogenic stimuli.

See also: **Glutamate**: Functional Reorganization of Inhibitory Circuits in Epilepsy: mGluR1/5 Signaling Mechanisms and Long-Term Plasticity; Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; Role of Kainate Receptors in Glutamatergic Synaptic Transmission: Implications for Acute and Chronic Seizure Generation; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis.

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Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation : Impact on Seizure Expression and Implications for Epileptogenesis

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Introduction

Epileptogenesis is the process in which normal brain develops a persistent propensity for the spontaneous production of seizure discharges. In adults, this process can take place following cortical strokes, head trauma, or an episode of status epilepticus; a latent period of months to years is typical before the chronic recurrent seizures are expressed. The changes that initiate this transformation are numerous: in many cases, there is neuronal cell death and rewiring of neural circuits via aberrant axonal sprouting. Gliosis and hemosiderin deposits may also be present and contribute to the process. Temporal lobe, especially hippocampus, seems particularly vulnerable to the epileptogenic process, and hippocampal sclerosis or other evidence of gross structural damage is a common finding in those who develop epilepsy following head trauma. However, there are patients who sustain relatively minor head trauma, with no obvious lesion on imaging studies, who go on to develop posttraumatic epilepsy. The cascade of events that finally culminates in epilepsy remains unclear. One common initiating factor is unequivocal: in all patients, there is massive glutamate release accompanying the inciting event. The primary focus of the studies undertaken in our laboratory is to understand the potentially-epileptogenic long-term sequelae of excessive glutamatergic activation, in hopes of finding ways to interfere with the epileptogenic process.

Background

Glutamate, the major excitatory neurotransmitter of the brain, acts on two classes of receptors: ionotropic (NMDA

would predict a persistent loss of the slow AHP – a phenomenon that has been described after i.c.v. kainate. Even in normal tissue, this loss would enhance excitability because the cell would be more responsive to all excitatory input. Additionally, since the slow AHP functions partly to curtail bursting, loss of this negative feedback could lead to an increased frequency and duration of the paroxysmal depolarization shifts seen in epileptiform activity. Thus, activation of KARs during seizure activity could have long-term consequences for neuronal electrical properties by sensitizing and exacerbating the responses to epileptogenic stimuli.

See also: **Glutamate**: Functional Reorganization of Inhibitory Circuits in Epilepsy: mGluR1/5 Signaling Mechanisms and Long-Term Plasticity; Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; Role of Kainate Receptors in Glutamatergic Synaptic Transmission: Implications for Acute and Chronic Seizure Generation; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis.

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Introduction

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Background

Glutamate, the major excitatory neurotransmitter of the brain, acts on two classes of receptors: ionotropic (NMDA

and AMPA/kainate) and metabotropic. The metabotropic receptors are G-protein-coupled and activate various second messenger systems, resulting in both short-term and long-term changes in cellular excitability. Eight subtypes of metabotropic glutamate receptors (mGluRs) have been identified and classified into three subgroups. Group I mGluRs are primarily postsynaptically localized and have mostly excitatory effects, while group II and group III mGluRs are predominantly presynaptic and suppress transmitter release. A controversial ninth mGluR subtype appears to be linked to phospholipase D (PLD) activation and does not belong to any of the three subgroups; this receptor can be activated by cysteine sulfinic acid. Work in my laboratory has focused on the group I and PLD-coupled mGluRs and their roles in the expression of synchronized activity in the hippocampal network.

The Group I mGluRs

The group I mGluRs (comprised of mGluR1 and mGluR5) are located in numerous regions throughout the brain, and are especially prominent in the CA3 region of the hippocampus, where they are expressed in pyramidal cells, interneurons, and glia. These receptors tend to be perisynaptically localized, suggesting that they are recruited primarily during enhanced synaptic activity, with the increased glutamate release resulting in spillover into perisynaptic zones. Such enhanced activity might occur during normal physiological functions such as learning and memory, as well as pathological events such as strokes, seizures, and head trauma. The group I mGluR subgroup consists of the only phosphoinositide-linked glutamate receptors identified to date. They are coupled to the activation of phospholipase C (specifically, PLCβ1), which catalyzes the production of diacylglycerol (resulting in protein kinase C (PKC) activation) and inositol triphosphate (resulting in intracellular calcium mobilization). The cascade of events initiated by either of these pathways could potentially contribute to the induction and/or maintenance of long-lasting excitatory effects mediated by group I mGluRs.

Effects of Group I mGluR Activation on Cellular Excitability

Activation of group I mGluRs elicits depolarization of hippocampal neurons through several mechanisms, including suppression of a variety of intrinsic potassium conductances, activation of a nonspecific cationic conductance, activation of the sodium-calcium exchange current, and induction of a novel voltage-gated inward current. Most of these effects are seen within milliseconds of receptor activation and are not necessarily persistent. Hence, these cellular effects would be expected to contribute to the early excitatory synaptic and network effects of group I mGluR activation.

Long-Lasting Effects of Group I mGluR Activation on Synaptic Efficacy and Network Excitability

At the synaptic level, the group I mGluRs have been shown to participate in hippocampal long-term potentiation (LTP) and long-term depression (LTD), and therefore activation of these receptors is likely to be important for cognition. Increased glutamate release has also been a reported effect of group I mGluR activation. Although increased glutamate release could be secondary to depolarization-induced increases in cell firing, it may also result from direct activation of presynaptic or glial mGluRs. Either way, increases in glutamate release could increase activation of perisynaptic mGluRs, thereby further increasing the mGluR response. At both the synaptic and network levels, one would expect this domino effect to assist in producing the types of plasticity necessary for learning and memory consolidation; however, in excess, one might expect such hyperexcitation at synapses and neuronal networks to have damaging effects. Consistent with this assumption, in vivo studies reveal that group I mGluR activation not only elicits seizures but can also result in excitotoxic neuronal injury.

It should then be not surprising that baseline expression of group I mGluRs has been correlated with differences in seizure susceptibility in rodents. Additional studies in knockout mice suggest that seizures expressed in patients with Fragile X syndrome may be the result of hyperexcitation of group I mGluRs. Indeed, group I mGluR antagonists have been demonstrated to have anticonvulsant efficacy in several models.

Our studies and findings (among the first to explore this area of interest) have been consistent with those of others: group I mGluR activation does indeed enhance the expression of seizure discharges in the hippocampal slice preparation, and group I mGluR antagonists can suppress the expression of these discharges. What struck us, however, as the most significant effect of group I mGluR activation in our in vitro experiments was that the resultant seizure discharges do not disappear upon removal of the agonist, indicating that a long-lasting modification of network excitability is induced by group I mGluR agonist exposure. This effect is different from those seen in other in vitro models in which pharmacologic manipulations are employed to elicit acute seizure discharges. Given these observations, we have hypothesized that these receptors may be involved in the initiation of epileptogenesis. Our laboratory has consequently embarked on studies to elucidate the underlying pathways involved in greater detail.

Methods

The Guinea Pig Hippocampal Slice

Our laboratory uses the guinea pig acute hippocampal slice preparation. Guinea pigs are used because, unlike mice and rats, their brains mature at a much earlier time point after birth; in fact, they are born at a later stage of development than humans, whereas mice and rats are born relatively premature. Hence, our studies are believed to be relevant to the adult human condition.

We have used 400 μm acutely-prepared transverse hippocampal slices for our experiments. The acute hippocampal slice preparation offers many advantages over other experimental systems. Unlike in vivo studies, one can precisely control the environment of the slice (temperature, pH, oxygenation, delivery of metabolites). Furthermore, carefully titrated pharmacological manipulations are easily carried out and prolonged intracellular recordings can be obtained. In addition, in contrast to the dissociated cell preparation, much of the normal circuitry of the hippocampus is retained in the slice preparation by virtue of the lamellar organization of this particular brain region, allowing for the expression of network-generated activity.

Electrophysiological and Pharmacological Approaches

Electrophysiological approaches used in our lab include intracellular recordings from pyramidal cells and concurrent extracellular field recordings to document synchronized activity; dual intracellular recordings may be performed as well. The interface chamber is used to maintain the acutely-prepared slice in a stable environment, maintaining the health of the slice for the duration of the experiment (typically 3–6 h of recording). In such a system, activity recorded may be evoked with a stimulating electrode placed in a pathway afferent to the recorded cell; alternatively, spontaneous activity may be observed at rest and in response to pharmacological manipulations. Pharmacologically-evoked activity may be elicited by bath perfusion of the agent or by brief focal application. Recordings are amplified and digitized, then stored for later analysis.

The Picrotoxin Model of Interictal Activity

Numerous studies have explored mechanisms of synchronization in the hippocampal slice, and these studies have revealed a critical role for AMPA and NMDA receptors in the generation of the paroxysmal depolarization shift, the intracellular correlate of the interictal spike. Picrotoxin is a commonly used agent for the generation of interictal-like activity in the hippocampal slice: via the block of $GABA_A$ receptor-mediated inhibition, spontaneous recurrent synchronized bursts are generated. Each of these bursts is typically less than half a second in duration (see, for example, control bursts in **Figure 1**) and is mediated entirely by AMPA/kainate and NMDA activation (the ionotropic glutamate receptors). Additional blockade of $GABA_B$ receptors has little effect on the length of these bursts (**Figure 1(a)** left), indicating that complete block of all GABAergic inhibition in the acute guinea pig hippocampal slice is insufficient to elicit ictal-length discharges. We therefore begin all our experiments with picrotoxin, which serves as a useful gauge of network integrity and allows us to begin our manipulations with uniformly healthy slices (so that interslice variability is minimized). This baseline treatment also allows us to eliminate the GABA receptor or interneuron as a site of action of our mGluR agonist.

Recent Results

Epileptogenic Propensity Resulting from Selective Group I mGluR Activation

In hippocampal slices, we have observed that exposure to selective group I mGluR agonists such as (*S*)-3,5-dihydroxyphenylglycine (DHPG) or (*S*)-3-hydroxyphenylglycine (3HPG) results in the expression of long synchronized oscillatory discharges (each burst 1–8 s in duration; **Figure 1**). We consider these oscillatory discharges to be ictaform because: (1) they are synchronized, (2) they exceed 1 s in duration, suggesting that a clinical correlate (i.e., seizure) would occur if such a burst was experienced in vivo, and (3) the oscillations comprising the discharges gradually change in frequency and amplitude, which is typical of a seizure discharge. Hence, this finding suggests that group I mGluR activation can be a trigger for the interictal-to-ictal transition. These data are supported by results of in vivo experiments from other laboratories, which reveal that group I mGluR activation elicits seizures, and that seizures in numerous models can be suppressed with group I mGluR antagonists.

Unlike other in vitro models of pharmacologically-induced epileptiform activity, the ictaform discharges elicited by transient exposure to group I mGluR agonist persist following washout of the agonist (**Figure 2**). Additional experiments have supported the contention that this observation represents true evidence of network plasticity induced by the activation of the group I mGluRs. Should such plasticity be induced in vivo, it would likely predispose the subject to experience recurrent unprovoked seizures. We therefore refer to this finding as 'in vitro epileptogenesis.' True epileptogenesis, of course, would take longer to occur and would persist indefinitely; our system only allows us to follow the phenomenon for several hours. Nevertheless, we believe that this finding has relevance to the earliest inciting events that trigger the epileptogenic process, and left unchecked would likely result in full-blown epileptogenesis.

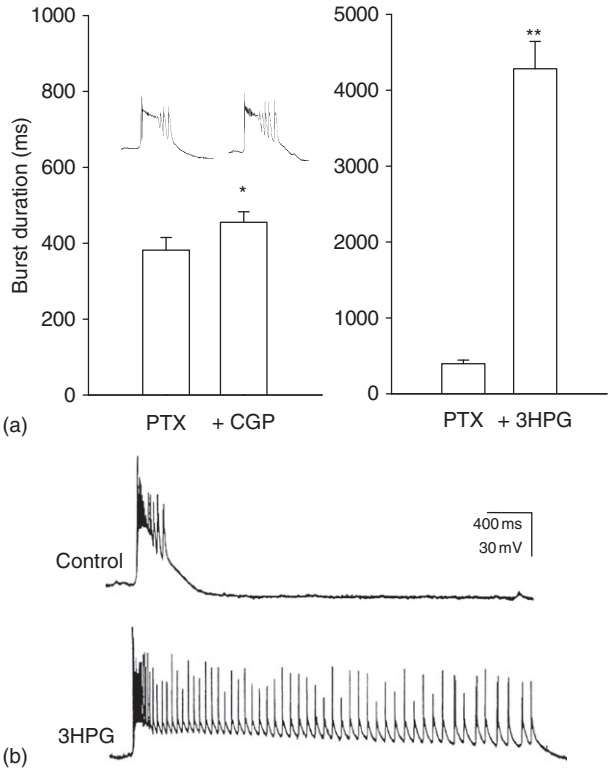


Figure 1 *Selective activation of group I mGluRs elicits interictal-to-ictal transition*. (a), *Left:* Summary data of effect of $GABA_B$ antagonist on length of picrotoxin-induced interictal bursts, revealing minimal lengthening, with bursts remaining below 540 ms in duration in all cases. Inset above graph displays bursts from one representative experiment before and after $GABA_B$ antagonist (adapted with permission from Huszár P and Merlin LR (2004) Contribution of $GABA_B$ receptor-mediated inhibition to the expression and termination of group I mGluR-induced ictaform bursts. *Epilepsy Research* 61: 161–165). (a), *Right:* Summary data of effect of selective group I mGluR agonist on PTX bursts (note difference in Y axis scale of the two bar graphs), with induced bursts ranging from 1–7 s in duration (adapted with permission from Merlin LR and Wong RKS (1997) Role of group I metabotropic glutamate receptors in the patterning of epileptiform activities in vitro. *Journal of Neurophysiology* 78: 539–544). *, $P < 0.05$; **, $P < 0.01$. (b): Representative recordings from a CA3 pyramidal cell in a guinea pig hippocampal slice. Initial interictal burst ('control') is a synchronized discharge expressed in the presence of picrotoxin, the $GABA_A$ receptor antagonist. After introduction of 3HPG, a selective group I mGluR agonist, synchronized bursts gradually lengthened, reaching ictal length (over 1 s duration) within 20–40 min (reproduced with permission from Merlin LR and Wong RKS (1997) Role of group I metabotropic glutamate receptors in the patterning of epileptiform activities in vitro. *Journal of Neurophysiology* 78: 539–544). PTX: picrotoxin, a $GABA_A$ antagonist; CGP: CGP35348, a $GABA_B$ antagonist; 3HPG: selective group I mGluR agonist.

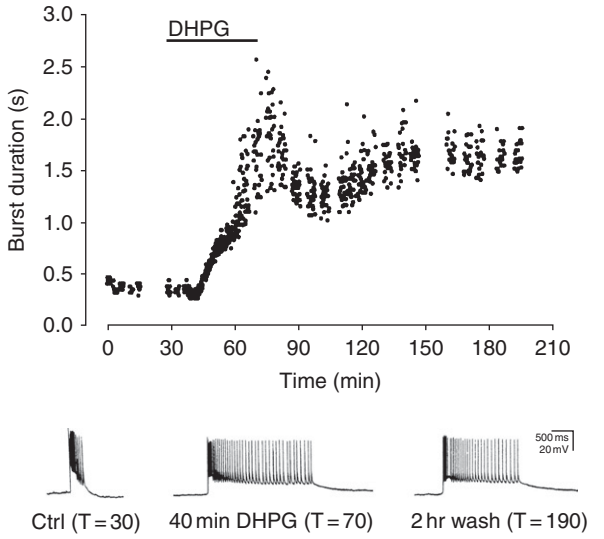


Figure 2 *Selective activation of group I mGluRs elicits persistent epileptiform activity*. Example of time course of change in burst length following 40 min exposure to DHPG, a selective group I mGluR agonist. Note that after removal of the agonist, ictal-length bursts continue to be expressed, suggesting initiation of epileptogenesis. Traces below graph are bursts recorded at times indicated on graph (reproduced with permission from Merlin LR (1999) Group I mGluR-mediated silent induction of long-lasting epileptiform discharges. *Journal of Neurophysiology* 82: 1078–1081).

Induction of In Vitro Epileptogenesis via Group I mGluR Activation: Key Features

The evaluation of the mechanisms for the network plasticity that underlie epileptogenesis is similar to the evaluation of synaptic plasticity mechanisms underlying LTP. One needs to tease apart the factors that are critical for induction (i.e., the initial trigger for the plasticity) vs. the factors that underlie the maintenance (i.e., the sustained expression of the enhanced activity). We have embarked on these studies; a summary of our findings to date is provided in **Table 1**. The agents that can suppress the expression of the seizures in the maintenance phase may be effective anticonvulsants for mGluR-induced seizures, but only the agents the can block induction are likely to have antiepileptogenic potential in patients whose epilepsy is induced by hyperstimulation of group I mGluRs.

Of the experimental findings on **Table 1**, the following are noteworthy: (1) the lack of involvement of NMDA receptor activation in the induction process, which is in marked contrast to many types of LTP; (2) the requirement of active protein synthesis for induction but not maintenance (the rapidity of the response suggesting no need for transcription, only translation); and (3) the ability of group I mGluR activation alone (in the absence of other glutamate receptor activation), and especially mGluR5 activation, to induce the persistent prolonged bursts. These findings suggest that AEDs that block AMPA or NMDA receptor-mediated excitation, while potentially effective at suppressing the expression of seizure discharges, will not suffice to prevent progression of the subclinical

Table 1 Induction vs. sustained expression of group I mGluR-induced epileptogenesis

Experimental protocol	*Effect on induction (i.e., blocks epileptogenesis?)*	*Effect on expression (i.e., suppresses seizures?)*
AMPA receptor blocker	Does not impede induction	–
NMDA receptor blocker	Does not impede induction	Does not suppress seizure expression or length of seizure discharges
Combined AMPA and NMDA receptor blockers	Does not impede induction	Blocks expression of both interictal and ictal-length synchronized discharges
mGluR1 antagonist	Does not impede induction	*Markedly reduces length of seizure discharges*
mGluR5 antagonist	*Blocks induction*	*Moderately reduces length of seizure discharges*
Protein synthesis inhibitors	*Blocks induction*	Does not suppress seizure expression or burst length
Activation of PLD-coupled mGluRs with CSA	*Blocks induction*	Does not suppress seizure expression or burst length
PKC inhibitor chelerythrine	Does not impede induction	Does not suppress seizure expression or burst length
$GABA_B$ receptor blocker	–	*Increases length of seizure discharges*

epileptogenic process potentially driven by group I mGluR activation.

Once this enhanced network excitability has been induced, the normal state cannot be restored; all we can do is reversibly suppress the expression of the seizure discharges. Hence the key to 'curing' the epilepsy is in prevention of epileptogenesis. To date, only two types of agents with potential clinical usefulness have been found effective in preventing mGluR-induced epileptogenesis in our model: an mGluR5-selective antagonist and cysteine sulfinic acid, an agonist of the putative (elusive) PLD-coupled mGluR. Although protein synthesis inhibitor is also effective, it is hard to envision a method by which to deliver such an agent without invoking excessive toxicities.

Maintenance of Group I mGluR-induced Epileptogenesis: What Underlies the Sustained Expression of Ictal Discharges?

One might expect NMDA receptor activation to be a participant in the expression of group I mGluR-induced prolonged bursts in light of the facts that (1) NMDA activation can indeed elicit burst prolongation in other systems, and (2) group I mGluR activation can enhance NMDA receptor-mediated responses. Surprisingly, blocking NMDA receptors had no impact on the length of the seizure discharges induced by group I mGluR activation. The only effective agent for reversibly shortening the persistent bursts is group I mGluR antagonist, suggesting that the transient activation of group I mGluRs persistently enhances the ability of these same receptors to participate in the ongoing expression of ictal discharges. This action could be the result of direct enhancement of receptor excitability or responsiveness to glutamate, increased number of receptors or insertion of receptors in the membrane, or their translocation to the synapse, increased anchoring protein (Homer), and/or reduced desensitization or constitutive receptor activation, to name a few possibilities. Once induced, neither protein synthesis inhibitors nor cysteine sulfinic acid can suppress the ongoing seizures, even though either agent is highly effective at blocking the epileptogenic process when present during attempted induction.

Further studies were performed to unravel the separate roles of mGluR1 vs. mGluR5 (the two members of group I) in the induction and expression of the group I mGluR-induced prolonged bursts. Although both receptor subtypes participate in the ongoing expression of persistent prolonged bursts, we revealed that mGluR1 antagonist is substantially more effective at suppressing the ongoing expression of the bursts, virtually restoring the bursts to interictal length; in contrast, while the mGluR5 antagonist could significantly shorten the bursts, they remained significantly longer than the initial picrotoxin-induced activity (**Figure 3**). These observations are in stark contrast to the mechanism of induction of the epileptogenesis, where mGluR1 antagonist alone was ineffective for the prevention of epileptogenesis mGluR5 antagonist could completely prevent the induction process.

Downstream Second Messengers: Interplay of PKC Isoforms in Induction vs. Suppression of In Vitro Epileptogenesis

The mGluR-mediated induction of long-lasting effects in the neuronal network is likely to involve the activation of various second messengers. Identification of the pathways involved could aid in the development of novel antiepileptogenic agents that target these pathways specifically. The PKC family is oftentimes involved in mGluR-mediated effects such as synaptic plasticity. Yet group I mGluR activation still elicits persistent ictaform discharges in the presence of chelerythrine, a relatively broad-spectrum

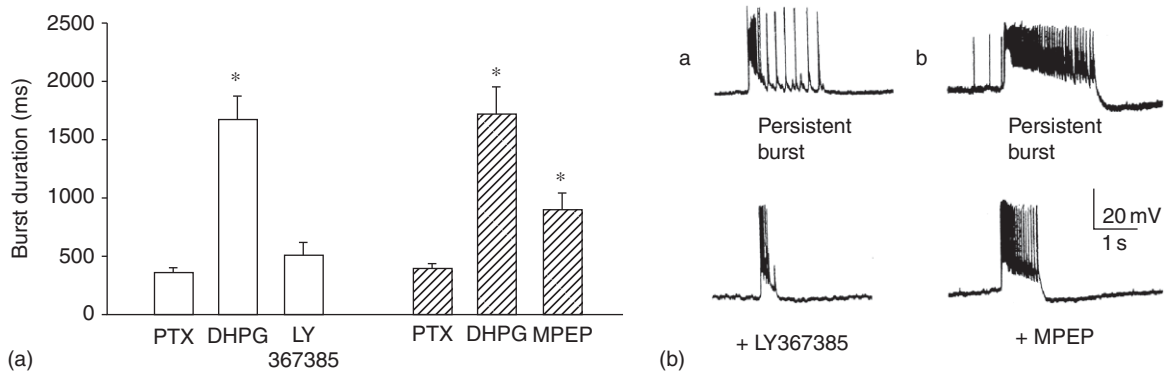


Figure 3 *Selective mGluR1 and mGluR5 antagonists significantly shorten ongoing DHPG-induced ictaform bursts*. (a): Summary data revealing effect of the group I mGluR agonist in inducing persistent burst prolongation, then comparing efficacy of mGluR1 antagonist (LY367385) vs. mGluR5 antagonist (MPEP) at suppression of the ongoing ictaform activity. The mGluR1 antagonist reversibly restores bursts to interictal length, while the mGluR5 antagonist, although having a significant suppressive effect of its own, is less effective and unable to prevent fully the expression of ictal-length bursts. (b): Recordings from CA3 pyramidal cells, revealing the persistent prolonged bursts before and after application of the mGluR1 (a on left) or mGluR5 (b on right) antagonist. Upper traces were recorded at 1 h washout of group I mGluR agonist, just before introduction of subtype-selective antagonist. Lower traces were after 20–30 min exposure to the antagonist. (reproduced with permission from Merlin LR (2002) Differential roles for mGluR1 and mGluR5 in the persistent prolongation of epileptiform bursts. *Journal of Neurophysiology* 87: 621–625).

PKC inhibitor, suggesting a lack of involvement of most PKC isoforms. In contrast, PKC activation via the PLD-linked mGluR seems to have an inhibitory effect on mGluR-induced epileptogenesis: cysteine sulfinic acid's suppressive effect on the induction process can be blocked with chelerythrine, revealing PKC dependence of the effect. Although these data suggest that PKC activation may be antiepileptogenic, preliminary data with phorbol esters contradict this hypothesis, as they alone, via activation of PKC, elicit prolonged ictaform discharges. These conflicting effects may turn out to be PKC isoform-specific, or depend on the microcellular compartment in which the PKC is activated.

Future Goals and Challenges

So where do we go from here? These findings provide core evidence that the group I mGluRs may be useful targets for anticonvulsant drugs as well as antiepileptogenic agents, but they may prove to be effective only in the subset of patients in which this pathway is involved. Therefore, one important problem to be addressed is identification of the patient population in which these data are relevant. There may be patients with genetic mutations that result in overexpression or hyperexcitability of the group I mGluR system, or its downstream partners such as Homer and assorted second messengers; one would expect such mutations to result in mGluR-driven seizures which would be responsive to group I mGluR antagonists or other relevant agents discussed herein. At present, we lump most epileptic patients on the basis of clinical seizure presentation and history, but we know little about the underlying physiological abnormalities in a given patient. Some patients may have hyperactive NMDA receptors; others may be in muscarinic overdrive; still others may have abnormalities of voltage-gated channels making the cells more excitable. In the foreseeable future we will undoubtedly develop the means to identify patients on the basis of physiological and/or genetic abnormality, and will then be able to select which AED is appropriate for which patient – on the basis of the patient's underlying mechanism instead of the hit-or-miss technique now in use by clinicians.

It is noteworthy that 30–40% of patients with epilepsy have not achieved control of their seizures, despite the large number of pharmaceutical agents in our armamentarium to date. Perhaps this situation suggests the models of epilepsy currently used for drug testing and development are not representative of all patients. Advances in genetic testing may allow us to determine whether these refractory patients share a common physiological mechanism underlying their seizures, one which is not currently targeted by the FDA-approved agents on the market. Among this group may be patients whose seizures are mediated by activation of the group I mGluRs. Our in vitro model of mGluR-induced epileptogenesis may serve as a novel system in which new categories of drugs can be evaluated.

Validating this model may involve in vivo studies. Examination of the role of the group I mGluRs in post-traumatic seizures and poststroke seizure would be of interest; the use of knockout mice in these studies may serve to clarify the importance of these receptors as well.

Of course, as our studies to date have been performed in guinea pigs, switching to mice is no small task, and knockout guinea pigs are not yet available.

Screening patients for mGluR or Homer mutations may not only be relevant to patients presenting with epilepsy (to allow for mechanism based drug selection) but may also serve to assist in the potential treatment of trauma patients with antiepileptogenic therapy. Studies need to be done to determine what makes individuals with seemingly equivalent brain injuries have different outcomes (e.g., with respect to posttraumatic or poststroke epilepsy). Perhaps patients have different inherent susceptibilities for the development of epilepsy, in some cases based on differences in their group I mGluR or PLD-coupled mGluR systems. We may someday be able to offer prophylactic antiepileptogenic therapies to patients on the basis of their individual susceptibilities.

Finally, group I mGluR activation itself need not automatically result in epilepsy. We have shown that activation of these receptors with nonselective, broad spectrum mGluR agonist will elicit similar-length seizure discharges, but will not induce a persistent epileptic state – these discharges promptly disappear upon washout of the broad-spectrum agonist. Therefore, further studies are needed to clarify the factors that determine whether ictogenic activity becomes epileptogenic, and clarify the mGluR-induced intracellular cascade involved in initiating epileptogenesis. It is remarkable that, unlike the persistent bursts induced by selective agonist, the nonpersistent long bursts elicited by nonselective mGluR agonist are completely unaffected by either PLD activation or $GABA_B$ antagonist. These data suggest that the generation mechanisms for mGluR-dependent persistent vs. nonpersistent seizure discharges may be significantly different. The mechanisms responsible for determining whether mGluR-induced epileptiform activity becomes persistent or remains nonpersistent have yet to be discovered, but the data suggest that it is inadequate for us to simply identify the group I mGluR subgroup as a target for antiepileptogenic treatments; we must pursue a deeper understanding of what determines whether or not the induced ictaform activity results in a persistent epileptic state. Identification of the PKC isoforms involved (via molecular/biochemical studies) in ictogenesis vs. epileptogenesis, and identification of the critical proteins underlying the induction process in this protein synthesis-dependent pathway, may help shed light on these matters. Such insight would provide hope that we may some day be able to offer help to patients at risk for developing epilepsy and prevent the epileptogenic process from proceeding, thereby preventing the development of epilepsy in that subset of patients in whom this pathway is the culprit.

See also: **Glutamate**: Functional Reorganization of Inhibitory Circuits in Epilepsy: mGluR1/5 Signaling Mechanisms and Long-Term Plasticity; Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability; Role of Kainate Receptors in Glutamatergic Synaptic Transmission: Implications for Acute and Chronic Seizure Generation; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity.

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Role of Kainate Receptors in Glutamatergic Synaptic Transmission: Implications for Acute and Chronic Seizure Generation

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Introduction

Temporal lobe epilepsies (TLEs) are frequently observed in adults (30–40% of all forms of epilepsies) and are often refractory to pharmacological medication. Though many morpho-functional changes have been linked to this syndrome, the physiopathological mechanisms responsible for the epileptic condition require further elucidation. A large part of our present understanding of the physiopathology of TLE comes from studies using acute and chronic animal models. The first and most investigated model of TLE relies on systemic or intracerebral injections of a neurotoxin and neuroexcitant: kainic acid (KA). KA is an agonist of a subtype of ionotropic glutamatergic receptors (KA receptors (KARs)) the activation of which induces prolonged depolarization of hippocampal neurons, leading to epileptiform discharges in the hippocampus and associated limbic structures, as well as secondarily generalized limbic seizures (to which we shall refer as *acute seizures*). In addition to these ictogenic properties, KA injections are also epileptogenic. Injected animals that survive the initiating status epilepticus show, after a seizure-free latent period of several weeks, recurrent spontaneous seizures of hippocampal origin and became *chronically epileptic*. A considerable effort has been made to understand the epileptogenic effect of kainate injections. A current widely accepted hypothesis is that the epileptogenic basis is established as a consequence of multiple factors, involving neuronal cell death and reactive axonal rewiring, following kainate-induced status epilepticus. This reorganization further leads to quantitative and qualitative alterations in both the synaptic and intrinsic properties of surviving neurons, which result in an increased excitability.

More recently, the availability of new pharmacological tools together with progress in molecular biology allowed fast progress in the understanding of the physiological properties of kainate receptors. Notably, a role was recently proposed for kainate receptors in fast glutamatergic neurotransmission in the hippocampus, together with AMPA and NMDA receptors. This article will summarize the basic properties of kainate receptors and kainate receptors mediated synaptic transmission and to which extend they shed new lights on the ictogenic and epileptogenic actions of KA injections.

Background

Structure and Diversity of Kainate Receptors

The first autoradiographic studies using radioactive kainate [^{3}H]-KA revealed the presence of high-affinity KA binding sites in the brain. These observations suggested the existence of specific KARs that could be major players in the generation of acute epileptic seizures. In the early 1990s, KAR subunits were cloned and identified as members of the ionotropic glutamatergic receptor family (together with AMPA and NMDA receptors). As is the case for AMPA receptors, KARs are tetramers formed from four subunits. The cloned KAR subunits are termed GluR5, GluR6, GluR7, KA1, and KA2. GluR5–7 subunits have a low affinity for KA and can form functional homomeric receptors when expressed in heterologous systems; KA1 and KA2 have a higher affinity for KA and must be associated with GluR5–7 subunits to form functional receptors. Post-translational modifications, such as mRNA editing or alternative splicing, further increase the diversity of KARs subunits. Alternative splicing is seen mainly on the C-terminal domain of GluR5–7 subunits, and the results of this alternative splicing can modify KAR subunit trafficking to the plasma membrane and their interactions with intracellular partners. GluR5 and GluR6 mRNA can also be edited at a Q/R site in the second pore-forming membrane domain. Unedited subunits have a neutral glutamine at this site, while edited subunits have a positively-charged arginine. The fully edited subunits have a ring of positive charges in the pore of the channel that result in a lower Ca^{2+} permeability.

Physiological Role of Kainate Receptors in Glutamatergic Synaptic Transmission

For a long time, the lack of specific antagonists for KA (versus AMPA) receptors has precluded the study of their specific physiological role. Indeed, most molecules used to block AMPA (versus NMDA) receptors (such as CNQX) also blocked KARs. Recently, the development of a series of 2,3-benzodiazepines (such as GYKI 53655 and GYKI 52466), which are noncompetitive AMPAR-selective antagonists, has allowed the study of native KARs. The use of genetically engineered mice which lack specific KAR subunits further accelerated these

studies. While the role of AMPARs in glutamatergic synaptic transmission is well established, the role of KARs has emerged more recently, mainly from studies of the mossy fiber synapse between dentate granule cells and CA3 pyramidal cells of the hippocampus. It was reported that in CA3 neurons, excitatory postsynaptic currents (EPSCs) could be evoked by mossy fiber synapses in the presence of the selective AMPAR antagonist GYKI 53655 – with strong-and/or high-frequency electrical stimulation of mossy fibers, but not after a single shock. It was concluded that these EPSCs were mediated by KARs because they were insensitive to NMDA receptors antagonists and were further blocked by a mixed AMPARs/KAR-santagonist, CNQX. Interestingly, EPSCs mediated by KARs ($EPSC_{KA}$), differed from $EPSC_{AMPA}$ not only in their pharmacology but also in electrophysiological characteristics – i.e., their smaller amplitude and slower kinetics. In subsequent studies, KARs were shown to be involved in synaptic transmission onto inhibitory interneurons of the hippocampus, and in many other structures of the brain such as cerebellar Golgi cells, Purkinje cells, interneurons and principal cells of the neocortex, and neurons in the amygdala.

From these studies emerged the notion that KARs could participate in glutamatergic synaptic transmission, but only when large amounts of glutamate were released during repetitive activation of afferent fibers. This limitation was thought to be due to an extra-and/or peri-synaptic location of KARs. Recently, we reexamined the conditions under which postsynaptic KARs could be activated, and observed that they are involved in 'miniature' synaptic transmission (i.e., release of the small amount of glutamate contained in individual synaptic vesicles or quanta). Indeed, in CA3 pyramidal cells and CA1 interneurons, miniature excitatory postsynaptic currents (mEPSCs) solely mediated by kainate receptors ($mEPSC_{KA}$) can be recorded in the presence of specific AMPAR antagonists; these $mEPSC_{KA}$ are blocked by further addition of the mixed AMPAR/KAR antagonist CNQX or by SYM 2081, a compound that selectively desensitizes KARs. This result strongly argues that KARs can be activated by small amounts of glutamate, and against the view that these receptors are located at extrasynaptic sites. Our view has recently been confirmed by electron microscopy experiments showing the presence of GluR6 and KA2 subunits within the postsynaptic density of mossy fiber synapses. Our study also confirmed the slow time course and smaller amplitude of $mEPSC_{KA}$s when compared to $mEPSC_{AMPA}$s. Finaly, we showed that KARs carry as much as half the total glutamatergic current in hippocampal neurons and KARs are thus key players in physiological glutamatergic synaptic transmission. Interestingly, we also observed the presence of miniature EPSCs mediated by the coactivation of both AMPA and kainate receptors ($mEPSC_{AMPA/KA}$) that are likely colocalized at the same postsynaptic site.

Another specific feature of KAR-mediated synaptic transmission (when compared to AMPAR-mediated synaptic transmission) is its cellular and synaptic specificity. Early autoradiographic studies suggested that kainate receptors were specifically enriched in the stratum lucidum of the CA3 area, the zone of mossy fibers termination onto CA3 pyramidal cells in the hippocampus. Indeed, high affinity [^{3}H]-KA binding sites were abundant within this area, and lesions of the mossy fiber pathway or of CA3 pyramidal cells resulted in a dramatic reduction of the mean density of [^{3}KA] binding sites. More recently, physiological experiments using selective AMPA receptors showed that $EPSC_{KA}$ couldn't be evoked in CA1 pyramidal cells of the hippocampus by stimulation of the Schaffer collaterals. Consistent with this result, no $mEPSC_{KA}$ or $mEPSC_{AMPA/KA}$ can be recorded in CA1 pyramidal cells – or in dentate granule cells of the hippocampus. In addition to this CA3 cellular specificity, KAR-mediated synaptic transmission is also pathway specific. Indeed, in CA3 pyramidal cells, postsynaptic KARs can be selectively activated by stimulation of the mossy fiber pathway but not stimulation of other glutamatergic afferents (such as the associative/commissural fibers). Furthermore, removal of the mossy fiber pathway in organotypic slice cultures suppresses the KAR-mediated component of miniature glutamatergic transmission.

Altogether, these studies demonstrate that AMPARs and KARs can be activated by the same amount of released glutamate, and that KARs are key players in glutamatergic synaptic transmission in the hippocampus (and probably in other brain structures). KAR-mediated synaptic transmission is intriguingly different from AMPAR-mediated transmission, in time course, amplitude and cellular and synaptic specificity.

Degenerative Changes and Mossy Fiber Sprouting in the Kainate Models of TLE

The most common anatomo-pathological alteration observed in TLE patients is the sclerosis of the Ammon's horn, characterized by the degeneration and loss of pyramidal cells and interneurons, and leading to the shrinkage of the hippocampal complex together with reactive sprouting of the mossy fibers. In rats treated with KA, seizures induce the death of CA3 pyramidal cells (**Figure 1**) and eventually CA1 (the latter most likely due to anoxic mechanisms). Hilar mossy cells, the cells of origin of the excitatory (glutamatergic) associational-commissural input to dentate granule cells, also degenerate as a consequence of seizures (**Figure 1**). In addition, many interneurons within the hilus also degenerate, thus decreasing the number of GABAergic terminals impinging upon the dentate granule cells (**Figure 1**). These degenerative changes occur within the first three days following the KA-induced SE insult, and are associated with typical reactive glial reaction

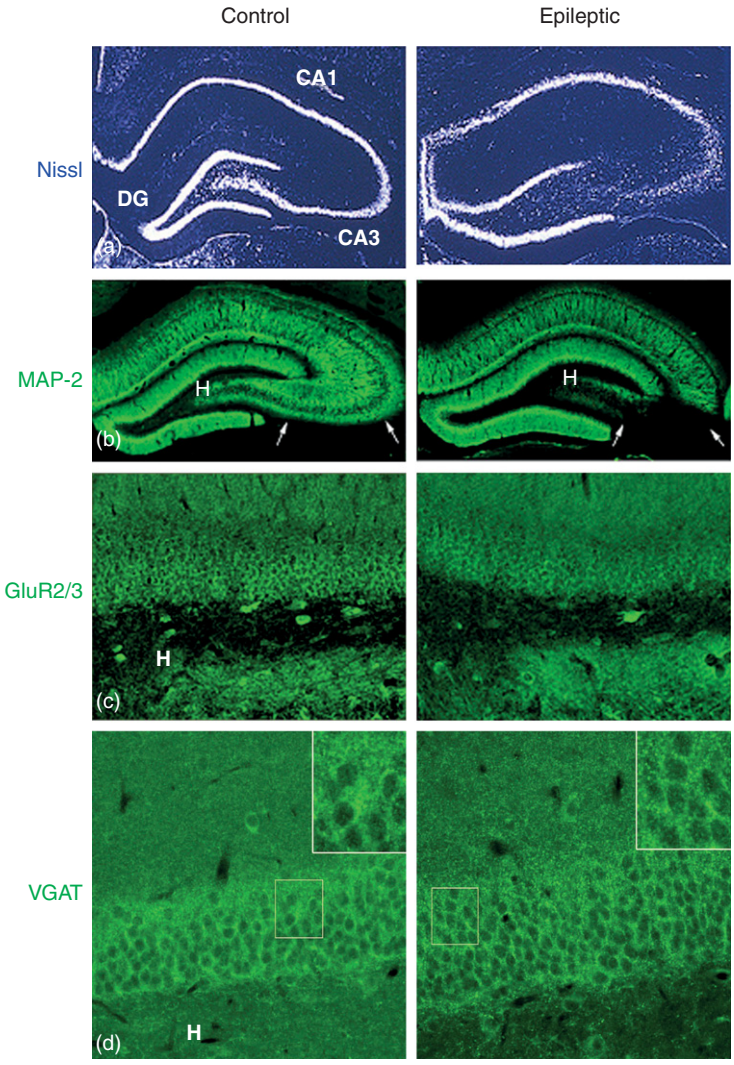


Figure 1 Degenerative changes in experimental TLE. Hippocampal sections from control (left) and chronic epileptic rats (right) sacrificed 30 days after injection of vehicle (a, c, e, g), kainate (b, c), or pilocarpine (f, h). (a) Nissl staining depicting a significant loss of CA3 neurons in chronic epileptic rats. (b) Microtubule associated protein MAP-2 immunostainings illustrate a lack of CA3 pyramidal neurons in chronic epileptic rats. (c) Immunostaining of glutamate receptors GluR2/3 decorates granule cells and hilar mossy cells. The number of the later was reduced in chronic epileptic rats. (d) Vesicular GABA transporter VGAT immunostaining reveals a partial loss of GABAergic terminals in the dentate gyrus of chronic epileptic rats. DG: dentate gyrus; m: molecular layer; g: granule cell layer; H: hilus. Squares in G and H were enlarged in their respective inserts. Bars = 100 μm (A–D) and 50 μm E–H).

characterized by the proliferation and hypertrophy of astrocytes and microglia-macrophage activation. As a consequence of this cell loss, remaining dentate gyrus granule cells lack: i) the glutamatergic input of mossy cells, which innervate the proximal third of granule cell dendrites, and ii) the GABAergic input from hilar interneurons. At time of cell degeneration (within the first few days after SE), animals do not display motor nor EEG seizures, but only low frequency interictal-like epileptiform activities. These results suggest that degenerative lesions per se are not sufficient for the emergence of spontaneous seizures.

Brain degeneration is frequently associated with the emergence of a reactive sprouting which is likely aimed at compensating for the absent synaptic inputs. In most case, this plasticity involves the formation of abnormal connections. This reorganization has been particularly well analyzed in the dentate gyrus of kainate-treated rats, in which collateral sprouting of mossy fibers takes place within the hilus; the newly formed mossy fiber axon collaterals cross through the granule cell layer and branch throughout the inner third of the molecular layer, thus replacing the glutamatergic terminals of degenerated mossy cells (**Figures 2** and **3**). Electron microscopy analysis clearly demonstrated that these terminals establish synaptic contacts with granule cell dendrites, and that these newly formed synaptic contacts display the typical features of conventional mossy fiber synapses (i.e., similar to the contacts between mossy fiber terminals and their normal

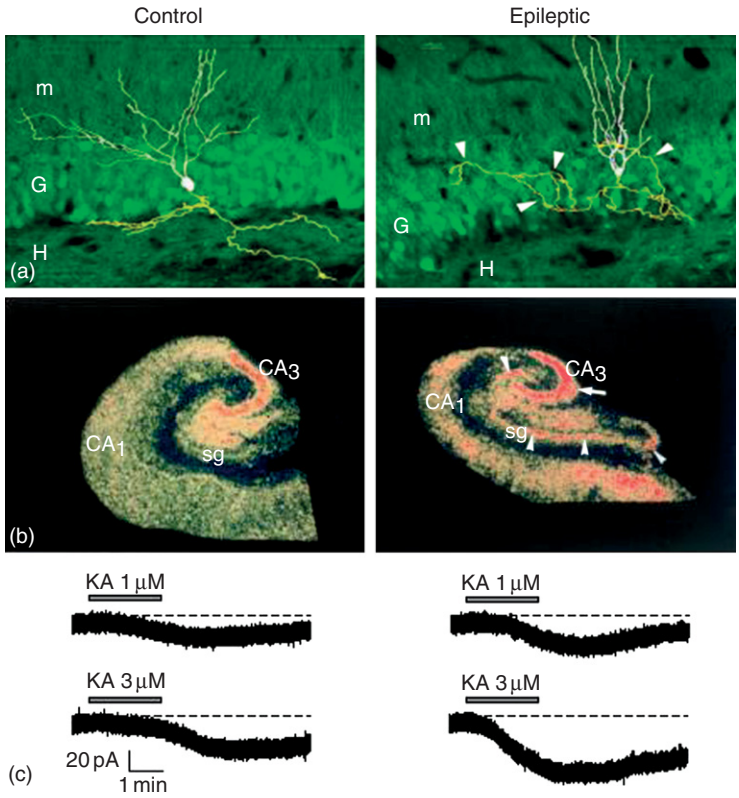


Figure 2 Sprouting of mossy fibers is linked to an increase of kainate binding sites. (a) Photomontage of sections immunostained with an antibody against the calcium binding protein CaBP (in green) and 'camera lucida' reconstruction of representative Golgi stained granule cells from control (left) and chronic epileptic (right) rats. Arrowheads depict axon collaterals passing through the granule cell layer and distributing within the inner molecular layer. (b) Representative autoradiographs depicting the distribution of kainate binding sites in the hippocampus from a child with generalized epilepsy (right) as compared to a nonepileptic one (left). Note that the binding for kainate was increased in the dentate gyrus (the subgranular layer: sg; arrow heads) but also in other hippocampal fields (e.g., the CA3 stratum oriens: arrow). (c) Kainate receptor-mediated current induced by bath application of kainate in a control granule cell (left) and a granule cell from epileptic rat (right).

target, the CA3 pyramidal cell dendrite), including boutons of giant size, containing a high density of synaptic vesicles, developing numerous asymmetric synaptic contacts with giant spines. As a consequence of this sprouting, a direct recurrent excitatory, granule cell-to-granule cell network emerges (see below).

The sprouting of mossy fibers is an event that starts after a relatively long delay after the initial insult, and requires considerable time to be completed – this period coinciding approximately with the time course of the latent period (~30 days). In the kainate model of epilepsy, the first signs of sprouting were observed 12 days after the KA injection, and 15 more days were required before maximal sprouting was observed. After this time, no major changes were observed, as the newly established contacts appeared stable (were still observed in animals sacrificed up to one year later). The recurrent mossy fiber network is functional, as shown first in the pioneering study by Tauck and Nadler; these investigators showed that the activation of a few granule cells, by antidromic activation of the mossy fiber pathway, resulted in interictal-like recurrent network activity that could not be evoked by similar stimulations in the dentate gyrus from control rats. This activity, initially described in the CA3 area, is generated by recurrent excitatory networks within a context of decreased inhibition. Focal stimulations, using glutamate uncaging, reveal the functionality of the recurrent mossy fiber network. In such experiments, the selective stimulation of a small group of granule cells induced an increase in the frequency of excitatory synaptic events recorded in distant granule cells in KA-treated epileptic animals but not in controls. More recently, Scharfmann and colleagues, using paired intracellular recordings, produced direct experimental confirmation for the presence of monosynaptic excitatory connections between dentate granule cells in epileptic rats. The functionality of the recurrent excitatory network is also reflected in the increased frequency of excitatory postsynaptic events recorded in dentate granule cells from epileptic rats (compared with control rats), which is correlated with the level of mossy fiber sprouting. Recurrent mossy fibers share several physiological properties with control mossy fibers, such as a strong

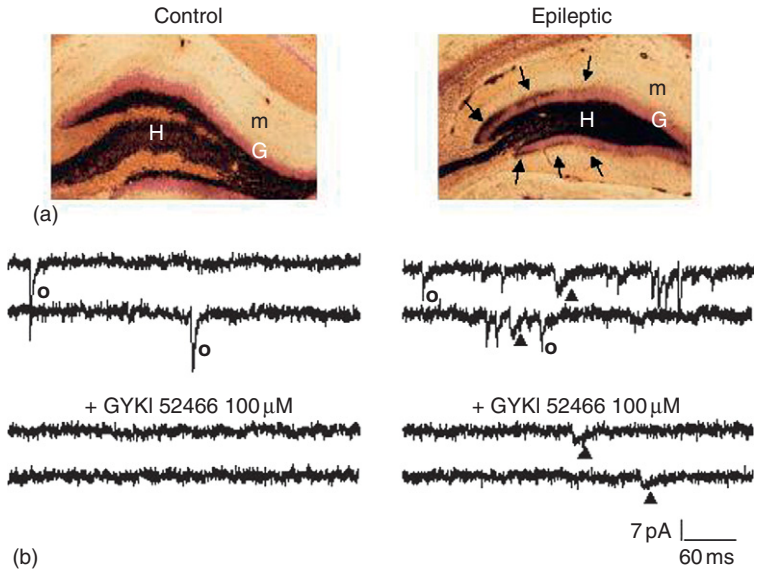


Figure 3 Ganule cells from epileptic rats generate aberrant slow kainate receptor-mediated synaptic events. (a) Timm staining of hippocampal mossy fibers of control (left) and chronic epileptic (rigth) rats. Arrows depict the inner molecular layer invaded by mossy fiber terminals. (b) Spontaneous EPSC recordings ($V_h = -70$ mV) from a control granule cell (left) and a granule cell from a chronic epileptic rat (right). Note that in control tissue all synaptic events are fast (open circle) and mediated by AMPARs because they are fully abolished by 100 μM GYKI 52466. In contrast, in the epileptic tissue, the EPSCs additionally includes slow kainate receptor-mediated events (close triangle) that are resistant to 100 μM GYKI 52466.

facilitation of synaptic transmission with a small increase in stimulation frequency ('frequency facilitation'), sensitivity to the mGluR2/3 agonist DCGIV, and the ability to release dynorphin.

Mossy fiber sprouting has also been observed in the hippocampus of human patients with TLE and hippocampal sclerosis, as seen in histochemical, immunochemical and autoradiographic analysis. Recent in vitro work, using surgically resected hippocampal slices from TLE patients, has shown that mossy fiber sprouting is correlated with the severity of induced seizures. Mossy fiber sprouting has also been observed in other forms of epilepsy. In a case of a child who died after severe epileptic seizures (Figure 2), the hippocampus exhibited no signs of significant cell death, but histochemical analysis demonstrated an increased density of mossy fiber terminals not only in the dentate gyrus but in the CA3 region as well.

Taken together, the clinical data strongly suggest that the hippocampus of TLE patients is generally characterized by the formation of this recurrent excitatory loop, which together with a decreased inhibition would increase the excitability of surviving neurons and facilitate the synchronization of the network-allowing the emergence of seizures.

Methods

Our group investigated the properties of dentate granule cells and networks, from chronically epileptic Wistar rats, using patch-clamp and extracellular recordings in hippocampal acute slices. The identification of recorded neurons is made possible by introducing biocytin into the recording pipette, and then reconstructing the recorded cell post-hoc using camera lucida or Neurolucida systems. To obtain chronically epileptic rats, an initiating period of status epilepticus is induced by intraperitoneal injection of kainate or pilocarpine. Morphological analysis of neuronal cell types and axonal terminals are carried out using histochemical (Nissl and Timm) and immunohistochemical techniques.

Results

Role of Kainate Receptors Activation in Acute Seizure Generation

Well before the cloning of kainate receptors, several arguments pointed to the activation of specific kainate receptors as one of the main determinants of the epileptogenic actions of kainic acid injections. The first argument for this hypothesis is the high density of high-affinity kainate binding sites in seizure prone regions like the hippocampus or the amygdala. A second argument is that measured concentrations of kainic acid in these regions after systemic injections (50–250 nM) were well below concentrations needed to activate other types of glutamatergic receptors like AMPA receptors. These low concentrations of KA, however, could induce powerful ictal-like activities when directly injected in the hippocampus in vivo or

in vitro. Furthermore, limbic motor seizures associated with kainic acid injection were not observed before the full development of the mossy fiber pathway (which mainly develops postnatal) and high affinity kainate binding sites in the hippocampus. Indeed, studies performed in our Institute have shown that the developmental profile of kainate-induced seizures in the intact isolated hippocampus in vitro was correlated to the development of postsynaptic kainate receptor-mediated current evoked in CA3 pyramidal cells. Accordingly, when the normal development of dentate granule cells and mossy fibers is impaired, KA binding sites are lost in the hippocampus and kainic acid applications fail to generate any seizure in the hippocampus in vitro. More recently, the link between kainate receptor activation and kainic acid-induced acute seizures in the hippocampus has been reevaluated using knockout mice for specific KAR subunits. In GluR6−/− mice, bath applications of kainate at submicromolar concentration (that specifically activates KARs over AMPARs) do not generate any current in CA3 pyramidal cells. Postsynaptic responses mediated by kainate receptors after stimulation of the mossy fiber pathway are also disabled, and no seizures are observed after systemic kainic acid injections using conventional concentrations. Seizures can be observed when high doses of kainate are used ($30\,mg\,kg^{-1}$), although it is not clear whether these seizures are the classical secondarily generalized seizures usually observed after kainate injections or are a tonic–clonic seizure type (usually observed after $GABA_A$ receptor antagonist injections). The lack of the GluR6 subunit also prevented the generation of epileptiform bursts after KA application onto hippocampal slices. These experiments suggest that postsynaptic KARs that include the GluR6 subunit in CA3 pyramidal cells are important for the induction of acute seizures after systemic KA injections. CA3 pyramidal cells form a network of strongly interconnected neurons, through recurrent excitatory connections, and have a low threshold for generating epileptiform burst activity under conditions of increased excitability (i.e., increase excitation or reduced inhibition). Such epileptiform activities would then propagate to the CA1 area, and from there to other associated limbic structures.

In addition to being expressed in principal cells of the hippocampus, KARs are also expressed in hippocampal interneurons. These receptors include the GluR5 subunit and can thus be selectively activated by specific agonists such as ATPA. Activation of these receptors leads to a prolonged depolarization of interneurons and results in a marked increase of the tonic inhibition of CA1 pyramidal cells. Thus, in the CA1 area, KA acting on GluR5-containing receptors would have an overall inhibitory effect; GluR5 agonists decrease the spontaneous discharge of CA1 pyramidal cells and reduce the propagation of seizures triggered by bicuculline in an in vitro preparation of interconnected neonatal hippocampi. GluR 5 is also expressed by interneurons in the CA3 area of the hippocampus. Studies have shown that the threshold for seizure generation in this area is decreased (in hippocampal slices) from GluR5−/− knock out mice. In contrast to this inhibitory effect mediated by Glur5-containing KARs, ATPA systemic injections induce clonic seizures in mice. This somewhat confusing effect might be due to activation of neurons of the basolateral amygdale, in which GluR5 containing receptors are involved in glutamatergic synaptic transmission.

KARs are also expressed on presynaptic mossy fiber axons and terminals from dentate granule cells. On presynaptic mossy fiber terminals, KARs have a dual effect on synaptic transmission: at low concentration (20–50 nM), kainate increases glutamatergic release onto CA3 pyramidal cells, whereas at higher concentrations KA (200–500 nM) decreases release. Thus, the low concentration of KA that reaches the hippocampus after systemic injections could bind to presynaptic KARs and enhance then the release of glutamate onto CA3 pyramidal cells, increasing the excitability of the CA3 hippocampal network. Kainate receptors are also expressed presynaptically by inhibitory GABAergic terminals of the hippocampus, were they have been shown to decrease the release of GABA. In accordance with this, specific antagonism of presynaptic GluR5-containing receptors prevents epileptiform activities induced by the muscarinic agonist pilocarpine. However, since KARs also directly depolarize interneurons through GluR5 containing receptors, the global effect of KA injections on inhibitory synaptic transmission in the hippocampus is not easy to predict.

Role of Kainate Receptors in Chronic Seizure Generation

We have seen that the activation of KARs by exogenously applied KA induces epileptic seizures. Do KARs (activated by endogenous glutamate) play a role in seizure generation in chronically epileptic rats? Recent experiments from our group support this hypothesis.

Early autoradiographic studies showed that mossy fiber sprouting is accompanied by a gradual increase of ^{3}H-KA binding sites, in the inner molecular layer of the dentate gyrus that reaches a maximum 30 days after the initial KA injection (**Figure 2**). These changes appear to be due to the expression of functional postsynaptic KARs, since we observed an increase in the amplitude of KAR-mediated currents induced by bath application of kainate (unpublished observations, **Figure 2**). The presence of aberrant postsynaptic KARs was further confirmed by the observation that stimulations in the inner one-third of the molecular layer of the dentate

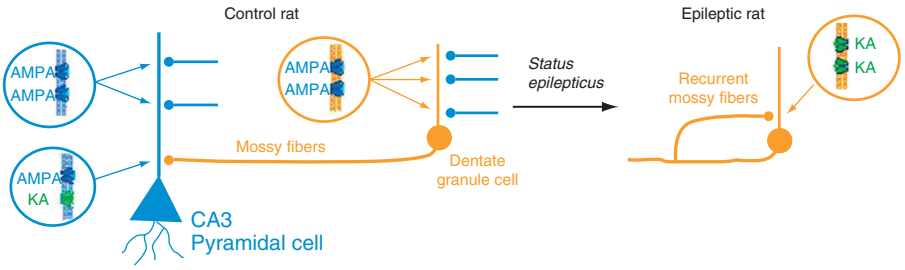


Figure 4 Schematic representation to illustrate that recurrent mossy fibers establish aberrant kainate receptor-operated synapses on granule cells from epileptic rats.

gyrus (the termination zone for recurrent mossy fibers) induces a slow KAR-mediated synaptic response in granule cells from chronic epileptic rats – but not from age-matched control rats were only fast $EPSC_{AMPA}$ could be evoked (Figure 3). The presence of slow $EPSC_{KA}$ was correlated with the appearance of recurrent mossy fibers since $EPSC_{KA}$ were not observed 4–5 days after the inaugurating status epilepticus (before mossy fiber sprouting). Furthermore, $EPSC_{KA}$ presented a pharmacological and physiological profile specific to mossy fiber synapses such as a strong facilitation of synaptic transmission with a small increase in stimulation frequency ('frequency facilitation') and sensitivity to the mGluR2/3 agonist DCGIV. Finally, $EPSC_{KA}$ could be evoked in granule cells from epileptic rats by focal stimulation of distant granule cells through the recurrent mossy fiber network whereas in control rats in the absence of the recurrent mossy fiber network similar stimulation failed to evoke any EPSPs in distantly recorded dentate granule cells. Together, these data demonstrate that mossy fibers sprouting induce the expression of functional postsynaptic kainate receptors in abnormal target cells (Figure 4). Finally, we observed that KARs are involved in the synchronized network-driven activities supported by recurrent mossy fibers in the dentate gyrus from epileptic rats. These data suggest that aberrant postsynaptic KARs located at mossy fiber synapses onto dentate granule cells could participate, together with the abundant recurrent axonal innervation, in the pathological synchronization of the dentate gyrus neurons.

Future Directions and Implications

The observations described in this article may have interesting implications for the treatment of human TLE, since changes in [^{3}H]-KA binding sites have been observed in the dentate gyrus of children with epilepsy (as compared with age-matched controls) (Figure 2). Furthermore, a specific increase in KAR subunit expression has been observed in granule cells from patients with TLE. Kainate receptors display a specific and selective distribution within the brain, particularly in temporal areas. Our recent observations support the notion that in chronic epilepsy, some cells (e.g., the dentate granule cells) abnormally express this glutamate receptor subtype at synaptic sites and that this abnormal distribution contributes to increased hippocampal excitability. Therefore, this receptor subtype constitutes an interesting pharmacological target, especially since its inhibition would not markedly perturb glutamatergic transmission at most synapses, but would reduce excitability of specific cell types and circuits involved in epileptogenesis.

Another important implication of synaptic KAR expression by dentate granule cells, is the resulting major changes in the kinetics of EPSPs received by these cells. EPSP kinetics can profoundly modify several fundamental neuronal functions, such as temporal integration of incoming synaptic inputs as well as temporal firing precision. Indeed, computational studies have shown that fast $EPSP_{AMPA}$s must be very synchronous, or generated at high frequencies, to induce spiking, whereas slow $EPSP_{KA}$s can generate a sustained depolarization (even at low frequencies) through their temporal summation. Changes in EPSP kinetics will also modify the interaction between synaptic and intrinsic properties in dentate granule cells and can modify the computational properties of these cells. Thus, beside effects on seizure generation, these modifications of KARs could contribute to cognitive dysfunctions that are frequently observed in patients with TLE. Future work should address these questions, using state of the art techniques to record and manipulate single cells and networks in intact in vivo preparations.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; **Glutamate**: Functional Reorganization of Inhibitory Circuits in Epilepsy: mGluR1/5 Signaling Mechanisms and Long-Term Plasticity; Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability; **Plasticity**: Axon Sprouting in Epilepsy; Synaptic Reorganization in the Hippocampal Dentate Gyrus; **Pyramidal Cells**:

Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain; **Transporters**: Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures.

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GRANULE CELLS

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Granule Cell Properties in Seizure Discharge
Phenotypic Plasticity of the Granule Cells and its Relation to Epilepsy
Properties of Dentate Granule Cells and their Relevance to Seizures

Granule-Cell Properties in Seizure Discharge

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Introduction

The dentate gyrus is thought to be a key structure for information transfer into the hippocampus proper. In chronic human and experimental epilepsy, manifold changes occur within the dentate gyrus. These range from changes in the abundance of identified neuron populations (i.e., neurogenesis of dentate granule neurons and loss of certain types of interneurons), axonal reorganization of excitatory and inhibitory neurons, and profound changes in the expression and function of neurotransmitter receptors and ion channels. This article begins with an introduction into the basic functional anatomy of the hippocampus. The following sections will provide a synopsis of the changes observed in chronic epilepsy, and present a number of current hypotheses on how these changes may conspire to produce hyperexcitability.

Background – Functional Anatomy of the Dentate Gyrus

The dentate gyrus receives its major input from the entorhinal cortex (perforant pathway) via glutamatergic synapses. The perforant path axons terminate on the dendritic tree of granule neurons within the molecular layer. The dentate granule cells (DGCs) project via the distinctive, nonmyelinated mossy fibers to the hilar and CA3 regions. Mossy fibers form en-passant synapses onto the proximal dendrites of the pyramidal neurons of the CA3 region. In the CA3 region, an intriguing feature of mossy fiber boutons is that they also contact numerous interneurons with filopodial-like processes. In fact, mossy fibers in the normal CA3 region contact many more inhibitory interneurons than CA3 pyramidal cell dendrites. Thus, the output of dentate granule neurons carries a powerful inhibitory component.

Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain; **Transporters**: Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures.

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In addition to innervating CA3 neurons, mossy fibers collateralize in the hilar region and innervate various target-cell types. These cells may be glutamatergic (i.e., mossy cells) or one of numerous classes of interneurons utilizing the inhibitory neurotransmitter γ-aminobutyric acid (GABA). GABAergic neurons in the hilus and in other areas of the dentate gyrus provide a remarkably strong inhibition in this region. They can be subclassified into numerous categories on the basis of their soma location and dendritic arborization patterns, their axonal termination patterns, as well as their characteristic spiking patterns following depolarization. In addition, neurochemical criteria have been used to refine the definition of interneuron subclasses. In this context, a major criterion with perhaps the highest degree of functional relevance is onto which principal neuron compartments interneurons form synapses (i.e., the somatic or axonal compartment, the dendritic compartment or both). A more detailed description of different interneuron classes is beyond the scope of this article (see Further Reading). Nevertheless, it is important to note that the presence of different types of interneurons allows GABA release onto DGC soma and dendrites with precise spatiotemporal control, mediating powerful feed-forward and feed-back inhibition.

Methods

A large number of techniques have been used to address the function and dysfunction of granule neurons. First, electrophysiological techniques have been used to record the membrane potential of granule neurons. Such techniques include sharp microelectrode recordings in which neurons are impaled with a thin glass microelectrode, or patch-clamp recordings. Such recordings allow us to determine the resting electrophysiological properties of granule cells (such as the membrane potential and passive electrical properties), the properties of individual action potentials, and their repetitive discharge behavior. In addition, these techniques also allow the investigator to record the response of granule cells to the activation of excitatory and inhibitory synapses (excitatory and inhibitory postsynaptic potentials). These techniques have been used to glean information on the intrinsic and synaptic properties of granule neurons under control conditions, and in chronic epilepsy.

Besides being eminently useful, microelectrode recording techniques permit recording from one, two, or at most three neurons simultaneously from a slice preparation. Alternative techniques have therefore involved the sampling of activity, at high-temporal resolution, in larger neuronal populations. One of these techniques is imaging with voltage-sensitive dyes (which have molecules that integrate into cell membranes upon perfusion into a slice). Upon excitation with light of appropriate wavelength, these fluorophores emit a fluorescent signal that is dependent on the membrane voltage. It has been shown that this emission can be calibrated to neuronal membrane voltage. These dyes complement microelectrode recording techniques, and open the exciting possibility to monitor the activity of neuronal ensembles at high speeds.

In addition to functional techniques, classical morphological techniques have been used to address altered morphology of granule neurons and interneurons in the dentate gyrus. These approaches include analyzing the morphology of these cells by filling them with tracers such as biocytin, and then reconstructing their dendritic and axonal arbors. In addition, the axonal projections of granule neurons can be readily labeled with the Timm staining procedure, which takes advantage of the high concentration of zinc in mossy fibers. Immunohistochemical techniques are widely used to assess the expression of marker proteins that allow classification of different cell types in the dentate gyrus. Finally, immunolabeling and molecular tools such as quantitative real-time RT-PCR, expression profiling, and immunoblotting have been used to address the specific molecular changes in ion channels, receptors, and other proteins in different cell types in the dentate gyrus.

Recent Results

Increased Neurogenesis of Dentate Granule Neurons

In discussing changes occurring in the epileptic hippocampus, we first focus on changes in the abundance of different neuron populations and their functional connectivity. One of the most striking changes in the epileptic dentate gyrus is the increased generation of dentate granule neurons. The subgranular zone of the dentate gyrus is one of the niches in the adult brain, which contain neuronal precursors that continue to produce new neurons throughout life. Neurogenesis in this region is strongly increased in animal models of mesial temporal lobe epilepsy (TLE). In human epilepsy, experimental protocols aimed at labeling newly generated neurons in vivo cannot be used. Nevertheless, dentate granule neurons in patients with TLE show increased expression of markers for cell division, as well as markers labeling neural progenitors and immature neurons. The increased generation of granule cells raises several interesting questions: (1) how do newly generated neurons integrate within preexisting neuronal networks, (2) do granule neurons generated following seizures integrate differently from those produced by 'normal' neurogenesis, and (3) is the effect of augmented neurogenesis anti- or proepileptogenic?

Systematic analyses using labeling of newly generated neurons with BrDU or viral transfection have revealed

that the majority of newly generated granule cells migrate within the granule-cell layer. In addition, in chronic human and experimental epilepsy, some newly generated neuronal precursors migrate aberrantly to the hilus and molecular layer after prolonged seizures and differentiate into ectopic DGCs. These findings raise the question whether aberrant migration of newly generated granule cells might contribute to granule-cell dispersion, a characteristic broadening of the granule-cell layer seen in some forms of experimental epilepsy, and in human TLE with Ammon's horn sclerosis. This question is still under debate. An alternative hypothesis contends that dispersion may be caused by aberrant localization of mature granule cells.

Regardless of the precise localization of newly generated granule cells, the question remains as to what their functional role in the epileptic hippocampus is. Recordings from putative newly generated ectopic granule cells in rodents have revealed that they share intrinsic properties with normally located granule cells. However, they seem to be easily recruited into seizure activity occurring in the CA3 region. This may be a specific feature of ectopic granule cells. A recent study has compared granule cells within the granule-cell layer generated following seizures to granule cells generated following behavioral stimuli. In this study, viral labeling techniques were used to unequivocally identify newly generated granule neurons. Intriguingly, aberrantly and 'normally' generated granule cells displayed similar intrinsic membrane properties. However, new neurons born following seizures differed with respect to synaptic drive and short-term plasticity of both excitatory and inhibitory afferents. The net effect of these differences in functional afferent connectivity would be consistent with the newly generated granule-cell population being less excitable on a network level. The role of newly generated granule neurons in seizure discharges on a network level, however, is not yet clear, and will also depend on the properties of efferent synapses formed by these neurons within the hippocampus.

Changes in the Functional Connectivity of Dentate Granule Neurons

What is the output of dentate granule neurons in chronic epilepsy? The mossy fiber axons of DGCs undergo characteristic changes both in experimental and human TLE. Sprouting of mossy fiber axons in the epileptic hippocampus results in the formation of aberrant, excitatory synapses of mossy fiber boutons onto the proximal dendrites of dentate granule neurons (Timm staining of the mossy fibers in control and epileptic hippocampus depicted in **Figure 1(a)** and **1(b)**, respectively). Mossy fiber sprouting is a characteristic feature of many experimental models of epilepsy, in particular, models that utilize prolonged status epilepticus for the induction of chronic epilepsy. It is also commonly observed in human TLE with Ammon's horn sclerosis.

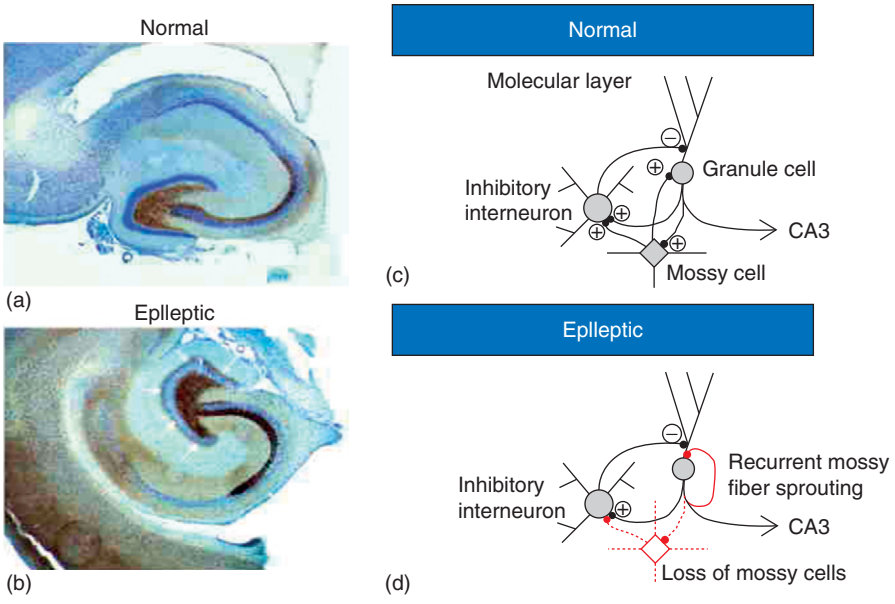


Figure 1 Mossy fiber sprouting and altered synaptic connectivity in the epileptic dentate gyrus. (a, b): Timm-stained sections of control hippocampus (a) and hippocampus from a chronically epileptic rat (pilocarpine model of epilepsy, b). The brown staining product labels mossy fibers (arrows in b). Note the de novo projection of mossy fibers within the inner molecular layer of the dentate gyrus. (c, d): Schematic diagram of altered connectivity in the dentate gyrus in chronic epilepsy. Changes in epilepsy (i.e., loss of mossy cells and recurrent sprouting of mossy fibers) are depicted in red. (+) and (−) indicate excitatory (glutamatergic) and inhibitory (GABAergic) synapses.

What is the functional consequence of mossy fiber sprouting? This question has fascinated neuroscientists for several decades. Morphological studies have shown that virtually all the synapses formed by mossy fibers in the granule-cell and inner molecular layers are with other granule cells, and electrophysiological recordings from pairs of granule cells have proven the existence of monosynaptic connections between granule neurons in epileptic hippocampus. These results suggest an interesting difference of aberrant and normal mossy fiber terminals: Whereas normal mossy fiber terminals in the CA3 region form many more inhibitory synapses than excitatory synapses, it seems as if recurrent mossy fiber synapses provide a monosynaptic, exclusively excitatory feedback pathway that is not normally present (see **Figure 1(c)** vs. **1(d)**). It is important to realize that the normal dentate gyrus is not devoid of excitatory feedback pathways. Here, excitatory feedback is derived via mossy fibers that activate mossy cells, which in turn project back to dentate granule neurons (**Figure1(c)**). Since mossy cells are lost in human and experimental epilepsy, this disynaptic excitatory feedback circuit is replaced by a more local, monosynaptic one in epileptic tissue (**Figure 1(d)**). Apart from creating a monosynaptic recurrent excitatory loop, mossy fibers release zinc when activated. Because granule cells in chronic human and experimental epilepsy express GABA receptors with increased zinc sensitivity, this has been hypothesized to cause a decrease of GABAergic inhibition during seizure activity, but direct evidence for this is lacking.

The cellular mechanisms underlying aberrant sprouting of mossy fibers have been partially identified. The loss of CA3 target neurons may cause mossy fibers to seek alternate postsynaptic targets. Another attractive hypothesis is that axons emanating from newly generated granule cells may grow abnormally into the inner molecular layer. While this may be the case, newly generated neurons are almost certainly not the only source of recurrent mossy fiber sprouting, because disrupting granule-cell neurogenesis by irradiation does not interfere with the development of mossy fiber sprouting in experimental epilepsy. It is likely that following induction of sprouting, the trajectories of outgrowing axons are determined by multiple components of the extracellular matrix, signaling molecules or synaptic adhesion molecules that mediate axon repulsion or attraction. Significant changes in many such molecules have been observed both in human and experimental epilepsy.

Loss of Interneuron Populations in the Epileptic Dentate Gyrus

In the face of increased generation of granule cells, what is the fate of interneuron populations in chronic epilepsy? This question is of great importance because interneurons have crucial roles in mediating feed-forward and feedback inhibition in the dentate gyrus. They have also been shown to be important in the generation of synchronous population activity. Numerous studies have used markers of interneurons to examine which subtypes are affected in chronic human and experimental epilepsy. In some animal models, loss of interneurons and inhibition takes place in the hippocampus. However, in the human hippocampus the majority of inhibitory interneurons appear to be preserved, even though loss of some subtypes (such as parvalbumin-containing interneurons) has been reported. Electrophysiological recordings from epileptic human hippocampus support the presence of strong inhibition, despite the loss of some subtypes of GABAergic neurons. This question has been further investigated by a quantification of the number of perisomatic inhibitory (symmetric) synapses made by parvalbumin-containing presynaptic terminals onto granule cells. In human epileptic specimens with Ammon's horn sclerosis, there was no reduction in the number of inhibitory synaptic contacts onto the granule-cell bodies or proximal dendrites. In fact, there was an increase in the coverage of axon initial segments by parvalbumin-containing inhibitory terminals. Thus, it seems as if a loss of parvalbumin-immunopositive interneurons may reflect a transient down-regulation of parvalbumin expression rather than a loss of interneurons. Alternatively, the reduced number of parvalbumin-immunopositive interneurons may sprout, resulting in the maintenance of inhibitory synapse numbers. There is some evidence for such reorganization of axonal systems of surviving interneurons. The precise anatomical localization and the quantitative contribution of different types of interneurons are much less clear than for principal neurons. This is primarily due to the presence of many distinct classes of interneurons, which makes it necessary to label and track individual axons of functionally and neurochemically identified cells.

Changes in the Elementary Properties of Synapses and Receptors

A large body of evidence exists supporting altered excitatory and inhibitory neurotransmission in the dentate gyrus. It should be stressed that the majority of these studies is based on recordings from granule neurons, without a determination whether these were newly generated neurons or not. Thus, the changes observed could be due to the fact that part of the neurons included in these datasets were newly born granule cells, or to altered properties of synapses onto preexisting granule cells. Assuming a homogeneous population on adult granule neurons, recordings of synaptic input will in most cases reflect a mixture of preexisting and newly formed synapses. Newly formed synapses appear to have properties that distinguish them from preexisting synapses, and

it will be important to extend our understanding of the elementary properties of these synapses.

Regardless of the mechanisms, excitatory glutamatergic synaptic transmission onto granule neurons has been shown to be altered both in chronic experimental and human epilepsy (see **Table 1**). A large body of evidence has accumulated supporting altered properties of the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor. Epilepsy-associated changes in NMDA receptor function include a prolongation of channel opening, an increase in agonist sensitivity, or a decrease in Mg^{2+} sensitivity. These changes have been proposed to contribute to hyperexcitability in the kindling model of TLE and in human epilepsy. They are unlikely to be caused by persistent changes in the expression of NMDA receptor subunits or splice variants. Instead, there is good evidence that there is an altered regulation of the NMDA receptor complex, most likely via a less-efficient dephosphorylation by the Ca^{2+}-dependent phosphatase 2B. In contrast to NMDA receptors, the subunits underlying α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor complexes show transcriptional changes in experimental epilepsy. The precise functional consequences of these changes on a network level are so far unclear. Glutamatergic synaptic transmission is powerfully modulated by metabotropic glutamate receptors and other presynaptic autoreceptors. Metabotropic glutamate receptors also undergo changes in human and experimental epilepsy. So far, at least eight mGluR subunits are known, which can be grouped according to sequence similarities and mode of action. Class I mGluRs (mGluR1 and mGluR5) are located predominantly postsynaptically, and cause a release of Ca^{2+} from intracellular stores. Class II (mGluR2 and 3) and III mGluRs (mGluR4, mGluR6-8) are predominantly located presynaptically and modulate neurotransmitter release. Presynaptic receptors were shown to be down-regulated in the lateral perforant path in chronic experimental epilepsy and epilepsy patients with Ammon's horn sclerosis, resulting in less mGluR-dependent depression of glutamate release. In contrast, postsynaptic types of mGluRs were shown to be up-regulated in chronic human and experimental epilepsy and may promote epileptogenesis. Changes in $GABA_B$ receptors include an up-regulation of postsynaptic $GABA_B$ responses in addition to a down-regulation of presynaptic $GABA_B$ receptors, resulting in enhanced inhibition in experimental TLE. Given the large changes in glutamatergic neurotransmission in the dentate gyrus, as well as profound changes in Ca^{2+} dynamics and handling in dentate granule neurons, it is not surprising that the potential of the perforant path-dentate gyrus synapses to exhibit synaptic plasticity is severely impaired in human epilepsy with Ammon's horn sclerosis, as well as in some models of experimental epilepsy.

The GABA system also displays a number of changes in chronic epilepsy (see **Table 1**). GABA binding to ionotropic $GABA_A$ receptors causes opening of the receptor ionophore, which is permeable to Cl^- and – to a lesser extent – to HCO_3. In the presence of a normal adult transmembrane Cl^- gradient, this channel opening results in the expression of an inhibitory postsynaptic current that hyperpolarizes the postsynaptic neuron. In the

Table 1 Summary of changes in voltage-gated ion channels and neurotransmitter receptors in dentate granule neurons in chronic epilepsy

	Changes in animal model	*Molecular mechanisms*
Voltage-gated ion channels		
A-type K^+ current (I_A)	–	–
Delayed rectifier K^+ current (I_K)	–	–
H-currents (I_h)	Up-regulation of I_h	Transcriptional up-regulation of HCN subunits, other mechanisms
Ca^{2+} currents	Altered Ca^{2+}-dependent inactivation of Ca^{2+} channels	Loss of calbindin-D_{28k}
Neurotransmitter receptors		
NMDA	Up-regulation of NMDA receptor function	Altered modulation by phosphatase 2B, resulting in prolonged NMDA receptor opening
AMPA	No large functional changes detected	Transcriptional changes
Metabotropic glutamate	Down-regulation of presynaptic Group III metabotropic glutamate receptors in the perforant path	Down-regulation of mGluR8 function
$GABA_A$	Increase of Zn^{2+} sensitivity, altered magnitude of tonic $GABA_A$ receptor-mediated currents	Change in subunit composition, change in synaptic vs. extrasynaptic localization of subunits
$GABA_B$	Up-regulation of postsynaptic $GABA_B$-mediated responses, down-regulation of presynaptic $GABA_B$-mediated actions	Altered expression of pre- and postsynaptic $GABA_B$ receptors, other mechanisms

NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; GABA, γ-aminobutyric acid.

dentate gyrus, profound transcriptional changes in $GABA_A$ receptor subunit expression take place. These changes result in altered function and pharmacology of the $GABA_A$ receptor complex, including an increased sensitivity to blockade by the divalent cation Zn^{2+}. This finding is important because recurrent mossy fibers contain large amounts of vesicular Zn^{2+}, and might release it during seizures. This release could potentially lead to a dynamic block of $GABA_A$ receptors and a collapse of inhibition. This attractive hypothesis has not been so far directly proven. Further changes in GABAergic synapses include a change in the precise localization of GABA receptors composed of specific subunit combinations at perisynaptic vs. postsynaptic locations. Such changes have been proposed to impair fast (phasic) synaptic inhibition at individual GABAergic synapses. A second type of inhibition, mediated by extrasynaptic GABA receptors persistently exposed to low ambient GABA levels (tonic inhibition), may also be increased as a consequence of such changes in subsynaptic localization of receptors.

Changes in Intrinsic Properties of Dentate Granule Neurons via Altered Expression of Voltage-Gated Ion Channels

In contrast to the manifold changes in synaptic properties, the intrinsic firing behavior of granule neurons seems to be more stable, especially when compared with other hippocampal subregions. Unlike the majority of CA1 neurons, which convert from regular-firing to a burst-firing mode in experimental epilepsy, DGCs retain a regular-firing mode. Even though there are no changes in firing mode of dentate granule neurons in different models of epilepsy, there may be epilepsy-related changes in some aspects of neuronal discharge. For instance, a reduction in spike after hyperpolarizations of medium duration were observed in a subgroup of granule cells from patients with Ammon's horn sclerosis. This loss of medium spike after hyperpolarization was quite likely related to the loss of spike frequency accommodation that is characteristic both of rodent granule cells as well as of granule cells recorded from epilepsy patients without Ammon's horn sclerosis. In addition, action potential thresholds were increased, and an enlarged fast after hyperpolarization was observed. Which changes in voltage- or calcium-dependent conductances may underlie these phenomena have not been determined. However, changes in many voltage-gated conductances have been reported both on the expression and functional level (see **Table 1**). For instance, voltage-gated sodium channels display changes in voltage-dependence; however, slow inactivation processes, the time-dependent properties of activation and inactivation, and recovery from inactivation are unaffected in the pilocarpine model of epilepsy. Voltage-gated transient (A-type) and persistent (delayed rectifier) potassium currents displayed no changes in the pilocarpine model of epilepsy. Changes in other types of currents both on the expression and functional level in granule cells have been reported. It should be noted that in many cases, differing results have been obtained with different models, and a correlation between expression and functional data is not always obvious.

An analysis of firing behavior with somatic recording electrodes, however, does not capture all aspects of intrinsic excitability, for instance, changes in distal dendrites. Changes in dendritic excitability may profoundly affect integration of dendritic excitatory input to an action potential output. Regrettably, recording from thin granule-cell dendrites is technically difficult, and so direct data on dendritic processing in granule neurons is scarce. However, there are several hints that dendritic processing may be affected in chronic epilepsy. For instance, there is a powerful up-regulation of HCN subunits within the dentate gyrus both in experimental epilepsy, and subgroups of epilepsy patients with Ammon's horn sclerosis. HCN subunits give rise to the H-current, a mixed cationic current activated by hyperpolarization, which is normally concentrated in the dendritic compartment and strongly influences dendritic integration in other types of principal neurons.

Collective Properties of the Dentate Gyrus in Seizure Discharge

How can these manifold sets of data be developed into a hypothesis regarding the function of the dentate gyrus in chronic epilepsy? This important issue has recently been directly addressed experimentally. Simultaneous voltage-sensitive dye imaging and patch-clamp recordings in hippocampal slices have allowed investigators to record the activity of different neuronal ensembles in the hippocampus at high-temporal resolution. These experiments have led to a significant advance in our understanding the role of the epileptic dentate gyrus in the context of the hippocampal network. So far, such experiments have addressed how inputs to the dentate gyrus from cortical areas are processed, and have confirmed that the normal dentate gyrus exerts a powerful control on information transfer from the cortex into the hippocampus proper. This idea has led to the dentate gyrus being termed the dentate 'gate.' Surprisingly, this control was virtually unchanged in chronically epileptic animals, contrary to previous hypotheses that the function of the dentate gyrus might be impaired. Thus, it seems as if the undoubtedly present changes that might contribute to a loss of dentate gating function (i.e., the earlier-mentioned changes in NMDA or GABA receptors) are offset, or homeostatically compensated, by other cellular changes that maintain the function of the dentate gate. It is interesting to note that these experiments have led to an alternate hypothesis regarding the ignition of seizure activity within the

hippocampus proper: It is likely that a direct cortical input to the CA1 region, the temporoammonic pathway, is weak in normal animals. In epileptic animals, the efficacy of this pathway in recruiting CA1 neurons is massively increased. It is likely that the dysregulation of this pathway provides an alternate route to permit activation of the CA1 and CA3 neuron by temporal afferents that bypasses the dentate gyrus.

Outlook

The dentate gyrus is one of the better-investigated structures in the brain. Nevertheless, there is much left to be learned about this structure. A continuation of the detailed dissection of the cellular properties of individual neuron types, as well as analysis of their synaptic connectivity will hopefully provide a more complete view of the functional matrix of the dentate gyrus. In addition, there are a number of novel issues and techniques that should be taken into account for future investigations.

First, the function of dentate granule neurons and most interneurons has been investigated virtually exclusively using somatic voltage- and current-clamp recording. In contrast, the properties of granule-cell dendrites are virtually unknown. Studies on CA1 and cortical neurons have shown that dendrites are structures with fascinating active properties, which strongly influence how different types of synaptic input converging on dendrites are integrated. These processes are not understood in DGCs.

Second, many studies to date have employed slice recording techniques to examine the functional alterations of granule cells and interneurons. These high-resolution approaches are indispensable to precisely analyze the properties and synaptic connectivity of different neuron types. However, it would be highly desirable to learn more about the in vivo behavior of identified neuron types during the initiation, maintenance, and termination of different types of seizure activity. These types of in vivo recording are increasingly being carried out to understand the relation of interneuron and principal cell firing during characteristic cortical activity patterns associated with cognitive processes. These techniques could be readily applied to epilepsy.

Finally, a general question to be answered in the future is what signalling pathways are evoked that convert granule cells from their normal physiological state to an 'epileptic' granule cell, a process termed *epileptogenesis*. Our knowledge of the transcriptional and translational control mechanisms that govern the coordinated production of proteins in neurons is rapidly expanding, and some intriguing novel studies have identified key pathways involved in epileptogenesis. Apart from the neurobiological importance of such mechanisms, they may open new avenues in the treatment and prevention of chronic epilepsy.

See also: **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; **Granule Cells**: Phenotypic Plasticity of the Granule Cells and its Relation to Epilepsy; Properties of Dentate Granule Cells and their Relevance to Seizures; **Interneurons**: GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex; Plasticity and Reorganization of GABA Neurons in Epilepsy; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization.

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Phenotypic Plasticity of the Granule Cells and its Relation to Epilepsy

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Introduction

Epileptic activity is generated when the balance of excitation and inhibition is impaired and when excitatory activity, normally conveyed by cells releasing glutamate, surpasses inhibition, which is exerted by interneurons releasing the inhibitory amino acid, γ aminobutyric acid (GABA). Sets of inhibitory interneurons not only control excitability but also fine-tune network activity, thus enabling generation of rhythms important for cognitive functions. However, the often-assumed view that glutamate excites and GABA inhibits is rather simplistic, and only true in part. Indeed, besides its well-known excitatory actions, glutamate can have inhibitory actions by depressing neurotransmitter release through the activation of presynaptic receptors, both with ionotropic or metabotropic activity. Alternatively, the inhibitory transmitter GABA can exert excitatory actions by directly depolarizing postsynaptic cells under certain circumstances, or by causing disinhibition – e.g., when one inhibitory interneuron inhibits another inhibitory cell. Irrespective of the direction of the actions exerted by these amino acids, it has been traditionally considered that some cells release glutamate and other cells release GABA, but not that a given cell can release both amino acids. Yet, in view of the presence of GABA in the glutamatergic mossy fibers (MFs, the axons of dentate gyrus granule cells), could it be that these cells release both neurotransmitters? If so, what impact would the simultaneous release of glutamate and GABA have on the excitability of the postsynaptic cell and, finally, on the overall excitability of the network under study? As evidence accumulates supporting the hypothesis that granule cells (GCs) corelease these amino acids, new possibilities of neural computation arise. Moreover, because a tight maintenance of the excitation–inhibition balance is needed to avoid the emergence of epileptic activity, such a mechanism would provide an effective means to rapidly auto-regulate the system.

Background

Granule-Cell Connectivity and Plasticity

The dentate gyrus (DG) is the main target of cortical input to the hippocampus. The principal cells of the DG, the GCs, project through the MF pathway to area CA3, where they release glutamate to excite pyramidal cells and local inhibitory interneurons. The latter, in turn, inhibit pyramidal cells to sustain feed-forward inhibition (**Figure 1**). Besides releasing glutamate, the GCs also contain and release dynorphin, enkephalin, Zn^{2+}, brain-derived neurotrophic factor (BDNF), and other peptides such as somatostatin, neuropeptide Y, neurokinin B, and cholecystokinin. Finally and surprisingly, the GCs also contain and release the inhibitory amino acid GABA. Most of the peptides that the GCs contain, as well as GABA and its synthesizing enzyme GAD_{67}, are regulated by activity – which is especially apparent after epileptic activity.

The activity-dependent plasticity of the GCs and their MFs, including the changes associated with epileptiform activity, is being actively explored with different experimental approaches. Particularly, in several models of experimental epilepsy, permanent and transient changes have been described at the molecular, functional, and anatomical level. In all experimental models of epilepsy, as well as in human epileptic patients, a series of plastic changes have been identified in the GCs. Among these plastic changes are alterations of the effects, metabolism, and content of Zn^{2+}; expression of neurotrophins and their receptors; sprouting of the MFs and formation of atypical GCs with basal dendrites; changes in the content and release of opioid peptides; induction of immediate early genes; regulation and transcription of opioid peptide genes and transcription factors; changes in the homeostasis of Ca^{2+} and ionic fluxes; and expression of GAD and GABA. Some of these changes appear to be adaptive and may serve to limit the risk of seizure generation, while others facilitate this process. Furthermore, some of these plastic phenomena seem not to be exclusively related to epilepsy but can occur after synaptic strengthening by long-term potentiation (LTP) induction.

In the DG-to-CA3 projection, each GC contacts ~15 pyramidal cells with 'giant boutons' and ~40–50 CA3 interneurons through philopodia and en passant terminals. Thus, it is clear that a major target of the MFs is onto inhibitory interneurons, thus involving the activation of feed-forward inhibitory circuits. The GCs also synapse proximally onto DG basket cells (providing local recurrent inhibition in the DG) and pyramidal-like neurons in the hilus; each GC makes 140–150 contacts of these latter types. Another important anatomical aspect of the MF pathway is that it

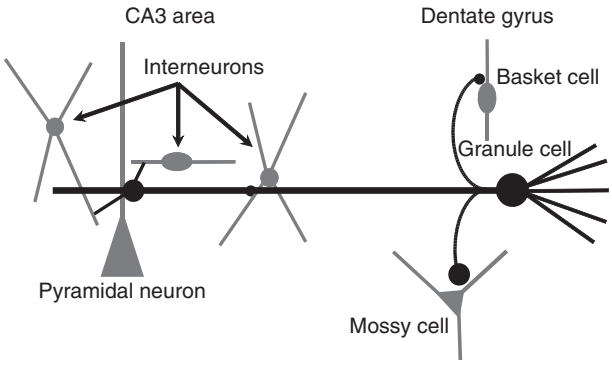


Figure 1 Diagram representing the multiple types of contacts made by granule cells. Within the dentate/hilar region collaterals of the mossy fibers innervate both mossy cells and inhibitory basket cells. In area CA3, the mossy fibers contact pyramidal neurons via large expansions and a large number of interneurons through either filopodial extensions from the large boutons or smaller en passant expansions along mossy-fiber axons. Reproduced from Jaffe DB and Gutiérrez R (2007) Mossy fiber synaptic transmission: Communication from the dentate gyrus to area CA3. In: Scharfman HE (ed.) *The Dentate Gyrus: A Comprehensive Guide to Structure, Function, and Clinical Implications*, Amsterdam: Elsevier.

is restricted to a narrow band running within the stratum lucidum, where the large terminals make contacts onto the most proximal portions of pyramidal neuron apical dendrites. In addition, there is a less extensive infrapyramidal MF projection onto the proximal basal dendrites of pyramidal neurons in the CA3b,c subregions.

Dual Glutamatergic–GABAergic Phenotype of Dentate Granule Cells

GABA was initially found to be present in the glutamate-containing terminals of the MFs of monkey and human hippocampi. However, since its synthesizing enzyme (glutamic acid decarboxylase, GAD) was not known to be expressed in GCs, it was thought that the amino acid was either transported from the extracellular space or originated from an alternative route of synthesis. The discovery of GABA in MFs led to the hypothesis that at least a component of the inhibitory synaptic potentials evoked in pyramidal cells by stimulation of the DG was of MF origin, and that the GABA released by the MFs could modulate the normal glutamatergic responses. Later work conclusively demonstrated the presence of GAD_{67} and GABA in human, monkey, and rat GCs, and that their expression was transiently up-regulated after seizures provoked by chemical convulsants, by the kindling method, and in a genetic epilepsy model.

Moreover, it was established that GAD_{67} could be transiently up-regulated in the GCs (**Figure 2(a)–2(c)**) and their MFs in an activity-dependent manner, even in the absence of epileptiform activity. Therefore, if the GCs had the necessary enzyme for the synthesis of GABA, and GABA itself was present, it seemed reasonable that GABA was probably used for fast neurotransmission. Soon after these demonstrations, the coexistence of glutamate and GABA in single MF boutons, and of their respective receptors in apposition to the presynaptic terminal, were confirmed. Finally, the mRNA of the vesicular GABA transporter (VGAT) was shown to be expressed in GCs in an activity-dependent manner (**Figure 2(d)**), although the transporter protein has not been detected in the MF, with the available antibodies. It was recently established that for the expression of the markers of the GABAergic phenotype in the GCs, calcium influx produced by glutamate, neurotrophin receptor activation, and protein synthesis were required.

Altogether, this evidence compellingly established that the adult GCs express a dual glutamatergic–GABAergic phenotype, whereby the latter is expressed in an activity-dependent manner. However, the expression of the GABAergic phenotype in GCs is constitutive during development. This observation has been one of the arguments that underlie the proposal that epileptogenesis recapitulates ontogenesis. Indeed, during the first three weeks of life, the GCs contain not only glutamate and its vesicular transporter, but also GABA, GAD_{67} and VGAT mRNA. These GABAergic markers abruptly disappear upon completion of early development and, as previously noted, can be reinduced in an activity-dependent manner.

As a result of these studies, investigators assumed that GABA synthesis in the MFs was a means to counteract the enhanced excitability caused by epileptic activity. But does the coexpression of a GABAergic phenotype in these glutamatergic cells turn them into inhibitory cells?

Methods

Electrophysiological Recordings

Adult Wistar rats (250 g) of control and epileptic groups (see later) were decapitated under deep ether anaesthesia, and combined entorhinal cortex–hippocampus slices (400 μm) were obtained and maintained in vitro at (35 ± 0.5)°C. They were constantly perfused with oxygenated artificial cerebrospinal fluid (ACSF) containing the following (in mM): NaCl, 124; KCl, 3; NaH_2PO_4, 1.25; $MgSO_4$, 2; $CaCl_2$, 2; $NaHCO_3$, 26; glucose, 10; pH 7.35. The drugs used were diluted in the ACSF – namely, the NMDA receptor antagonist (DL)-2-amino-5-phosphonovaleric acid (APV, 30 μM, Tocris); the non-NMDA receptor antagonist 6-nitro-7-sulphamoylbenzo(f)quinolaxine-2,3-dione (NBQX, 10 μM, Tocris, Tocris-Cookson, St. Louis, MO, USA); the $GABA_A$-receptor antagonist bicuculline methiodide (20 μM, Sigma, Sigma Aldrich, St. Louis, MO, USA); the group II metabotropic GluR agonist (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl) glycine (DCG-IV, 1 and 10 μM, Tocris); the group III

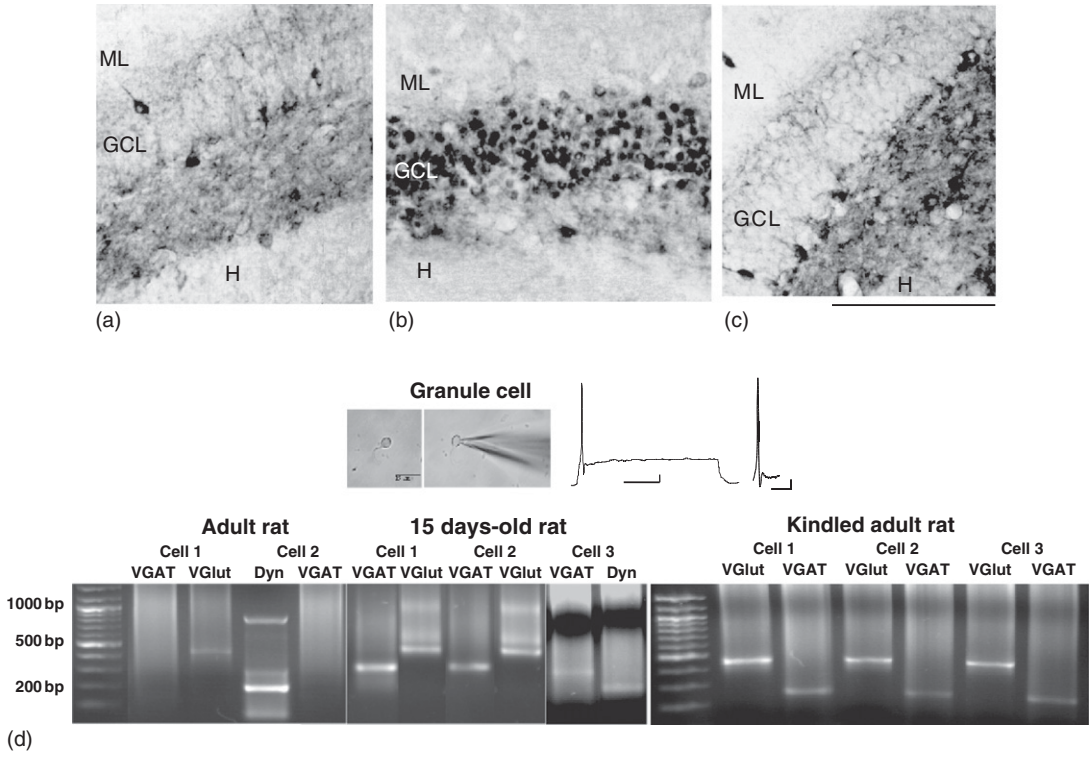


Figure 2 The granule cells express the markers of the GABAergic phenotype in an activity-dependent manner. (a) Immunohistochemical detection of GAD67 is normal in interneurons, but not in granule cells of the adult rat. (b) After seizures, the granule cells transiently up-regulate their expression of the enzyme. (c) A month after the last seizure, GAD67 is again undetected. The calibration bar represents 50 μm. (d) With single granule-cell PCR, we determined the expression of the glutamate (VGlut) and vesicular GABA transporters (VGATs), and dynorphin (Dyn) as a specific marker for granule cells. Granule cells of 15-day-old rats express Dyn, VGlut, and VGAT mRNAs. Adult healthy rats only express VGlut and Dyn mRNAs. After seizures, the expression of VGAT mRNA is up-regulated. (a—c) Modified from Ramírez and Gutiérrez (2001) Activity-dependent expression of GAD_{67} in the granule cells of the rat hippocampus. *Brain Research* 917: 139–146. (d), Modified from Gómez-Lira G, Lamas M, Romo-Parra H, and Gutiérrez R (2005) Programmed and induced phenotype of the hippocampal granule cells. *Journal of. Neuroscience* 25: 6939–6946, with permission from Society for Neuroscience.

metabotropic GluR agonist L(+)-2-amino-4-phosphonobutyric acid (L-AP4, 10 μM); the $GABA_B$ receptor agonist baclophen (3 μM, Sigma); and the protein inhibitor, cycloheximide (60 μM; Sigma).

Intracellular activity of pyramidal cells and interneurons in CA3 was recorded with glass microelectrodes (50–80 mΩ) filled with potassium acetate (2 M) and an AxoClamp 2B amplifier. (Molecular Devices, Foster City, CA) For evoking synaptic potentials, single-pulse electrical stimulation of the granule-cell layer of the DG was delivered at an intensity that evoked an excitatory postsynaptic potential (EPSP) with 60–70% of the amplitude required to make the cell fire action potentials. For extracellular recordings, we used glass microelectrodes (10–15 mΩ) filled with NaCl (155 mM). Field spontaneous activity was exported from pClamp8 and analyzed with ad-hoc programs written in MATLAB 7.0 (R14, MathWorks, Inc. Natick, MA). Samples of spontaneous field activity of either 10 or 16 s were acquired every 20 s throughout the experiments (usually lasting >2 h); they were digitized at 400 Hz and notch filtered at 60 Hz. Windowed Fourier analysis was applied using a Hanning window with 50% overlap. Through a graphical user interface, we were able to simultaneously visualize spectrograms and to calculate specific peak values and mean values for specific user-defined frequency windows.

Kindled and Acutely Induced Seizures

The rats were anaesthetized with ketamine (60 mg/kg i.p.) and implanted with bipolar stainless steel electrodes (80 KΩ) into the left basolateral amygdala (AP 2.5; L 5; H 8.5). Starting 7–10 days after the operation, the animals were stimulated daily with a 1-s train of square pulses (0.1-ms pulse duration, 60 Hz, 500 μA) until five generalized convulsive seizures (stage V) were evoked. Acute seizures were induced with a single i.p. administration of pentylenetetrazol (PTZ, 70 mg/kg, Sigma).

Kindling In Vitro

A bipolar (tip separation, 25 μm), glass-insulated platinum wire (50 μm) electrode was placed over the perforant path

or the granule-cell layer of the DG for stimulation. The stimulation protocol consisted of three trains of 0.1-ms pulses at 100 Hz; the train duration was 1 s and the intertrain interval was 1 min. These trains were delivered every 15 min, without varying the stimulus intensity. Between the kindling stimulation trials, synaptic responses were evoked with single pulses at 0.1 Hz delivered to the granular cell layer of the DG. The response to these single pulses was acquired and analyzed off-line with the program pClamp6 (Axon Instruments). For the experiments in which protein synthesis was inhibited, the slices were incubated in cycloheximide (60 μM) 15 min before initiation of the kindling stimulation protocol and for as long as it lasted (3 h). Intracellular recordings began at least 30 min after the removal of cycloheximide from the bathing medium, and could last several hours. The kindling stimulus intensity used for these experiments was previously determined, and set at a value needed to obtain 50% of the maximum amplitude of the DG population response to test pulses.

Immunohistological Studies

For the detection of GAD_{67} immunoreactivity, the slices of kindled epileptic rats, of PTZ-treated rats, or slices stimulated in vitro were processed simultaneously with control slices. They were fixed in phosphate buffer, 0.1 M, at pH 7.4 with paraformaldehyde at 4% for 48 h and transferred to a buffer with sucrose (30%) for 24 h. The slices were then frozen and cut at 30 μm in a microtome and collected in PBS (0.9% NaCl). The slices were incubated free-floating in PBS with 0.3% H_2O_2 to block endogenous peroxidase activity. After rinsing for three periods of 10 min in PBS, they were incubated in 10% horse serum (Gibco), 1% gelatin (Sigma) and 0.2% Tween-20 (Sigma), to avoid unspecific GAD staining. Slices were then incubated in the presence of GAD_{67} antibody (Chemicon) at 1:1000 (controls) or 1:1500 (stimulated) in PBS and horse serum (5%; 4°C) during 36 h with agitation. After four 10-min washing periods, they were incubated in the presence of an anti-rabbit biotinylated IgG (1:800; Amersham Pharmacia Biotech). The slices were then rinsed 4 times and processed with the ABC kit (Vectastain, Vector Laboratories) for 1 h at room temperature. After four 10-min washing periods, the slices were developed with diaminobenzidine (Sigma; 10 mg per 25 ml PBS (10 mM)) for 10 min and contrasted with nickel sulphate (30%).

Single-cell RT-PCR

The GCs were dissociated and used for whole-cell electrophysiological recordings. After recording, the cytosolic content of the cells was extracted by applying gentle negative pressure with the recording pipette; contents were placed in a PCR tube containing 8 μl of buffer 5× (one-step RT-PCR kit, Quiagen, Valencia, CA), 1.6 μl of dNTPs (final concentration 10 mM; One-Step RT-PCR kit Quiagen), 50 pmol of each oligonucleotide (to amplify the selected RNAs; see below), 40 units of RNAse inhibitor (Invitrogen, Carlsbad, CA), and 3 μl of the mixture of 'Omniscript' reverse transcriptase, 'Sensiscript' reverse transcriptase, and 'HotStarTAq' DNA polymerase (one-step RT-PCR kit, Quiagen), yielding a final volume of 40 μl. The RT-PCR reaction was then carried out in a thermocycler (PCR express, Hybaid, Ashford, UK) as follows: 30 min at 50°C; 15 min at 95°C; 30 s at 94°C, 30 s at 52°C, 1 min at 72°C (40 PCR cycles), and, finally, 10 min at 72°C. A second PCR reaction was then conducted with nested oligonucleotides. The product of the first amplifying protocol (3–5 μl) was used as a template for the second PCR protocol, in a final volume of 25 μl. The PCR solution contains PCR buffer 10× (Ammersham Biosciences, Piscataway, NJ), 10 μM of dNTPs (Invitrogen), 1-unit Taq DNA polymerase (Ammersham), and 50 pmol of each oligonucleotide. The reaction was carried out as follows: 2 min at 94°C, 30 s at 94°C, 30 s at 55–58°C, and 1 min at 72°C (35 PCR cycles). The specific oligonucleotides for each gene were custom-designed (Invitrogen) based on the indicated references. The oligonucleotides were for VGAT (sense, antisense, nested (antisense)); dynorophin (sense, antisense, nested (sense)); VGlut-1 (sense, antisense, nested). The products were visualized by electrophoresis in agarose gels (1.5%) with ethidium bromide. The expected weight of each final PCR product is VGAT, 250 bp; VGlut-1, 450 bp; and dynorphin, 200 bp. For controls, we conducted multiple sham experiments in which the surrounding bath solution was collected and processed in parallel. We did not detect the expression of mRNA in cells where we omitted the reverse transcriptase from the RT reaction.

Recent Results

Activation of the Mossy Fibers Provokes $GABA_A$ Receptor-Mediated Monosynaptic Responses in their Target Cells of CA3

The electrophysiological evidence of the presence of GABAergic transmission from the MFs to CA3 is in total agreement with the aforementioned immunohistochemical and molecular biological data. When the GABAergic markers are expressed (e.g., during development or after the induction of hyperexcitability), stimulation of the MFs – in the presence of glutamate receptors blockers – produces $GABA_A$ receptor-mediated monosynaptic responses both in pyramidal cells and interneurons of the CA3 area. It has been proposed that the MFs are, in fact, primarily GABAergic during the first days of life; subsequently, as development proceeds, more and more fibers are recruited to the dual use of GABA and glutamate, constituting a dual-phenotype pathway.

Finally, by the beginning of the fourth week of life, the GABAergic phenotype shuts off.

It has been known for many years that the activation of the MFs monosynaptically excites pyramidal cells and local inhibitory interneurons, and that the interneurons in turn inhibit pyramidal cells. Thus, activation of the DG provokes monosynaptic excitation and disynaptic inhibition on CA3 neurons that, with intracellular recordings, are evidenced as sequences of excitatory and inhibitory postsynaptic potentials. The perfusion of the NMDA-type glutamate receptor antagonist 5APV and the AMPA-kainate-type receptor antagonist NBQX blocks all synaptic responses, demonstrating that the inhibitory transmission in the MF-to-CA3 projection is disynaptically mediated (**Figure 3(a)**). However, under the same experimental conditions, the stimulation of the MFs in developing rodents leads to monosynaptic GABAergic responses in CA3 pyramidal cells and interneurons. Further, these GABAergic responses have the same pharmacological and plastic properties as those reported for glutamatergic MF transmission – i.e., they exhibit strong frequency-dependent facilitation, and they are presynaptically inhibited by the activation of metabotropic glutamate receptors (mGluR).

As already mentioned, in the healthy adult rat, in which the GABAergic markers are not expressed in MFs, MF GABAergic transmission cannot be observed. Interestingly, in rats subjected to kindling, stimulation of the MF in the presence of glutamate receptor blockers provokes a monosynaptic bicuculline-sensitive inhibitory postsynaptic potential (IPSP) (**Figure 3(b)**). This monsynaptic IPSP has the same latency as the control EPSP and is strongly inhibited by the activation of mGluR. Moreover, this response is transient; it is observed 24–48 h after the last-kindled seizure but not if the experiment is carried out a month after the last seizure (**Figure 3(c)**). If seizures are again produced, these monosynaptic GABAergic responses reappear. Experiments also showed that the kindled epileptic state was not necessary for this monosynaptic IPSP to be expressed, as a

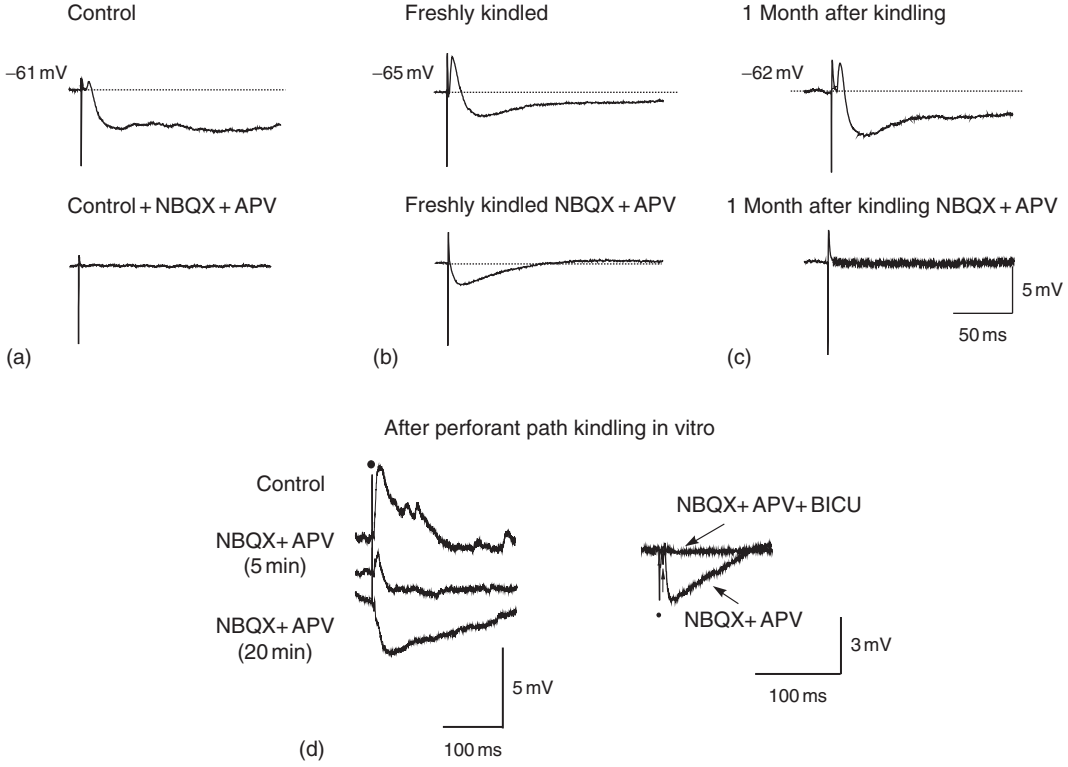


Figure 3 Synaptic responses of CA3 pyramidal cells to DG stimulation in preparations from healthy rats (a) and 24 h (b) and a month after the last generalized kindled seizure (c). In cells from freshly kindled rats, DG stimulation under NBQX + APV perfusion elicited IPSPs, which were blocked by bicuculline (lower trace). A month after the last seizure, DG stimulation in the same conditions no longer evokes GABAR-mediated responses. Note the correspondence of these responses with the expression of GAD_{67} depicted in **Figure 1** (d) Repeated application of an LTP-like protocol (in vitro kindling) to a hippocampal slice from a healthy rat produces the emergence of GABAR-mediated monosynaptic transmission from the mossy fibers. Traces are averages of six responses. (a—c), Modified from Gutiérrez and Heinemann (2001) Kindling induces transient fast inhibition in the dentate gyrus-CA3 projection. *European Journal of Neuroscience* 13: 1371–1379, with permission from Blackwell Publishing. (d), Modified from Gutiérrez (2002) Activity-dependent expression of simultaneous glutamatergic and GABAergic neurotransmission from the mossy fibers in vitro. *Journal of Neurophysiology* 87: 2562–2570, with permission from American Physiological Society.

single seizure could provoke its emergence. Furthermore, the repeated application of an LTP-inducing protocol in the perforant path or in the MF themselves – which does not produce epileptiform activity – provokes the emergence of MF GABAergic monosynaptic transmission (**Figure 3(d)**). These experiments demonstrated that a certain level of sustained excitation, and not the presence of epileptic activity, is required for the GCs to express the GABAergic phenotype and GABAergic transmission. These experiments also showed that this process requires protein synthesis. Activation of the postsynaptic cell is not required for the induction of MF monosynaptic GABAergic transmission; i.e., the novel incorporation of $GABA_A$-R in the postsynaptic site does not account for the appearance of GABAergic responses on MF stimulation.

As previously noted, the putative release of glutamate and GABA from the MF takes place onto both their targets in CA3 (the pyramidal cells and the interneurons) and also onto hilar interneurons. In fact, for each contacted pyramidal cell, the MFs contact 50 interneurons. This difference can have profound functional significance given that feed-forward inhibition is a powerful mechanism that controls CA3 excitability. And as discussed here, at least in some respects the release of glutamate and GABA onto interneurons seems to be different from the release onto pyramidal cells.

Intracellular recordings from interneurons in CA3 of healthy adult rats revealed that MF-evoked EPSP/IPSP sequences can be totally blocked in the presence of glutamatergic antagonists (**Figure 4(a)** and **(4b)**), thus demonstrating that inhibitory transmission onto interneurons, as onto pyramidal cells, is disynaptically mediated. However, as previously described in pyramidal cells, MF activation in the presence of glutamatergic antagonists provokes monosynaptic IPSPs with the same latency as the control EPSP after seizures or LTP-like stimulation (**Figure 4(c)** and **4(d)**). However, although MF monosynaptic GABAergic transmission is expressed onto interneurons under these conditions, the effect of the MF glutamatergic transmission surpasses the inhibitory one. In animals subjected to seizures or tetanic stimulation, activation of the MF pathway provokes a high amplitude and long-lasting EPSP that totally obscures the underlying IPSP in interneurons. In contrast, pyramidal cells in these preparations display an enhanced IPSP that partially shunts the EPSP. Thus, the interneurons are more readily excited than pyramidal cells. The pyramidal cells thus receive a strong inhibitory input from the interneurons within CA3, in addition to the monosynaptic MF GABAergic signal. Activation of MF at high frequencies provokes the more pronounced IPSPs in pyramidal cells to be potentiated, and their summation leads to pyramidal-cell hyperpolarization. In interneurons, high frequency stimulation leads to summation of EPSPs and readily provokes interneuron spiking (**Figure 4(g)** and **4(h)**). These experiments show that although both pyramidal cells and interneurons receive the same dual MF input, the released glutamate-GABA ratio onto the target cells can be different. This differential response of pyramidal cells and interneurons of CA3 prevents disinhibition of CA3 and, as discussed later, can modify the interneuronal network-mediated oscillatory activity.

There is another mechanism that controls the release of neurotransmitters at the MF synapse. Glutamate and GABA released from the MFs can exert presynaptic inhibition of nearby MFs, the terminals of which contain mGlu, and $GABA_A$ and $GABA_B$ receptors. Activation of different presynaptic mGluRs differentially inhibits glutamate or GABA release, and it is also likely that the density of presynaptic mGluRs and $GABA_A$ and $GABA_B$ receptors differ in large MF terminals, in en passant boutons, and in filopodial extensions (**Figure 4(e)** and **4(f)**; see also **Figure 6**).

Functional Implications with Regard to Epilepsy

It has been proposed that the DG serves as a filter that limits the transfer of information from the entorhinal cortex to the pyramidal layer of the hippocampus. However, in kindled animals, this filtering mechanism seems to be altered. Clearly, in the hippocampus of epileptic rats, one of the compensatory changes is the expression and release of GABA from the DG. Therefore, in the adult rat, the transient expression of a GABAergic phenotype in the otherwise glutamatergic GCs seems to be a response to counteract enhanced excitation. Stimulation of the DG, or direct activation of MFs, produces IPSPs in pyramidal cells and interneurons; when recorded extracellularly, $GABA_A$ receptor-mediated field responses can also be detected, indicating that a considerable number of boutons of a well-defined GABAergic pathway simultaneously release GABA onto a large number of receptors. This observation shows that the MF pathway constitutes a 'GABAergic-also' pathway after seizures, and that GABA release has a strong impact on the CA3 area as a whole. This enhanced GABAergic transmission from the DG-to-CA3 could be involved in the anterograde amnesia that appears for some time after seizures, particularly after status epilepticus, which can hamper storage of information in the hippocampal network. Indeed, changes in the phenotype of MFs may have an impact on the capability of the network to generate rhythmic activities such as theta and gamma oscillations. In fact, the MF GABAergic input to CA3 has been shown to modulate its spontaneous oscillatory activity after seizures. Inhibiting release of MF GABA by the activation of mGluRs provokes the enhancement of β/γ activity in CA3 by a presynaptic mechanism (**Figure 5**).

These experimental results suggest that, after seizures, the MF projection exerts a tonic inhibitory control on

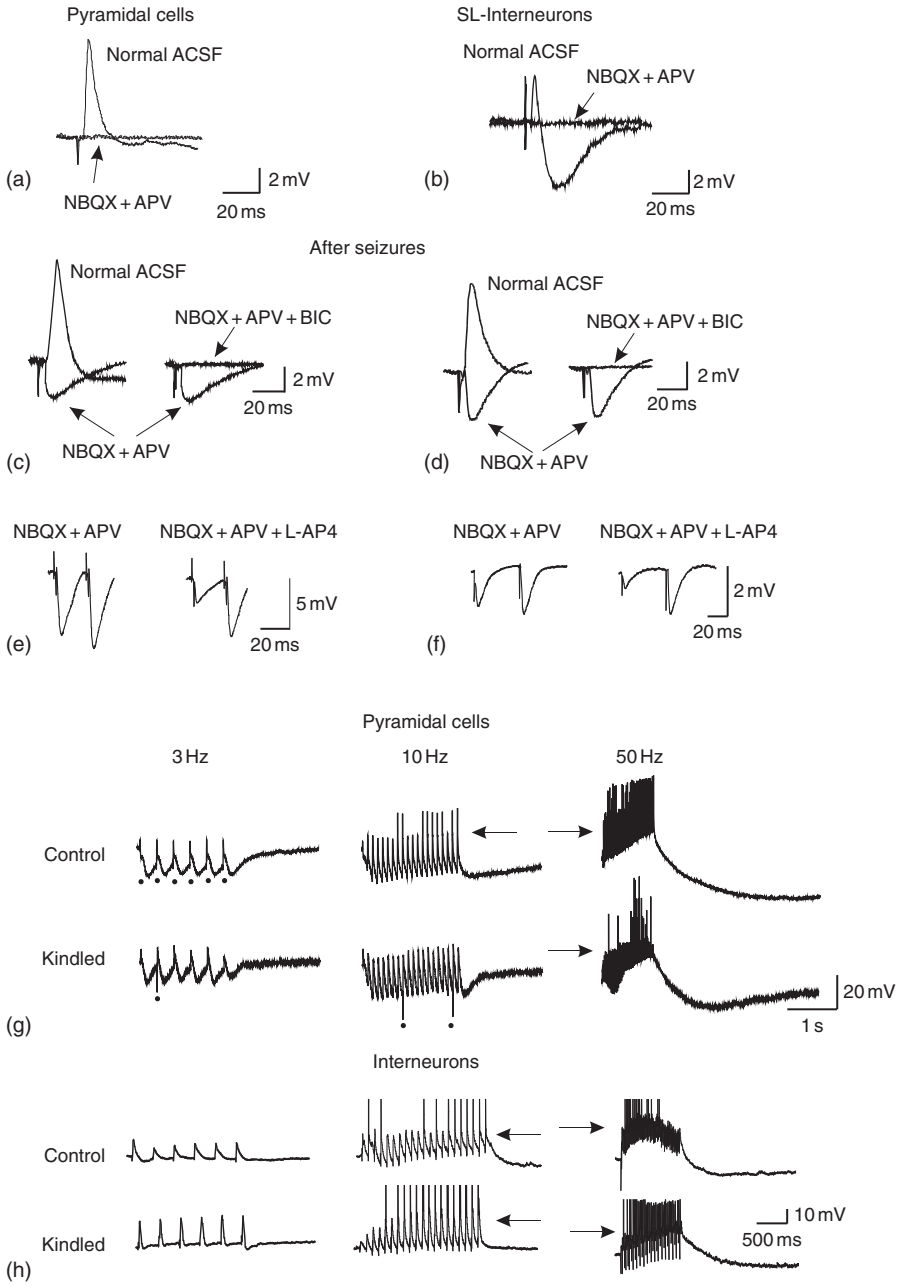


Figure 4 Mossy fibers simultaneously release glutamate and GABA onto all its target cells in CA3 area. In electrophysiologically and anatomically identified pyramidal cells (a) and Stratum lucidum interneurons (b) from control preparations, MF stimulation produced EPSP/IPSP sequences that were blocked by iGluR antagonists. In preparations from rats that were subjected to seizures, the same stimulation produced a monosynaptic $GABA_A$-R-mediated IPSP in the presence of NBQX and APV. This IPSP was blocked by the addition of bicuculline (c, d). A paired-pulse stimulation paradigm that produced potentiation of the second response was used to reveal the presynaptic inhibition of the $GABA_A$-R-mediated responses by activation of mGluR-III with L-AP4. The first response to the pair of pulses was depressed by L-AP4, while the $IPSP_2$:$IPSP_1$ ratio was enhanced, both in pyramidal cells (e) and in interneurons (f). Synaptic responses of CA3 pyramidal cells (g) and interneurons (h) to mossy-fiber activation at 3, 10, and 50 Hz in preparations from a control and from a kindled rat. Pyramidal cells from kindled rat presented less action potentials per train than those from control rats, whereas interneurons from kindled rats reached threshold for action potentials more easily than those from control rats. The arrow shows the point above which the responses were action potentials. The dots indicate stimulus artifacts. All traces are averages of three successive trials. (a–f), Adapted from Treviño M, Vivar C, and Gutiérrez R (2007) β/γ oscillatory activity in the CA3 hippocampal area is depressed by aberrant GABAergic transmission from the dentate gyrus after seizures. *Journal of Neuroscience* 27: 251–259, with permission from Society for Neuroscience. (g, h), Modified from Gutiérrez and Heinemann (2001) Kindling induces transient fast inhibition in the dentate gyrus-CA3 projection. *European Journal of Neuroscience* 13: 1371–1379, with permission from Blackwell Publishing and from Vivar C (2006) Plasticity of mossy fiber transmission onto the interneurons of the CA3 area of the hippocampus, doctoral dissertation, CINVESTAV.

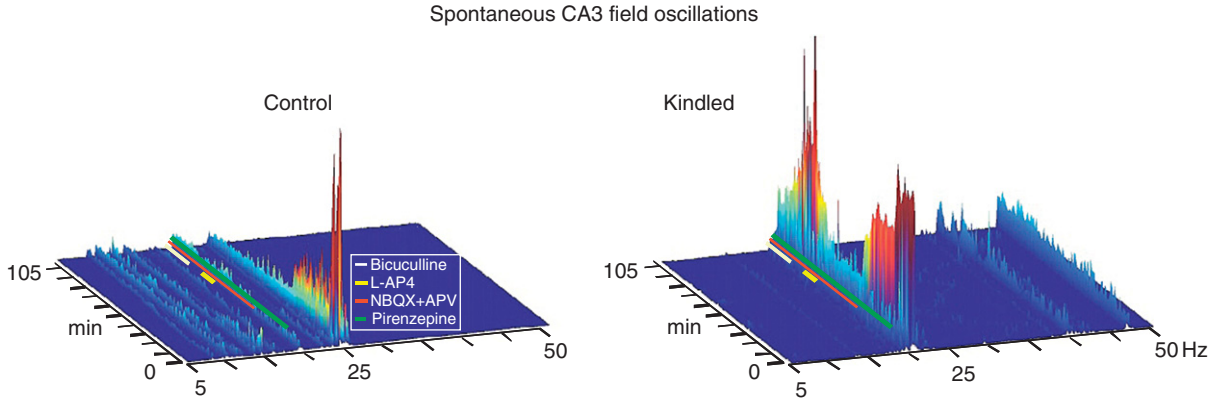


Figure 5 Mossy-fiber GABAergic input affects spontaneous field oscillations in the CA3 area after seizures. 3D layout of the spectrograms (from 5 to 50 Hz, traces of 4 s, *x* axis) were obtained throughout the experiment (FFT of traces of 10 s of duration, acquired every 20 s, *z* axis), in different pharmacological conditions. Spectral power is indicated in a color scale (*y* axis) where warm colors depict high power and cold colors depict low power. Colored lines over the mesh indicate the addition of pirenzepine (blue), NBQX + APV (red), L-AP4 (yellow), and bicuculline (white) to the normal ACSF. Note that the mGluR-III agonist, L-AP4, which presynaptically inhibits GABA release from the mossy fibers potentiates β/γ activity. In contrast, L-AP4 is ineffective in control preparations, where GABA release from the mossy fibers is not present. This is indicative of a tonic inhibition of the mossy fibers on the oscillations originated by the GABA interneuronal network in CA3. Adapted from Treviño M, Vivar C, and Gutiérrez R (2007) β/γ oscillatory activity in the CA3 hippocampal area is depressed by aberrant GABAergic transmission from the dentate gyrus after seizures. *Journal of Neuroscience* 27: 251–259, with permission from Society for Neuroscience.

CA3. A possible effect of the release of GABA from the MF would be to enhance GABA concentration in the extracellular space, which is likely to enhance tonic GABA currents. It has been shown that tonic GABA currents represent the most powerful inhibitory mechanism controlling the excitability of hippocampal cells. Oscillatory activity in area CA3 is intrinsically generated by the interneuronal GABA network, and MF input exerts its inhibitory control precisely on this network (**Figure 6**). Consequently, not only interneurons within CA3, but also the principal cells of the DG may be engaged in setting the tone for oscillations – to be expressed or suppressed – in the hippocampus (**Figure 6**).

In human temporal lobe epilepsy and respective animal models, MFs rearrange such that they also innervate neighboring glutamatergic projection cells (GCs) in addition to their 'normal' physiological innervation of mossy cells and basket cells in the hilus. These connections provide for a strong coupling between different lamellae in the hippocampus and thus may contribute to seizure spread. Yet to be explored is whether recurrent MFs, which develop after epilepsy and that innervate other GCs, also release GABA. Interestingly, aberrant, recurrent, sprouted MFs have been thought to underlie enhanced excitability of the DG. However, if these aberrant MFs were also to release GABA, it is unclear what the impact of this recurrent dual transmission would be on the activity and excitability of the DG. Corelease of GABA and glutamate after acute seizures would presumably prevent (or reduce the likelihood of) the activation of neighboring GCs and may also prevent activation of hilar mossy cells. Reduced activation in these cells may help to explain reduced seizure susceptibility, which is frequently observed after temporal lobe seizures.

Future Goals

The data reviewed compellingly show that the GCs can transiently express a dual glutamatergic–GABAergic phenotype during development, and also in adult animals following high levels of activity, particularly seizures. The morphological evidence that GABA can be exocytosed at MF synapses to act on postsynaptic $GABA_A$ receptors is as strong as that indicating that glutamate is released. However, the available body of electrophysiological evidence, although compelling, is indirect. Simultaneous recordings of synaptically connected granule and pyramidal cells are needed to unequivocally prove this hypothesis. Although this task is technically difficult (single GCs only contact few pyramidal cells), accomplishing it would help to clarify whether glutamate and GABA are released from single or different MF terminals and/or from single or different release sites. Another intriguing issue of GCs plasticity is the nature of the vesicular transporter for GABA in the MFs. Such a transporter has not been found in MF terminals, despite the presence of its mRNA and the modulation of vesicular transporter mRNA by activity, as determined by single-cell RT-PCR. This result suggests that in some GABAergic cells, as in the GCs, a yet unidentified GABA transporter exists and is expressed in an activity-dependent manner.

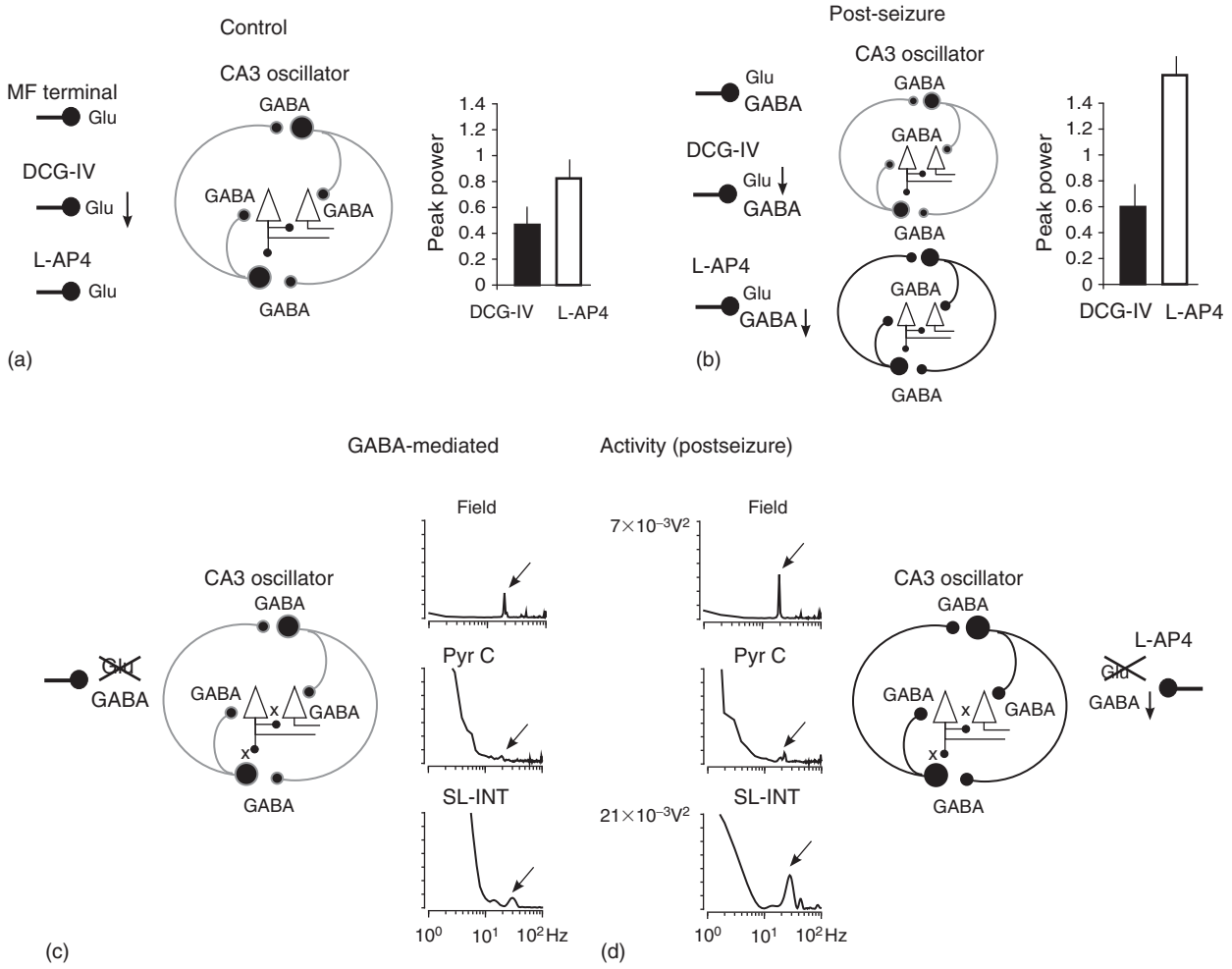


Figure 6 β/γ activity in the CA3 region is inhibited by mGluR-sensitive, GABA-mediated aberrant transmission from the mossy fibers. (a) CA3 is an endogenous oscillator, which receives a glutamatergic input from the MF. Activaiton of group II mGlu presynaptic receptors inhibits glutamate release from the MF and strongly depresses oscillatory activity, while activation of group III mGlu presynaptic reptors with L-AP4, which inhibite GABA release from the MF, produces a noticeable enhancement of the β/γ power. This uncovers the tonic inhibitory control of the MF on the oscillatory activity. This control is exerted on GABA-mediated oscillations because it is observed in the presence of iGluR blockers. (c) Indeed, in the absence of glutamatergic input, GABA-mediated oscillatory β/γ activity (arrows) can be observed in field recordings and in subthreshold spontaneous membrane oscillations of stratum lucidum interneurons of area CA3 after seizures. (d) Activation of group III mGluR relieves the inhibition of GABA-mediated β/γ oscillations (arrows) in the CA3 field, which is primarily reflected in the subthreshold spontaneous membrane oscillations of stratum lucidum interneurons. Reproduced from Gutiérrez R, *et al*. (2003) Plasticity of the GABAergic phenotype of the 'glutamatergic' granule cells of the rat dentate gyrus. *Journal of Neuroscience* 23: 5594–5598, with permission of the Society for Neuroscience.

Finally, we are still to answer the important questions about what molecular 'switch' is responsible for turning off the GABAergic phenotype in GCs as development proceeds, and conversely, what turns on GABAergic expression after epileptic activity? A number of candidate molecules (mostly transcription factors) regulating the expression of GABAergic markers have been described, and it is likely that one or some of them are involved in the regulation of this phenotypic plasticity in the GCs. This intriguing field of investigation will certainly give us important insights, not only into the generation and maintenance of epileptic activity, but also into a powerful neuronal communication tool.

Acknowledgements

I thank the collaborators, whose data contributed to this review. Research in my laboratory was funded by Consejo Nacional de Ciencia y Tecnología, the Third World Academy of Sciences, the Alexander von Humboldt Foundation, and Fundación Miguel Alemán, A.C.

See also: **GABA**: Plasticity of GABAergic Systems during Epileptogenesis; **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Plasticity**: Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; The Role of Short Term

Synaptic Plasticity in Development and Spread of Seizures.

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Properties of Dentate Granule Cells and their Relevance to Seizures

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Introduction

The hippocampus is required for declarative memory, the type of memory accessible to conscious recollection. For an individual to distinguish similar memories, he must encode distinct representations of the temporal and spatial relationships comprising events. This component of memory formation, referred to as pattern separation, depends on the activity of dentate granule cells. These neurons comprise the first stage of information processing within the hippocampus. Excitatory input from all parts of the cerebral cortex is fed through the entorhinal cortex and then dispersed onto a large number of granule cells having a low (0.1–1 Hz) baseline firing rate. This arrangement enables individual granule cells to receive and relay only a small, distinct fraction of the total input. Granule cells are electrically compact. Their dendritic properties support the precise temporal integration of synaptic signals at frequencies up to the top of the gamma range. The granule-cell firing rate increases (to 10–40 Hz) during behavioral tasks that activate memory formation. Granule-cell axons, the mossy fibers, transfer these discrete information sets to the pyramidal cells of hippocampal area CA3. In the rat, each granule cell innervates an average of only 14 CA3 pyramidal cells and each pyramidal cell receives a single mossy-fiber synapse apiece from only 50 granule cells. In contrast to this sparse projection, each granule cell innervates 40–50 CA3 interneurons. The ability of granule cells to achieve pattern separation derives, in part, from strong mossy fiber-evoked feed-forward inhibition that prevents the spread of excitation from the few targeted CA3 pyramidal cells to the rest of the synaptically interconnected pyramidal-cell population. Pattern separation also depends on the relatively low probability of glutamate release from mossy-fiber boutons when they are activated at frequencies that correspond to baseline granule-cell firing. The net effect of granule-cell output, which favors inhibitory drive onto area CA3 during low-frequency activity, switches to net excitation at higher discharge frequencies, such as those at which granule cells fire during memory formation. Frequency-dependent facilitation is of greater magnitude and operates over a much broader dynamic range at mossy-fiber synapses on pyramidal cells than on interneurons. Moreover, some mossy-fiber synapses on CA3 interneurons

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undergo frequency-dependent depression. Finally, intense synaptic bombardment of granule cells by the perforant path produces a sizable depolarization that can spread to mossy-fiber boutons and facilitate transmitter release. These cellular properties allow granule cells to serve as high-pass filters for the transfer of cortical information to the rest of the hippocampus.

The same cellular properties that subserve pattern separation also allow dentate granule cells to resist the propagation of seizures from the highly excitable entorhinal cortex to the equally excitable CA3 area. The low baseline firing rate of granule cells may be explained by strong synaptic and tonic γ-aminobutyric acid (GABA) inhibition, a high resting membrane potential, high K^+ conductance, low Ca^{2+} conductance, little or no h current (hyperpolarization-activated cation current – I_h), and a virtual lack of monosynaptic recurrent excitation. The near absence of mossy-fiber → granule-cell synapses allows each individual neuron to serve as a functional unit for encoding a distinct fraction of the total perforant path input and discourages synchronous discharge. In the normal brain, intense activation of granule cells by the perforant path is required for seizure propagation through the dentate gyrus, a phenomenon termed 'maximal dentate activation'. In epileptic brain, dentate granule cells are more easily recruited into epileptiform activity even though their excitability may appear unchanged in some tests. Changes in the intrinsic properties of granule cells, changes in granule-cell innervation, and the addition of hyperexcitable ectopic granule cells to dentate gyrus circuitry could explain these findings. To the extent that abnormal granule cell function contributes to seizure propagation, pharmacotherapy directed against these abnormalities may improve seizure control.

Background

The term 'granule cell' refers to a neuron with a small (8–12-μm diameter) round-to-oval cell body. Dentate granule cells in rodents usually have two spiny apical dendrites that branch extensively in the molecular layer. Rodent granule cells generally lack a basal dendrite, although ~2% have a recurrent basal dendrite, defined as a process opposite to the apical dendrite that is directed into the hilus and then eventually curves back into the molecular layer. However, ~25% of primate granule cells have a basal dendrite. The innervation of dentate granule cells is strongly laminar: Each excitatory and inhibitory afferent projection forms synapses on a distinct and usually nonoverlapping region of the plasma membrane. The major excitatory afferent projection, the perforant path, arises from the layer II stellate cells of the entorhinal cortex and forms synapses on the distal two-thirds of the apical dendrites. The perforant path conveys processed information from all regions of the cerebral cortex. The proximal third of the apical dendrites is innervated by the associational–commissural pathway, which originates from the mossy cells of the dentate hilus. This pathway provides polysynaptic feedback excitation from mainly more caudal regions of the ipsilateral dentate gyrus and from the contralateral dentate gyrus. Monosynaptic innervation from other granule cells replaces the associational–commissural projection in many persons with temporal lobe epilepsy and in animal models of this disorder (where many hilar mossy cells are lost). A rudimentary projection of this type has been demonstrated in the caudal dentate gyrus of normal rodents. Because granule-cell basal dendrites serve as targets for mossy fibers, monosynaptic recurrent excitation may be a more prominent feature of dentate granule cells in humans than in rodents. Granule cells receive synaptic inhibition from six distinct local GABA neuron populations. One interneuron population, the basket cells, has a unique role in generating the gamma frequency oscillations thought to be important for pattern separation. Dentate granule cells also receive sparse innervation from several subcortical regions of the brain, including the supramammillary nucleus of the hypothalamus, the medial septal–diagonal band complex, the nucleus locus coeruleus, and the dorsal raphe nucleus. There is currently no evidence for a back projection from CA3 pyramidal cells, except for the disynaptic pyramidal-cell → mossy-cell → granule-cell pathway. Pyramidal cells of the caudal CA3 area do send a projection to the molecular layer of the dentate gyrus, raising the possibility of monosynaptic feedback excitation. It has not been established, however, whether these fibers innervate granule cells or interneurons.

Dentate granule cells project to CA3 pyramidal cells and interneurons of area CA3 and the dentate gyrus. Short- and long-term plasticity at mossy-fiber synapses allow this pathway to impart mainly excitatory or mainly inhibitory drive to area CA3, depending on the frequency and synchrony of granule-cell discharge.

In addition to excitatory and inhibitory synaptic inputs, granule-cell excitability is regulated by membrane ionic currents, most importantly by tonic GABA current, the slow delayed rectifier K^+ current (I_K), the BK- and SK-type Ca^{2+}-dependent K^+ currents, and T-type Ca^{2+} current. Tonic GABA current is produced by ambient extracellular GABA and released GABA that escapes the synaptic cleft. It is thought to depress the baseline excitability of granule cells. The generation of an intrinsic granule-cell burst depends on the activation of slow inward currents by the action potential. Because of the high granule-cell resting membrane potential, action potentials activate low-threshold T-type Ca^{2+} current, which contributes prominently to the spike afterdepolarization characteristic of these cells. A sufficiently large afterdepolarization can

trigger additional action potentials comprising a cellular burst. The effect of T-type Ca^{2+} current is counteracted mainly by activation of BK-type or high conductance Ca^{2+}-dependent K^+ current. SK-type or low conductance Ca^{2+}-dependent K^+ current and I_K also suppress bursting behavior in these cells. BK-type current mainly limits the size of the spike afterdepolarization, whereas SK-type current is mainly responsible for spike frequency adaptation. The high resting membrane potential is maintained by K^+ conductance. Membrane potential is a linear function of extracellular K^+ concentration between 3 and 6 mM and increases at a slower rate above 6 mM. Modification of any of these channels may predispose to breakdown of the granule cell's filtering function and the emergence of seizures.

Methods

Our studies use rats that had developed status epilepticus after administration of the muscarinic receptor agonist pilocarpine. We normally allow status epilepticus to self-terminate without administration of an anticonvulsant. Animals that experience prolonged (6–8 h) status epilepticus exhibit very extensive and uniform hilar lesions followed by robust recurrent mossy-fiber growth, as observed in many persons with temporal lobe epilepsy. The pilocarpine model also replicates the accumulation of ectopic granule cells observed in those persons. Rats that experience pilocarpine-induced status epilepticus invariably develop unremitting spontaneous seizures after a delay of 1–3 weeks. In these respects, the pilocarpine model produces more consistent outcomes than the alternatives, such as the kainic acid or electrical stimulation models. Finally, pilocarpine-induced status epilepticus kills fewer CA3 pyramidal cells than does kainic acid or electrical stimulation, better replicating the lesion most commonly observed in surgically resected hippocampi and facilitating the placement of a stimulating electrode in the mossy-fiber bundle of hippocampal slices.

Most of our studies are performed during the chronic epileptic period 10 weeks or longer after status epilepticus. By this time, the granule-cell network appears to be fully mature. Some analyses are also performed 4–6 days after status epilepticus, before the new circuitry can be detected. Hippocampal slices are prepared for electrophysiological studies. Extracellular recording, whole cell and cell-attached patch clamp recording, and perforated patch recording are used, as appropriate, to define granule-cell properties, the physiology and pharmacology of mossy-fiber → granule-cell synapses, and the role of the novel circuitry in granule-cell epileptiform activity. In parallel studies, histological, ultrastructural, immunohistochemical, and immunocytochemical approaches are used to describe and quantitate the cytopathology of the lesion and the anatomical response to the lesion.

Results

Research in our laboratory and in others has described profound changes in the innervation and neurotransmitter mechanisms of dentate granule cells in animal models of epilepsy. In lesional models, excitatory innervation from other granule cells replaces the degenerated associational–commissural projection. In addition, epileptogenesis is associated with enhanced perforant path synaptic transmission. Paired-pulse facilitation of perforant path synaptic transmission is depressed, implying a greater probability of glutamate release. The NMDA receptor-mediated component of the perforant path EPSC is prolonged, suggesting a change in receptor kinetics and leading to crosstalk among perforant path synapses. In the kindling model of epilepsy, NMDA channels in granule cells have a greater mean open time, longer burst duration, longer cluster duration, and enhanced sensitivity to high-energy phosphates, whereas their sensitivity to Mg^{2+} decreases. Enhancement of NMDA receptor function has been shown to promote the synchronized discharge of granule cells.

Of the inhibitory projections to dentate granule cells, innervation from HIPP (soma located in the hilus, projection to the perforant path terminal zone) cells is most likely to be lost. HIPP cells coexpress somatostatin and neuropeptide Y with GABA. They account for ~40% of all hilar GABA interneurons and probably contribute 40–50% of the feedback (mossy fiber-evoked) inhibition to granule cells. HIPP cell axons form synapses mainly on the necks of granule-cell dendritic spines. Because perforant path synapses are found on the head of those spines, the HIPP cell innervation is believed to regulate the efficacy of perforant path innervation. Loss of this regulation is expected to augment cell firing in response to perforant path activation. Although some findings suggest axon sprouting by surviving GABA interneurons, electrophysiological studies have not demonstrated any significant recovery of inhibition attributable to this mechanism. Granule cells do recover, at least to some extent, from the loss of feedback inhibition and enhanced excitability that result from the loss of GABA interneurons. Suggested mechanisms for recovery include the repetitive activation of surviving interneurons by reverberating mossy-fiber activity, a somewhat increased number of mossy-fiber synapses on those interneurons, increased interneuron excitability, facilitated GABA release, and increased tonic GABA current. The net effect of these changes may be to restore baseline granule-cell excitability to near normal. However, granule-cell function must, in fact, be grossly abnormal because of the rewiring and

altered neurotransmitter mechanisms needed to achieve this state.

In contrast to synapses and neurotransmitter receptors, intrinsic properties of dentate granule cells appear little changed during epileptogenesis. The resting membrane potential, input resistance, and action potential threshold are unchanged. Similarly, we found no change in T-type Ca^{2+} current. The retention of spike frequency adaptation and lack of facilitated cellular bursting suggest little change in Ca^{2+}-dependent K^+ current. There is, however, some loss of the intracellular Ca^{2+}-binding protein calbindin-D_{28k}. Loss of this protein increases the Ca^{2+}-dependent inactivation of voltage-dependent Ca^{2+} currents, thus limiting Ca^{2+} influx. In addition, granule cells increase the expression of HCN1 h channel subunit during epileptogenesis. Expression of h current might be expected to enhance granule-cell excitability. It is unknown, however, whether functional h channels are actually expressed. Granule cells normally express HCN1, but attempts to evoke I_h have been unsuccessful. Evidently, either h channel subunits do not normally form functional assemblies in these cells or the assembled channels fail to insert into the plasma membrane.

Perhaps the most interesting change in dentate granule cells of epileptic brain is the appearance of functionally different granule-cell populations due to enhanced neurogenesis. Seizures are especially powerful stimuli for granule-cell neurogenesis. Most of the neurons generated after seizures migrate into the granule-cell body layer and differentiate normally. However, a sizable number migrate aberrantly into the dentate hilus. Granule cells that are born after seizures and migrate normally appear to be less excitable than control granule cells. In contrast, we find that hilar ectopic granule cells (HEGCs) retain some properties of immature granule cells despite their morphological differentiation. These properties maintain the high excitability characteristic of the immature state. Most HEGCs possess intrinsic burst capability related to a large T-type Ca^{2+} current (Figure 1). Other properties that enhance excitability include a relatively low mean resting membrane potential, high input resistance, reduced spike frequency adaptation, sparse synaptic inhibition, and abundant mossy-fiber innervation, even on the cell body. At near physiological temperature and in the presence of a modestly elevated extracellular K^+ concentration, HEGCs were reported to burst in synchrony with CA3 pyramidal cells. Although the significance of this observation is unclear, it suggests that HEGC bursting may be driven by extrinsic as well as intrinsic mechanisms.

Future Goals

Properties of dentate granule cells control cell firing in such a way that cortical information is transmitted effectively to CA3 pyramidal cells only when required for memory formation. At other times, granule cells are relatively quiescent. Epileptogenesis disrupts some of these control mechanisms. Both excitatory and inhibitory innervation, neurotransmitter receptor expression, and calcium currents change in ways that could either promote or inhibit seizures. Further effects on granule-cell innervation and cellular properties may yet be discovered. Clearly, dentate granule cells in epileptic brain function differently from those in nonepileptic brain. How do these differences impact pattern separation and ultimately cognitive function? To what extent do the requirements for

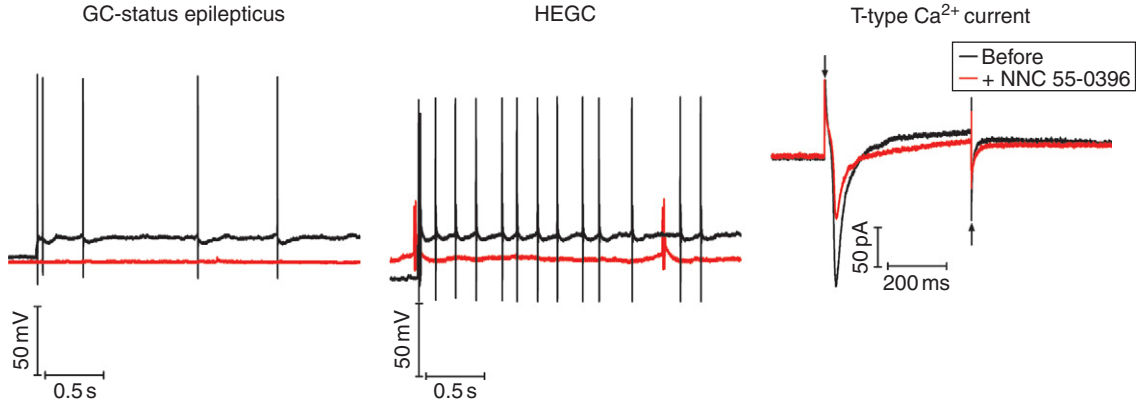


Figure 1 (Left) Normally located dentate granule cell is silent at resting V_m (red trace) and fires repetitive action potentials when depolarized to −48 mV (black trace) 10+ weeks after pilocarpine-induced status epilepticus. Marked spike frequency adaptation is evident. (Center) HEGC bursting at resting V_m (red trace) converts to repetitive action potential firing when the cell is depolarized to −48 mV (black trace). Spike frequency adaptation is reduced when compared with normally located granule cells. (Right) Large T-type Ca^{2+} current evoked in HEGC. Cells were voltage-clamped at −90 mV for 4 s before applying a 500 ms voltage step to −60 mV (between arrows). The superfusion medium contained blockers of high voltage-activated Ca^{2+} channels, voltage-dependent Na^+ channels, voltage-dependent K^+ channels, and ligand-gated ion channels. The selective blocker NNC 55-0396 (10 μM) reduced the peak current.

seizure propagation through the dentate gyrus change as a result of altered granule-cell function?

The addition of hyperexcitable HEGCs adds a new dimension to dentate gyrus physiology. HEGCs receive monosynaptic innervation from normally located granule cells and in most cases from the perforant path. Their mossy fibers project to normally located granule cells and probably to all the normal granule-cell targets. They therefore become thoroughly integrated into dentate gyrus circuitry. How does the introduction of spontaneously firing or bursting neurons into this circuitry alter pattern separation and seizure propagation? What factors beside T-type Ca^{2+} current contribute to their high rate of activity? Do they receive monosynaptic innervation from CA3 pyramidal cells and, if so, how does that alter circuit function? Why do they retain the hyperexcitable phenotype of immature granule cells when newly generated granule cells that migrate normally do not?

See also: **Granule Cells**: Granule Cell Properties in Seizure Discharge; Phenotypic Plasticity of the Granule Cells and its Relation to Epilepsy; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain; **Plasticity**: Axon Sprouting in Epilepsy; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization.

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H

HORMONES AND GENDER

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Female Sex Steroids and Neuronal Excitability

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Introduction

17β-Estradiol (E_2) and progesterone (P_4) are members of a class of hormones known as steroid hormones, which are derived from cholesterol, and secreted primarily by the gonads. As illustrated in Figure 1, P_4 – the primary endogenous progestin – is metabolized by the 5α-reductase enzyme to 5α-dihydroprogesterone (DHP), which in turn is reduced by the 3α-hydroxysteroid dehydrogenase enzyme to 3α-hydroxy-5α-pregnan-20-one (3α,5α-THP). Notably, P_4 and its metabolites have disparate mechanisms of action for their functional effects.

E_2, a metabolite of testosterone, also can have different effects and mechanisms than its precursor. Both clinical reports and basic research suggest that estrogens and progestins can alter neuronal excitability. Yet, the nature of their effects depend upon a number of factors – not only the precise molecule/hormone being investigated, but also the absolute concentration of and proximate changes in that hormone, and its potential to activate a diverse number of targets.

The following sections will present and discuss data in support of a role of ovarian hormones in modulating seizures. First, we will discuss data demonstrating an association between endogenous variations in both E_2 and P_4 and how these variations correlate with changes in seizure susceptibility. Second, effects of E_2 alone on ictal activity will be presented. Third, the mediation of seizure activity by progestins will be discussed. And fourth, the putative mechanisms by which estrogen and progestins have their anticonvulsant effects will be presented. Throughout our discussion, we include evidence that the hippocampus is an important brain area involved in hormonal modulation of seizures.

Background

Endogenous Variations in Ovarian Hormones

Clinical reports of cyclic variations in seizures among women with epilepsy support a role of ovarian hormones in the mediation of seizures. Catamenial epilepsy refers to

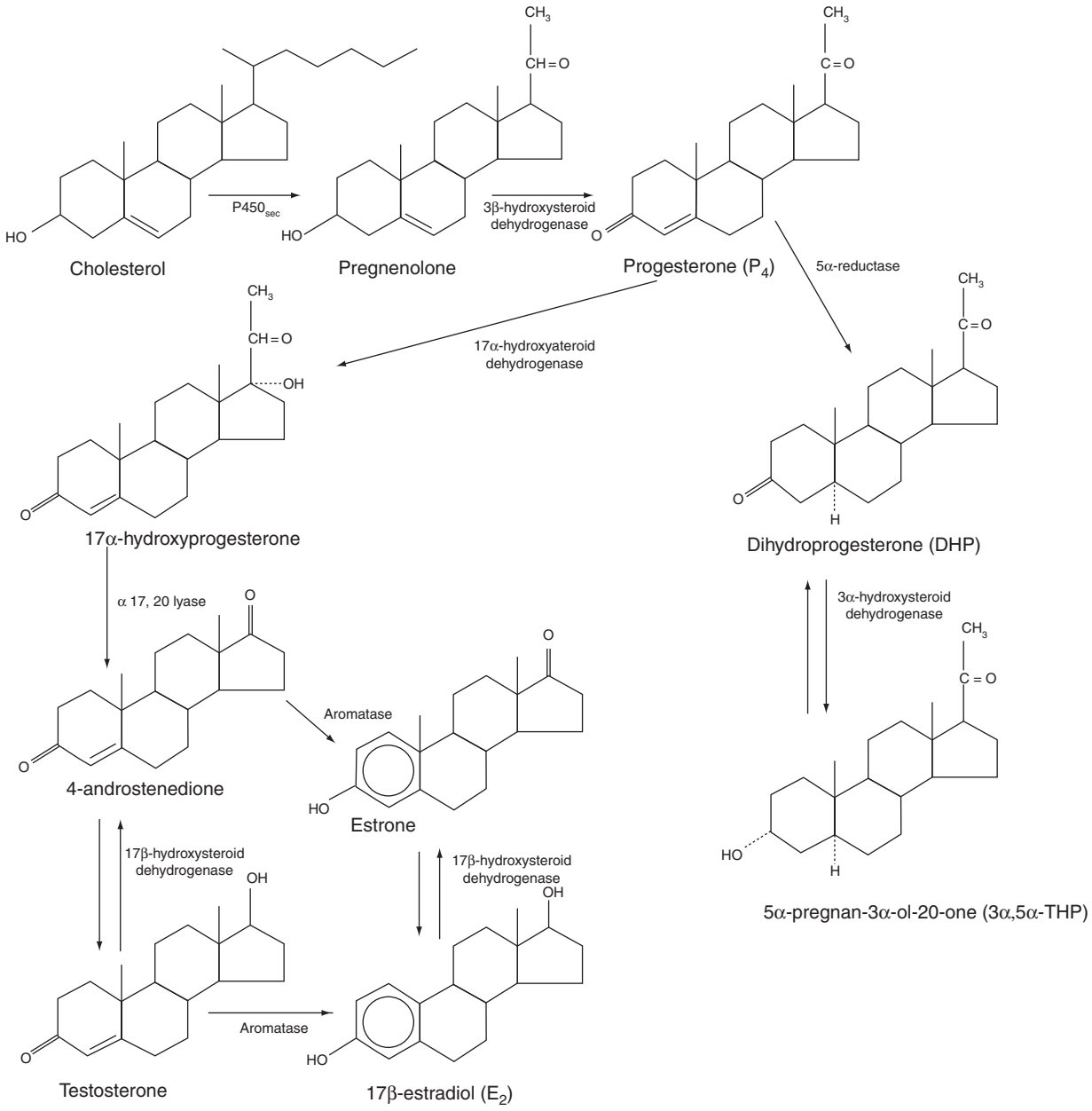


Figure 1 Diagram of formation of progestins, estrogen, and testosterone from cholesterol.

alterations in seizures associated with the menstrual cycle. Women with catamenial epilepsy typically have fewer seizures during the mid-luteal phase when P_4 levels are highest (**Figure 2**). Various patterns of catamenial epilepsy have been observed, including cases in which seizure frequency is increased during the perimenstrual phase (due to the withdrawal from P_4), during the preovulatory phase (due to increased E_2), or during an inadequate luteal phase cycle (due to lower than normal P_4 secretion during the second half of the cycle). Thus, there is heterogeneity in the manner in which catamenial epilepsy is expressed.

Prior research using animal models also supports the role of ovarian hormones in the modulation of seizures. Across the estrous cycle in rats, seizures are typically reduced during the afternoon of proestrous, when E_2 and P_4, are at their peak, rather than in diestrous when these same hormones are at their lowest (**Figure 3**, top).

In addition, findings from human and animal studies suggest there are typically opposing effects of E_2 (proconvulsant) and P_4 (anticonvulsant) on seizures. Hence, antiseizure effects during proestrous may be due to P_4's attenuation of E_2-mediated effects, via actions at various neurotransmitter systems, such as increases in

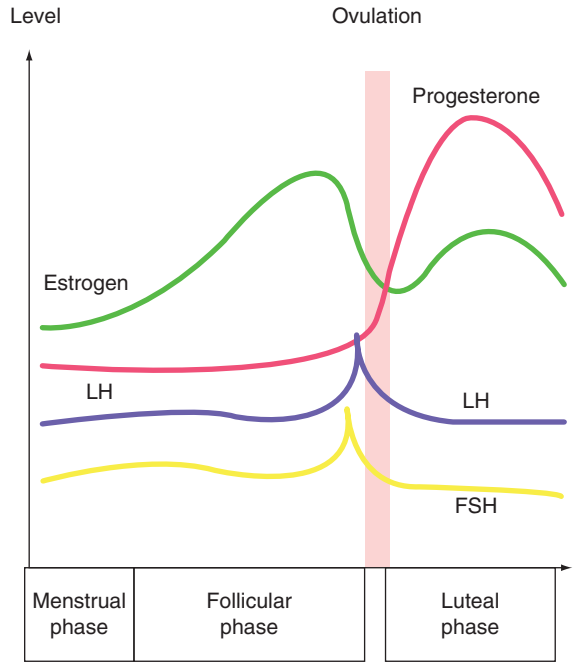


Figure 2 Schematic of estrogen, progesterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) across the menstrual cycle.

glutamatergic activity, that are implicated in epileptiform processes. Although these data clearly demonstrate an association between ovarian hormones and seizures, the relative influence of progestins versus estrogens requires further discussion.

Estrogen's Effects on Seizures

Emphasis has been placed on seizures increasing when there are natural, cyclic elevations in the E_2-to-P_4 ratio. Among women with epilepsy, menopause is often associated with an increase in seizures, which may be due in part to higher E_2 concentrations that are unopposed by P_4 (due to a decline in ovarian P_4 secretion). E_2-based oral contraceptives have also been shown to increase ictal activity among young women with epilepsy. Further, intravenous E_2 administration produced increases in seizures or epileptiform activity (or both) in women. These clinical findings suggest that E_2 has proconvulsant effects in women.

Findings from animal models also suggest that E_2 has proconvulsant effects. Systemic administration of E_2 lowers seizure thresholds in electroshock, kindling, pentylenetetrazol, kainic acid, and ethyl chloride seizure models. Further, central or intravenous E_2 administration to rabbits increases spontaneous electrical activity. However, in low, physiological concentrations, E_2 can have anticonvulsant effects (Figure 3, top). These experimental results, together with the above clinical observations, suggest that E_2 can increase neuronal excitability but that this effect is typically due to more robust changes in, or higher concentrations of, E_2.

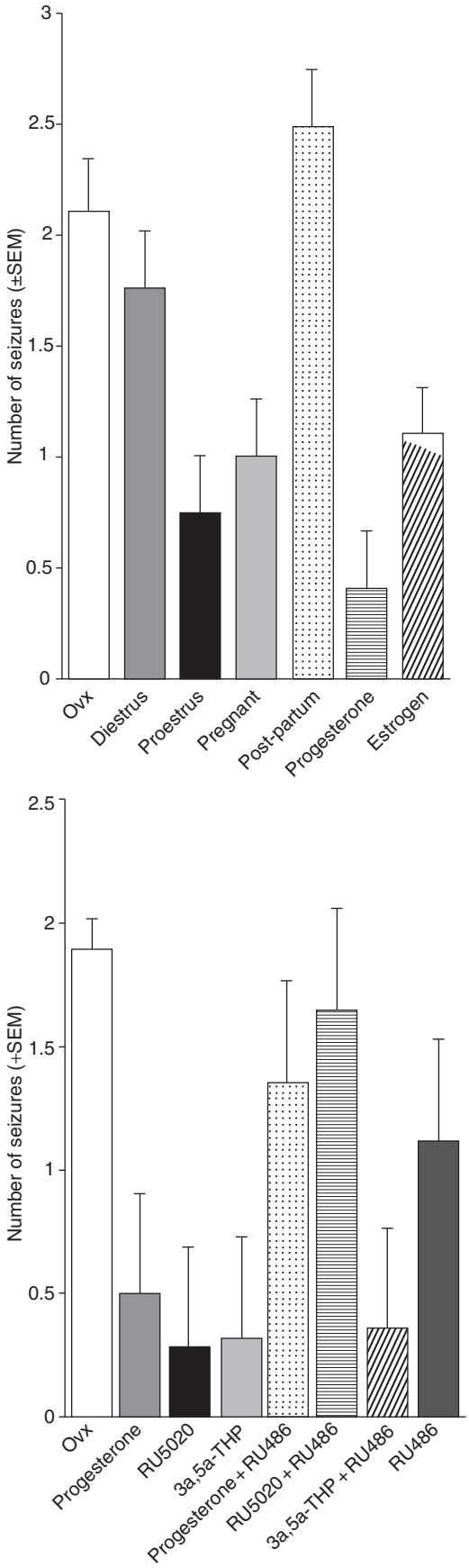
Progestins' Effects on Seizures

In contrast to E_2, progestins typically have anticonvulsant effects. Women with epilepsy often experience a decrease in seizure incidence during pregnancy, when progestin levels are ~10 times higher than at any other point in the lifespan. Progestin concentrations fall precipitously immediately following parturition, and this change is often associated with an increase in seizures. P_4 administration can help reduce seizures of women with catamenial epilepsy. Intravenous administration of P_4 increases serum P_4 to levels similar to those seen during the luteal phase, and decreases interictal spikes. Oral administration of natural P_4 improves control of intractable complex partial seizures. Two explanations for P_4's beneficial therapeutic effects have been posited. First, steroid hormones and antiepileptic drugs are both metabolized by hepatic enzymes. P_4 therapy offers an increase in the substrate for these liver enzymes, thus reducing their efficiency in degrading antiepileptic drugs and thereby enhancing the therapeutic efficacy of those drugs. Second, direct depressant actions of P_4 metabolites may underlie P_4's anticonvulsant effects. In support of this latter possibility, oral synthetic progestins, which do not readily metabolize to depressant progestins, have not demonstrated antiseizure effects in clinical investigations. Thus, metabolism of P_4 appears to be important for some of its anticonvulsant effects.

Progestins also have antiseizure effects in animal models. Pregnant rats, with high endogenous levels of progestins, have fewer seizures than do rats that are 1–2 days postpartum, when progestin levels are lower than during pregnancy (Figure 3, top). Removal of the primary endogenous source of P_4, the ovaries, increases seizures; reinstatement of P_4 produces the opposite effects, reducing kainic acid-, pentylenetetrazol-(PTZ), and perforant pathway-induced seizures in ovariectomized (ovx) rats. Direct administration of P_4 to the brain also has been shown to have antiseizure effects. In cats, application of P_4 to the cortex inhibited electrical discharges in a penicillin focus. In rats, application of P_4 to the hippocampus reduces PTZ-induced seizures.

Methods

There are a number of animal models of seizure disorders and our lab has used several of these over the years. We have used perforant path stimulation as a model of partial seizures. More often, we have used the chemoconvulsants, such as kainic acid or PTZ, as models of generalized



seizure disorders. We have found the effects of steroid hormones on ictal activity to generalize well across these models. A number of years ago, the reliability and availability of kainic acid became problematic. Hence, we switched to PTZ. Because of inconsistencies reported by other laboratories when using PTZ, we take a number of steps to offset such effects. PTZ is made up immediately prior to testing and animals are weighed immediately prior to testing to ensure correct dosing of PTZ. Animals are administered PTZ via intraperitoneal injections and some myoclonic activity is typically observed within 1 min. We monitor animals for 10 min following PTZ administration, recording the latency to, and number of, myoclonic twitches, forelimb clonus, tonic–clonic seizures, and hindlimb extensions. The seizure progression produced with our PTZ regimen (70 mg kg^{-1} for rats, 85 mg kg^{-1} for mice) is consistent, and reliably observed and quantified, using a modification of the scale devised by Racine (mouth and facial movements, head nodding, forelimb clonus, rearing, and rearing and falling). We observe consistent effects of estrogens and progestins on each stage of seizure progression (myoclonus, tonic–clonic seizures, and survival). The data presented in the subsequent section are based on measurements of latency and incidence of tonic–clonic seizures.

Recent Data

Mechanisms of Ovarian Hormones for Modulation of Seizures

Given the consensus that E_2 and P_4 influence ictal activity in women, and in animal models, it is important to elucidate the mechanisms by which ovarian hormones exert these effects. E_2 and progestins can have multiple mechanisms of action for their functional effects. One framework in which to think about actions of E_2 and progestins is classical versus nonclassical mechanisms. Steroid hormones have actions at intracellular steroid receptors specific for their class of hormones. E_2 can act at estrogen receptors (ERs) and P_4 at progestin receptors (PRs). Actions at intracellular receptors regulate the transcription of target genes, and as such are relatively slow,

Figure 3 Top panel represents average number (±SEM) of seizures of ovariectomized (white bar), diestrous (gray bar), proestrous (black bar), pregnant (light gray bar), postpartum (dotted bar), progesterone-administered (horizontal stripe bar), and estrogen-administered (diagonal stripe bar) rats. Bottom panel represents average (±SEM) of seizures of ovariectomized rats administered vehicle (white bar), progesterone (gray bar), RU5020 (black bar), 3α,5α-THP (light gray bar), progesterone + RU486 (dotted bar), RU5020 + RU486 (horizontal stripe bar), 3α,5α-THP + RU5020 (diagonal stripe bar), and RU486 (dark gray bar).

typically requiring at least 15 min, but often much longer. Actions at intracellular steroid receptors were long thought to be the only mechanism by which steroid hormones could produce changes in behavior and physiology. However, more recent research has demonstrated that steroid hormones can have actions via other substrates. Both E_2 and progestins can have rapid actions that are independent of activation of intracellular receptors. In support of that view, E_2 activates a number of kinases, including protein kinase A and mitogen-activated protein kinase, and may also act via a membrane ER. Progestins also have effects via nonclassical mechanisms. Two of the most investigated substrates for progestins include $GABA_A$ and NMDA receptors. Data from our laboratory, presented later, suggests that progestins can mediate seizure processes via both classical and nonclassical mechanisms.

Classical Actions of E_2 for Antiseizure Effects

As discussed earlier, E_2 is generally considered to have proconvulsant effects. However, recent data suggest that E_2 can be anticonvulsant and that such effects may be due, at least in part, to actions at intracellular ERs. Notably, there are two ER isoforms that have been characterized. Actions at ERα, the originally identified ER, are typically associated with modulation of reproductive physiology and behavior. E_2's actions via the recently identified ERβ are associated more with nonreproductive behaviors and processes. In our laboratory, we have examined effects on seizures of low dosages (0.1 mg kg^{-1}, s.c.) of selective ER modulators (SERMs) to begin to elucidate the mechanisms by which E_2 may have antiseizure effects. Administration of the ERβ-selective SERMs, coumestrol or DPN, decreases seizures to levels similar to those seen with low-dose E_2 administration, while ERα-selective SERMs, 17α-E_2 and PPT, are no more effective than vehicle at reducing seizures (Table 1). However, as discussed earlier, E_2 has multiple modes of action. Hence, it is likely that E_2 has effects via other mechanisms, including actions at membrane receptors and/or activation of signal transduction pathways. Indeed, E_2's ability to alter brain-derived neurotrophic factor in the hippocampus has also been shown to play a role in E_2's modulation of seizure activity. These data suggest that one mechanism through which E_2 may have its antiseizure effects is via actions at ERβ.

Table 1 Number of full seizures of rats administered vehicle, E_2, ERβ-selective SERMs (coumestrol, DPN), or ERα-selective SERMs (17α-E_2, PPT)

	Number of seizures
Vehicle	6.0 ± 0.3
E_2	1.7 ± 0.5
Coumestrol	2.8 ± 0.8
DPN	1.8 ± 0.5
17α-E_2	5.1 ± 0.4
PPT	5.5 ± 0.4

Classical Actions of P_4 for Antiseizure Effects

To reveal the extent to which progestins have classical actions at PRs to mediate ictal activity, we examined effects of progestins with varying degrees of affinity for PRs on seizure activity. Ovariectomized rats were administered P_4, RU5020, 3α,5α-THP, or vehicle. P_4 and RU5020 have actions at intracellular PRs, while 3α,5α-THP does not. However, P_4 can be metabolized to 3α,5α-THP, while RU5020 cannot. Hence, comparing effects of these progestins allowed us to begin to parse out the role of each of these ligands and substrates for antiseizure effects. P_4, 3α,5α-THP, and RU5020 had similar effects in reducing tonic–clonic seizures (compared to vehicle) (Figure 3, bottom). We have also examined effects of blocking PRs (in conjunction with progestin administration) on seizures. Rats were administered P_4, 3α,5α-THP, RU5020, or vehicle concomitant with administration of the PR antagonist, RU38486. Coadministration of RU38486 with P_4 and RU5020 had modest effects to increase seizures relative to vehicle-administered controls; however, there was no effect of RU38486 administration together with 3α,5α-THP on ictal activity compared with 3α,5α-THP alone (Figure 3, bottom). We have also examined P_4's antiseizure effects in PR knockout mice (no functional PRs). P_4 administration to PR knockouts has moderate effects to reduce seizures compared with P_4 administration to wildtype mice with functional PRs (Figure 4, top).

These data suggest that P_4 can have antiseizure effects in part via its actions at PRs.

Nonclassical Actions of Progestins for Antiseizure Effects

A focus of our research has been to identify the functional role of steroids' actions via classical steroid receptors versus other substrates. Reproductive behavior of rodents requires both traditional actions at steroid receptors and nonclassical actions of steroids. The finding that steroid hormones have actions at substrates other than intracellular steroid receptors broadened the putative role that steroid hormones might play in physiology and behavior. Steroid hormones' effects and mechanisms were no longer relegated only to brain areas containing intracellular steroid receptors or hypothalamically mediated reproductive behaviors. Indeed, steroid hormones have salient neurotrophic effects, especially in the hippocampus, and clinical studies have shown that circulating levels of 3α,5α-THP contribute more to antiseizure effects than do P_4 concentrations.

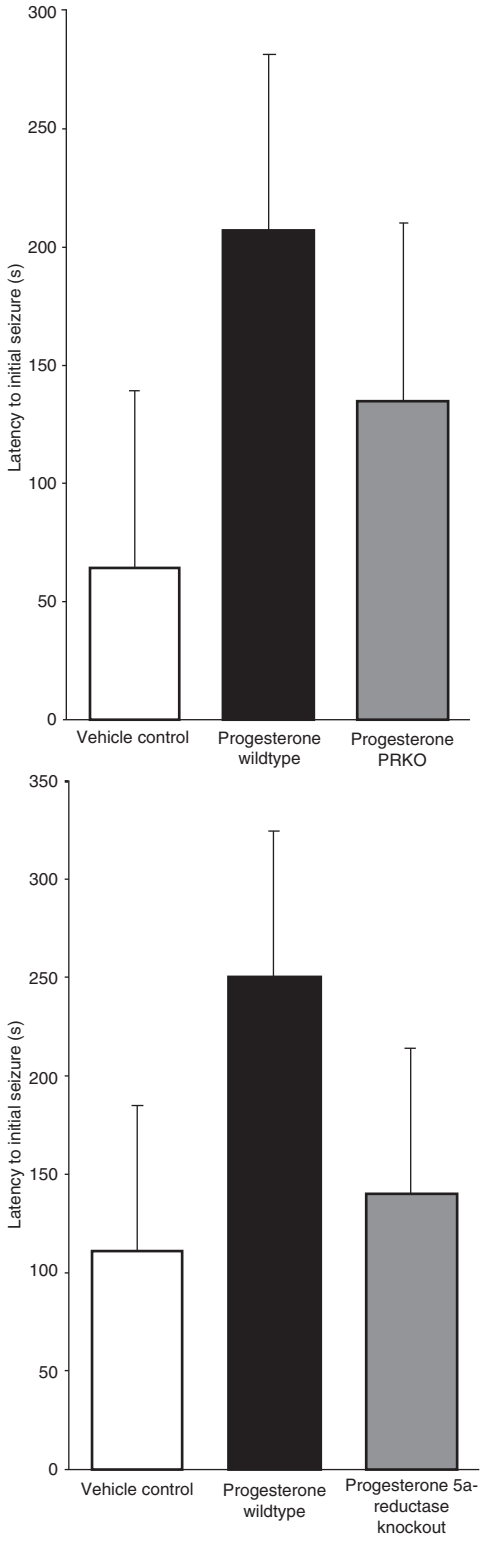


Figure 4 Top panel represents average number (±SEM) of seizures of vehicle control (white bar), progesterone-administered wildtype (black bar), and progesterone-administered PRKO (gray bar) mice. Bottom panel represents average number (±SEM) of seizures of vehicle control (white bar), progesterone-administered wildtype (black bar), and progesterone-administered 5α-reductase (gray bar) mice.

Our investigations of putative nonclassical mechanisms of progestins' antiseizure effects have focused primarily on the hippocampus. P_4 administration to 5α-reductase knockout mice that are genetically unable to metabolize P_4 to 3α,5α-THP is not more effective than to vehicle-administered rats at decreasing seizure activity (**Figure 4**, bottom). In rats, administration of systemic or intrahippocampal finasteride, which attenuates formation of 3α,5α-THP from P_4, blocks P_4's antiseizure effects (**Figure 5**).

Thus, actions of 3α,5α-THP in the hippocampus may underlie some of its antiseizure effects.

The notion that 3α,5α-THP may be a modulator of P_4's antiseizure effects is important given that P_4 and

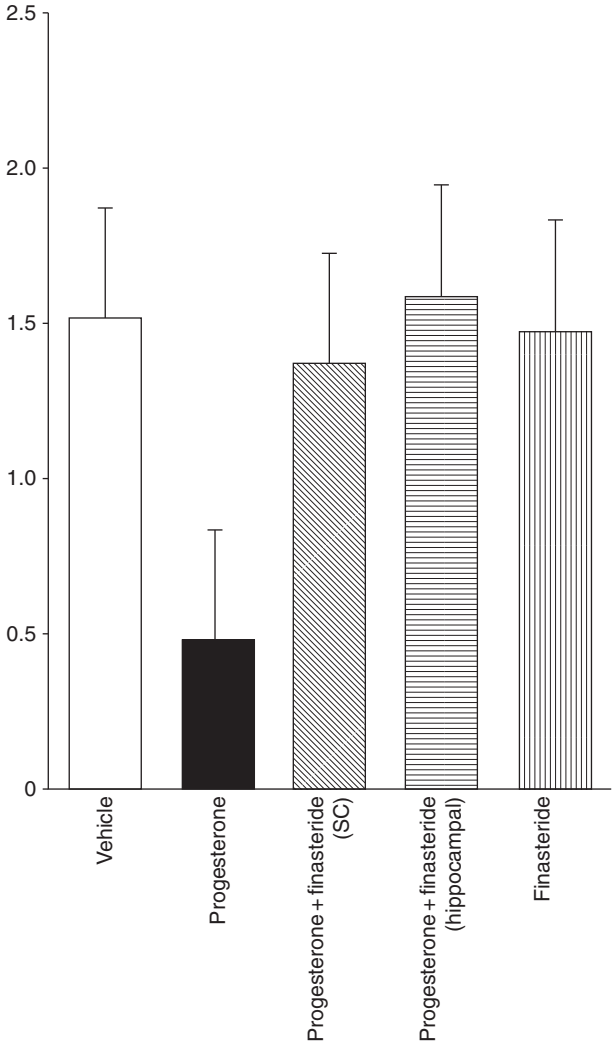


Figure 5 Average number (±SEM) of seizures of ovariectomized rats administered vehicle (white bar), progesterone (black bar), progesterone + systemic finasteride (diagonal striped bar), progesterone + intrahippocampal finasteride (horizontal striped bar), and vehicle + finasteride (vertical striped bar).

3α,5α-THP have different mechanisms of action. Notably, 3α,5α-THP is a very potent modulator of $GABA_A$ receptors and can alter the function of NMDA receptors but is devoid of activity at PRs. In contrast, P_4 binds well to PRs but is only weakly active at $GABA_A$ receptors and NMDA receptors. Hence, our investigations of progestins' nonclassical mechanisms for antiseizure effects have focused on $GABA_A$ and NMDA receptors. Administration of bicuculline, a $GABA_A$ receptor antagonist, to the hippocampus of hormone-primed rats attenuates P_4's antiseizure effects (**Figure 6**, top).

Rats administered MK801, a nonselective NMDA receptor antagonist, to the hippocampus had significantly more seizures than did vehicle-administered rats (**Figure 6**, bottom). These data suggest that progestins' antiseizure effects may involve both $GABA_A$ receptors and NMDA receptors. In conjunction with prior findings, it seems that antiseizure effects of progestins involve multiple classical and nonclassical substrates in the hippocampus.

Stress, 3α,5α-THP, and Seizures

Exposure to stressors can induce changes in hypothalamic–pituitary–adrenal (HPA) axis function, the primary mediator of neuroendocrine stress responses. Notably, 3α,5α-THP acts to dampen stress responses via its agonist-like actions at $GABA_A$ receptors. Given that stress may alter seizure susceptibility, in part due to altering secretion of 3α,5α-THP, we have examined effects of altered HPA function on seizure susceptibility. Adrenalectomized (ADX) or sham-ADX rats were administered P_4 or 3α,5α-THP and observed for seizure activity following PTZ administration. P_4 and 3α,5α-THP significantly reduced the number of seizures of sham, but not ADX rats, compared with vehicle administration (**Table 2**). These data suggest that some of progestins effects on seizures may involve the HPA axis as a target. Given that 3α,5α-THP is secreted in response to stress, and seizures can increase 3α,5α-THP, it is possible that 3α,5α-THP levels before and/or in response to seizures may influence their incidence or sequelae. Notably, P_4 therapy and ganaxolone, the B isomer of 3α,5α-THP, are currently under investigation in clinical trials for treatment of some seizure disorders.

Future Goals

The data presented and discussed here support a role of ovarian hormones in the modulation of seizure processes. Estrogens typically have proconvulsant effects, while progestins are generally anticonvulsant. However, it is important to note that E_2 and progestins do not act independent of one another. E_2 can increase the conversion of P_4 to 3α,5α-THP. When administered in low, physiological concentrations that are sufficient to enhance P_4's conversion, anticonvulsant effects of E_2 are observed. Hence, it is

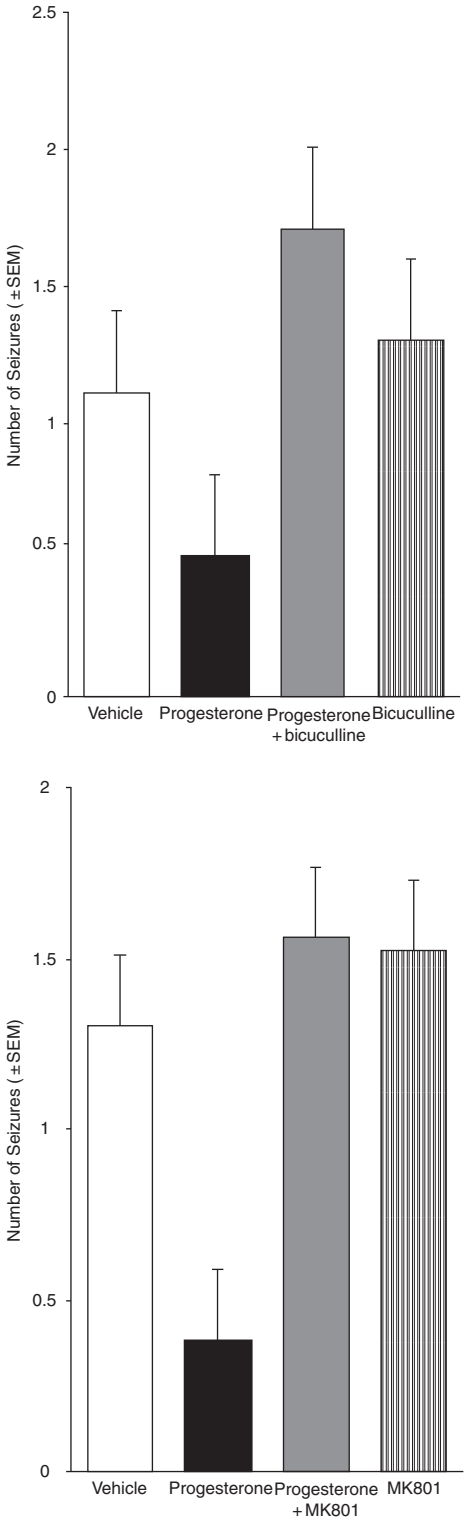


Figure 6 Top panel represents average number (±SEM) of seizures of ovariectomized rats administered vehicle (white bar), progesterone (black bar), progesterone + intrahippocampal bicuculline (gray bar), and vehicle + bicuculline (vertical striped bar). Bottom panel represents average number (±SEM) of seizures of ovariectomized rats administered vehicle (white bar), progesterone (black bar), progesterone + intrahippocampal MK801 (gray bar), and vehicle + MK801 (vertical striped bar).

Table 2 Number of tonic–clonic seizures of sham-ADX or ADX rats administered vehicle, P_4, or 3α,5α-THP

	Number of seizures	
	Sham-ADX	*ADX*
Vehicle	1.4 ± 0.4	2.0 ± 0.1
P_4	0.4 ± 0.1	1.6 ± 0.4
3α,5α-THP	0.3 ± 0.2	1.5 ± 0.4

important to fully understand how estrogens and progestins influence one another and their subsequent effects on seizure processes. It is also important to begin to elucidate the sources of 3α,5α-THP that modulate seizures, given that stress can induce 3α,5α-THP secretion. Further, androgens also vary in females and can alter ictal activity. In addition, antiepileptic drugs can alter steroid hormone metabolism and thereby the efficacy of oral contraceptives and hormone therapies. As such, understanding the role of steroid hormones and seizures, and the relationships between steroid hormones and therapeutic drugs (and their influence on seizures) are of great importance.

See also: **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Male Sex Steroids and Neuronal Excitability; Sex and Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility.

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Hormonal Modulation of Neuronal Excitability

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Introduction

Sex hormones influence neuronal excitability, depending on the hormone type or its metabolite. Hormone-induced changes in neuronal activity are believed to be an underlying mechanism for several disorders, such as migraine, depression, and epilepsy (e.g., catamenial epilepsy). The hormone-related disorders can occur in both sexes but are mostly studied and are best characterized in women owing to marked changes in women hormonal state throughout their lifespan. Hormonal fluctuations during the menstrual cycle mark the catamenial occurrence of seizures in some women. Another important factor in neuronal excitability among women is the loss of peripherally generated sex hormones at menopause. This profound hormonal decline is also associated with alterations in seizure patterns. Since the brain is a sexually dimorphic structure, the effects of sex hormones on neuronal

Table 2 Number of tonic–clonic seizures of sham-ADX or ADX rats administered vehicle, P_4, or 3α,5α-THP

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important to fully understand how estrogens and progestins influence one another and their subsequent effects on seizure processes. It is also important to begin to elucidate the sources of 3α,5α-THP that modulate seizures, given that stress can induce 3α,5α-THP secretion. Further, androgens also vary in females and can alter ictal activity. In addition, antiepileptic drugs can alter steroid hormone metabolism and thereby the efficacy of oral contraceptives and hormone therapies. As such, understanding the role of steroid hormones and seizures, and the relationships between steroid hormones and therapeutic drugs (and their influence on seizures) are of great importance.

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Hormonal Modulation of Neuronal Excitability

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Introduction

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excitability are sex-specific. Sex hormones exert their action on neurons either by changes in gene expression or by regulating membrane excitability through their own membrane receptors, as well as via direct action on neurotransmitter receptors and ion channels.

Background

Hormones influence neuronal activity either through excitatory or inhibitory mechanisms. Classical effects of sex hormones are mediated by activation of intracellular receptors, which are specific for individual hormones and can be found either in the cytoplasm or within the nucleus. Binding of a hormone to intracellular receptors mediates regulation of gene expression at the nuclear level. The subsequent effects have delayed onset but long duration, which extends beyond the period of the hormone presence in the tissue. In contrast, steroid hormones also produce rapid changes in neuronal excitability by activation of membrane-bound receptors as well as directly regulating neurotransmitter receptors or ion channels. Direct effects of steroid hormones on membranes last only when the hormone is bound to the receptor.

The importance of hormonal action in the brain is documented in reports showing the local *de novo* production of steroid hormones by neurons and glia in the central nervous system (CNS). These 'neurosteroids' are known for their nongenomic acute effects, especially at NMDA receptors and at synaptic and extrasynaptic $GABA_A$ receptors, and are responsible for 'fine tuning' of neuronal excitability. Also, neurosteroids play an important role in neuronal survival in developing as well as in aging brain.

The role of steroid hormones in neuronal excitability is very complex and depends on many factors, including the type of hormone, sex, age, regional distribution of hormone receptors, treatment duration, time interval for initiation of hormonal treatment following gonadal removal, or route of administration.

In our laboratory, we concentrate on the effects of the main female sex hormones, β-estradiol and progesterone, on neuronal activity. Although it is believed that progesterone decreases while estrogen increases neuronal excitability, this issue remains controversial. Bath application of β-estradiol as well as progesterone increases long-term potentiation (LTP) and enhances spreading depression induced by microinjection of KCl in neocortical slices. In the hippocampus, bath application of β-estradiol has no effect on pyramidal cell excitability in slices from female rats, but increases excitability of pyramidal neurons in slices from male rats. Interestingly, excitability of pyramidal neurons also shows sex-dependent sensitivity to bath administration of testosterone. While brain slices from males do not show any change in hippocampal excitability following testosterone, slices from female rats have estrous cycle stage-dependent responses; in slices obtained from females at diestrus, testosterone increases the excitability, but in slices from females at proestrus, testosterone decreases excitability.

Methods

Adult female Sprague Dawley rats from Taconic farms have been used. Rats were ovariectomized under Methofane anesthesia one week prior to hormone supplementation.

Region-specific Effects of β-Estradiol and Progesterone on Low $Mg^{2\pm}$-induced Epileptiform Activity

Ovariectomized female rats were injected with two subcutaneous injections of 17β-estradiol benzoate (EB) 24 hours apart. The dose of EB was 2 μg/0.1 ml/rat, which restores β-estradiol levels within physiological range. Combined entorhinal cortex-hippocampal slices were obtained 24 hours following the last EB (or oil control) injection. Another group of ovariectomized females received 500 μg/0.1 ml/rat of progesterone (P) 2 hours prior to slice preparation. Finally a group of ovariectomized females received a combination of both treatments (EB + P) and slices were prepared 2 hours after P administration. EB and progesterone were dissolved in peanut oil, which was injected (0.1 ml) in appropriate ovariectomized controls (instead of hormone treatment). To prepare slices, rats were sacrificed under CO_2 anesthesia, brain quickly removed from the skull and combined entorhinal cortex-hippocampal slices (400 μm thick) were cut on a vibratome in ice-cold artificial cerebrospinal fluid (ACSF). ACSF composition was (in mM): NaCl – 126; KCl – 5; NaH_2PO_4 – 1.25; $MgCl_2$ – 2; Ca Cl_2 – 2; $NaHCO_3$ – 26; glucose – 10; pH = 7.3; bubbled with 95% O_2/5% CO_2. Slices were then transferred to an interface chamber perfused with pre-warmed (34 °C) ACSF, and pre-incubated for 1 hour. Extracellular recording micropipettes (resistance 1–5 MΩ) were filled with 2 M NaCl. Recording electrodes were positioned either in layers IV-V of the medial entorhinal cortex or in the CA1 pyramidal layer of hippocampus. Following baseline recording, the slices were introduced to Mg^{2+}-free ACSF. Onset of epileptiform discharges was determined.

Effects of EB on Paired Pulse-induced Population Excitatory Postsynaptic Potentials in the Dentate Gyrus Granule Cells

Ovariectomized female rats were injected with either one or four injections of EB 24 hours apart. Controls received

oil instead of EB. The dose of EB was 2 μg/0.1 ml/rat. Combined entorhinal cortex-hippocampal slices were obtained 24 hours following the last EB/oil injection, as described previously. A stainless steel bipolar stimulating electrode was positioned in the mixed perforant path (over the outer and middle third of the molecular layer). Paired square pulses (50–400 μA, 150 μs duration) were delivered using a stimulator coupled to a stimulus isolation unit, at interstimulus intervals of 10–1000 ms. A glass recording micropipette (1–5 MΩ) was filled with 2 M NaCl and placed in the middle third of the external blade of the dentate gyrus granule cell layer (stratum granulosum) to record extracellular evoked responses (population spikes). Population spike amplitude was used as a measure of granule cell responses to perforant path paired-pulse stimulation (Figure 1). For each interstimulus interval, 6–10 responses were averaged to obtain stability of the responses. The interval between each paired stimulation was 30 s. Digitized data were computer analyzed using Axoscope software.

ANOVA and Student *t*-test were used for statistical analyses. Level of significance was always preset to $P < 0.05$.

Results

Region-specific Effects of β-Estradiol and Progesterone on Low $Mg^{2\pm}$-induced Epileptiform Activity (Table 1)

Removal of Mg^{2+} from the bathing medium induces epileptiform-like discharges (Figure 2) by activation of NMDA receptors, enhancement of transmitter release, and reduction of surface charge screening, all of which leads to an increase in neuronal excitability. In Most slices from female rats (as in slices from males), in the entorhinal cortex, low Mg^{2+} first induces seizure-like events characterized by long duration discharges with a negative DC shift. Additionally, in about 20% of slices from females, the discharges start as short bursting activity. Both types of initial discharges spontaneously develop into status epilepticus-like activity, characterized by 1–10 sec recurrent discharges and persisting as long as the slices are perfused with the low Mg^{2+} ACSF. In the entorhinal cortex, the onset of seizure-like events was delayed by progesterone treatment. However, EB or the combination of EB with progesterone had no effect on the seizure-like event onset. The progression of seizure-like discharges into the status-like pattern was not influenced by any hormonal treatment paradigm. In the CA1 hippocampal region, the epileptiform-like activity induced by low Mg^{2+} in slices from female rats starts as recurrent brief discharges (as in slices from male rats). In contrast to the entorhinal cortex, however, these short-duration discharges in the CA1 region simply increase frequency and amplitude over time, but maintain their initial appearance as 'interictal' activity. Progesterone accelerated the onset of seizure-like events in the CA1 region; EB or EB with

Table 1 Region-specific effects of female sex hormones on the onset of low Mg^{2+}-induced epileptiform-like activity

	EB	*P*	*EB + P*
EC	No effect	Accelerated	No effect
CA1	No effect	Delayed	No effect

EC: entorhinal cortex; CA1: hippocampal region; EB: β-estradiol benzoate.

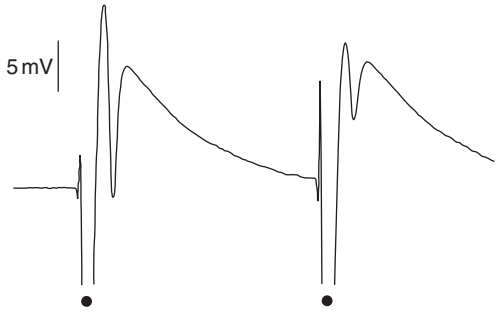


Figure 1 In vitro paired pulse responses recorded in the dentate gyrus granule cell layer following perforant path stimulation. Examples of averaged responses (population spikes) recorded after the delivery of 10 paired stimuli at interstimulus interval (ISI) 20 ms. There was a 30 s interpair interval to ensure tissue recovery. Calibration 5 mV. Dots mark truncated stimulus artifacts.

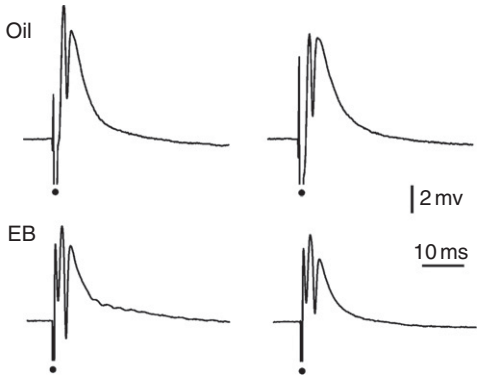


Figure 2 In vitro paired pulse responses recorded in the dentate gyrus granule cell layer following perforant path stimulation. Examples of averaged responses (population spikes) recorded from an oil or estrogen (EB) injected rat after the delivery of 10 paired stimuli at interstimulus interval (ISI) 300 ms. Note the suppression of the second response compared to the first response in the estrogen-injected rat but this difference is not present in oil-injected ovariectomized rat. There was a 30 s interpair interval to ensure tissue recovery. Calibration 2 mV and 10 ms baseline. Dots mark truncated stimulus artifacts.

Table 2 Mixed perforant path paired pulse stimulation: EB effects on granule cell excitability

	1xEB	*4xEB*
Early inhibition	No effect	No effect
Intermediate facilitation	No effect	No effect
Late inhibition	No effect	Enhanced

EB: β-estradiol benzoate.

progesterone treatment, however, had no effect on the onset of the epileptiform-like activity.

These data show that low dose EB does not influence neuronal excitability in the entorhinal cortex or in the CA1 hippocampal region. In contrast, progesterone has biphasic and region-specific effects on neuronal excitability in this model. Since progesterone was administered just 2 hours prior to slice preparation, its effects on neuronal activity are likely to be mediated by membrane nongenomic effects of progesterone metabolites. While some of progesterone metabolites enhance $GABA_A$ receptor mediated transmission, other metabolites may decrease the GABA-mediated Cl^- currents. Interestingly, in the CA1 region, bath application of some progesterone metabolites induces depolarizing $GABA_A$ receptor-mediated postsynaptic potentials. This effect may explain accelerated onset of the low Mg^{2+}-induced discharges in the CA1 region of progesterone-treated females.

Effects of EB on Paired Pulse-induced Population Excitatory Postsynaptic Potentials in the Dentate Gyrus Granule Cells (Table 2)

The paired pulse paradigm in vitro is a convenient way to test the physiological balance of excitatory/inhibitory functions. The paired pulse curve is calculated as a ration between the second response (R = population spike amplitude) and the first response (R2/R1) at increasing interstimulus intervals. The curve consists of 3 components referred to as: 1. early inhibition/depression (10–50 ms) characterized by prevailing $GABA_A$ receptor-mediated events; 2. intermediate facilitation (70–125 ms) when kainate/AMPA and later also NMDA receptor-mediated events prevail; and 3. late inhibition/depression (150–1000 ms) characterized by slow G-protein-mediated transmission mediated by e.g., $GABA_B$, neuropeptides, and metabotropic glutamate receptors.

A single EB injection did not change the paired pulse curve compared to oil controls. Chronic EB administration (for 4 days) enhanced the late paired pulse inhibition. These data suggest that the EB-mediated suppression of dentate granule cell excitability involves activation of gene expression, which requires longer EB treatment.

Future Goals

Effects of steroid hormones on neuronal excitability are complex and not well understood. The main controversy arises from the fact that the steroid hormone-induced effects are sensitive to many variables, and each laboratory chooses a slightly different paradigm for its experiments. Thus, it is hard to compare the data available in the literature.

In this article, I've emphasized the more controversial findings in order to stimulate discussion and appeal for more studies, which would help to increase our understanding of hormonal effects in the CNS. Such knowledge is very important, especially for developing treatment strategies for people with epilepsy. Also, epileptic patients (both men and women) often suffer with reproductive disorders not only as a result of seizures but also due to their antiepileptic treatments. The proconvulsant effect of estrogen is widely advertised, but some bench studies and also some clinical data do not support this view. Further, estrogens have been associated with neuroprotective effects, and several animal studies suggest that β-estradiol may be beneficial to prevent development of hippocampal sclerosis in rat models of temporal lobe seizures following status epilepticus.

Better knowledge of how sex hormones affect neuronal activity may help to prevent, or at least better manage seizures sensitive to hormonal changes, especially during puberty, the menstrual cycle, and menopause.

Acknowledgement

Supported in part by grants NS-36238 and NS-20253 from NINDS.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormones and Seizures; Hormones and Absence Epilepsy; Hormones and Epilepsy; Male Sex Steroids and Neuronal Excitability; Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; Steroid Hormones and Sex Differences in Seizure Susceptibility; Sex Differences in Seizure Sensitivity.

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Hormones and Absence Epilepsy

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Introduction

Puberty, menarche, menses, and menopause are often critical times when reproductive hormones may have a profound impact on seizures. An example is the shift from absence epilepsy to generalized tonic–clonic seizures that has been noted during puberty in some children with childhood absence epilepsy. There is however no direct evidence to substantiate the suggestion that this switch is due to changes in reproductive hormones. Data from research in laboratory animals show that gonadal hormones can exert a profound influence on neuronal excitability, seizures, and epilepsy. Similarly, environmental stressors are known to facilitate various types of epilepsy; it seems likely, therefore, that adrenal stress hormones are involved in regulation of seizures.

These observations have led to a focus on corticosteroids and ovarian steroid hormones, estrogen and progesterone, to clarify how these steroid hormones influence seizures in patients under stress and in women with epilepsy. The prevailing view on gonadal hormones is that estrogen is proconvulsant, whereas progesterone is anticonvulsant. The cyclical changes of these ovarian hormones appear to play a key role in the genesis of catamenial seizures. However, estrogen and progesterone are not the only reproductive hormones to be considered in evaluating excitability, seizures, or epilepsy in the female.

Testosterone also modulates seizure susceptibility, and it has been recently hypothesized that its pro- or anticonvulsant action depends on the concentrations of its specific metabolites.

It seems unlikely that these steroid hormones would exert single and uniform actions given our current understanding of their complex pharmacological and physiological relationships. Their modulatory effects on brain excitability and seizures are likely to depend on the endocrine state of a subject, their relative concentration, metabolism, and many other factors. Some recent advances have helped clarify past confusion in the literature: acute pharmacological studies show that progesterone dose-dependently increases spike-wave discharges (SWDs), but during pregnancy – when progesterone concentration is high – SWDs are reduced. Although the outcomes of the first study suggest a positive relationship between concentrations of progesterone and the number of absence seizures, the pregnancy study shows a negative relationship between concentration of progesterone and absence seizures. Clinical and animal research, designed with the relevant endocrinological and neurobiological issues in mind, will help advance this field in the future.

Background

Hormones and Their Action

Hormones are classically classified according to their chemical structure into peptide hormones (e.g., growth hormone, insulin, and antiduretic hormone), steroid

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Hormones and Their Action

Hormones are classically classified according to their chemical structure into peptide hormones (e.g., growth hormone, insulin, and antiduretic hormone), steroid

hormones (e.g., estrogen, progesterone, and testosterone), and tyrosine derivates (e.g., thyroxine and adrenaline). Peptide hormones are stored in vesicles and released by exocytosis in response to appropriate stimuli; steroid hormones are lipophilic, not stored in vesicles for secretion, and their rate of release is determined by their rate of synthesis; tyrosine derivates are synthesized by specific pathways in thyroid and adrenal medulla.

Circulating hormones of the gonads, adrenals, and thyroid gland have many effects on the brain. Some of these effects are permanent, while others are reversible. Many effects occur via the stimulation of gene expression through well-characterized intracellular receptors, whereas other effects take place rapidly at the surface of nerve cells via different type of neurotransmitter receptors. Many of these central effects are not confined to reproduction.

Twenty years ago, neurosteroids – steroids which are synthesized in the central and the peripheral nervous systems – were discovered. Neurosteroids are precursors (pregnenolone, dehydroepiandrosterone, and their sulfates) and metabolites (allopregnanolone, allotetrahydrodeoxycorticosterone) of steroid hormones, which influence the excitability of neurons predominantly by nongenomic mechanisms. They can be synthesized de novo in brain tissue mainly from cholesterol (e.g., pregnenolone or dehydroepiandrosterone) or are produced from hormones transported to the brain from peripheral sources (e.g., allotetrahydropregnanolone or allotetrahydrodeoxycorticosterone). Several of these steroids accumulate in the brain after local synthesis or after metabolism of adrenal or gonadal steroids. Progesterone is also a neurosteroid, and a progesterone receptor has been identified in peripheral and central glial cells. Pregnenolone appears to be among the most quantitatively important neurosteroids in the rat brain, and is a major precursor of the neurosteroid progesterone.

Neurosteroids may have the same action in the brain as steroid hormones; however, they exert their effects mainly through nongenomic mechanisms. Nongenomic steroid effects are principally characterized by their insensitivity to inhibitors of transcription and protein synthesis. The most obvious experimental evidence for the nongenomic effect is the rapid (i.e., within minutes) onset of action. These very rapid effects of steroids, mainly affecting intracellular signaling, are incompatible with the genomic mode of action. Instead, the rapid effects of steroid hormones are likely to be mediated through ion-gated neurotransmitter membrane receptors with pharmacological properties distinct from those of the intracellular steroid receptors. Neuroactive steroids may modulate – at low concentrations – the $GABA_A$ receptor, as allosteric coagonists or antagonists of GABA. Thus, their mechanism of action appears to be dependent on the specific structure of the $GABA_A$ receptor (with its subunits forming ligand-gated ion channels). At higher concentrations, they can also act as allosteric modulators of neurotransmitter receptors, such as NMDA, glycine, nicotinic, muscarinic, and sigma receptors.

Steroid hormones modulate seizure propensity in all types of epilepsy, including absence. Fluctuations in plasma and brain concentrations of steroid hormones and their neuroactive metabolites, over different stages of the reproductive cycle, play an important role in modulation of neuronal excitability. There is a unique relationship, however, between absence epilepsy and GABA. The pharmacological profile of absence epilepsy is opposite to the profiles typical of other types of seizures. Absences are associated with hyperfunction of GABAergic inhibitory system, while other seizure types are usually associated with a hypofunction of the GABAergic system. This counter-intuitive relationship between GABA and absence tends to complicate studies investigating the effects of neurosteroid hormones on absence; indeed, those that exhibit GABA-mimetic properties are proepileptic (i.e., they exacerbate absence seizures). Further, pharmacological studies have shown that whereas systemic administration of GABA-mimetic drugs enhances absence seizure activity, local administration of the same drugs may suppress SWDs. These observations make it difficult to predict the effect of steroids hormones such as progesterone (known to modulate the GABAergic system) on the occurrence of absence. It is necessary, therefore, to investigate local effects of these hormones directly.

Method

The majority of the studies reported here were carried out on WAG/Rij rats, a genetic model of absence epilepsy. GAERS (genetic absence epileptic rats from Strasbourg, originally described by Christian Marescaux and Marguerite Vergnes) and WAG/Rij rats (Wistar Albino Glaxo rats bred in Rijswijk) are most commonly used to study intrinsic, network, and behavioral features of absence epilepsy. All individuals of both strains show an age-dependent increase in the number of SWDs. The SWDs are accompanied by small but clear clinical signs typical of absence epilepsy. The SWDs show a circadian distribution, with a minimum at the onset of the sleep phase (of a 24 h sleep-wake cycle) when rats have a preponderance for deep slow wave sleep, and a maximum during the dark period. Both rat strains are well-validated models of absence epilepsy.

We have examined the relation between absence episodes and hormone levels in rats that are implanted with EEG electrodes in the cortex; effects of hormonal manipulations are assessed based on the frequency and duration of SWDs in freely moving animals over the course of many days. Blood samples are taken, to determine circulating hormonal levels, via the jugular vein (in chronic studies) or via incisions at the end of the rat's tail (in acute studies); up to 300 µl collected for these analyses (via RIA).

Recent Results

Progesterone and Related Neurosteroids

The most widely studied steroid hormone is progesterone. We addressed the question of whether (and how) progesterone affects absence seizure in two ways.

First, we examined the time course of absence seizures over the ovarian cycle in adult female WAG/Rij rats. In this way, we could establish the extent of physiological fluctuations of SWDs as a function of the fluctuations of the ovarian hormones progesterone and estradiol. These hormones are known to be elevated at specific days and hours of the ovarian cycle. The data of the long-term EEG recording of SWD in female WAG/Rij rats are presented in Figure 1.

These data show a consistent circadian pattern for the number of SWDs, with an acrophase during the first hours of the dark period and a nadir during the first hours of the light period. While this circadian distribution is in full agreement with earlier studies, it also shows a newly-observed additional increase in SWDs at the shift from the light to the dark phase of the proestrus day. The latter finding strongly suggests that a physiological increase in the concentration of plasma progesterone on the proestrus day enhances SWDs. The outcome of an acute pharmacological study pointed in the same direction. Progesterone rapidly enhanced, in a dose- and time-dependent manner, the number of SWDs. Similarly, an increase in SWDs during proestrus days was found in a rat model for atypical absences (adult rats show atypical SWDs after an injection of a cholesterol synthesis inhibitor AY9944), and an acute injection of progesterone increased SWDs in these animals. The relevance of these data is twofold: First, changes in progesterone in physiological concentrations affect the number of spontaneously occurring SWDs. Second, experimentally-administered progesterone increases SWDs in two different models of absence epilepsy – which is in agreement with a single case study in humans which showed increased absence occurrence in a woman when she was administered progesterone. The direction of the effect of progesterone is in contrast to the often found anticonvulsant action of progesterone on other seizure types, both in animal models and in catamenial seizures.

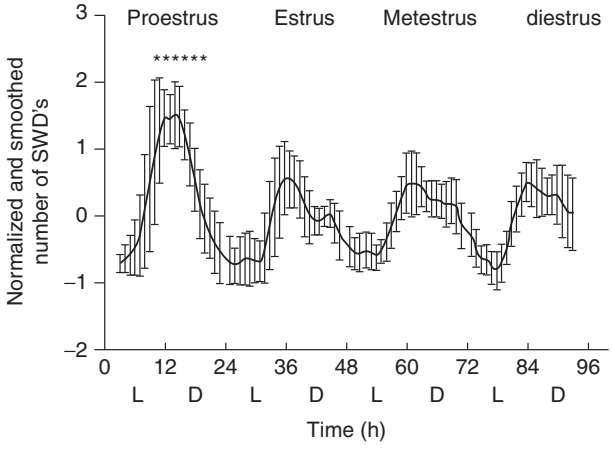


Figure 1 Hourly number of SWDs in the 4 days of the estrous cycle in WAG/Rij rats ($n = 8$). The hourly number (mean ± SD) of SWDs was normalized (mean, 0; variance, 1) per subject and smoothed by a centered moving averaging filter with span 4. Note the day effect at the first few hours of the dark period at proestrus. *Journal of Epilepsy Research*, with permission from Elsevier.

Subsequent research was designed to establish whether the effects of progesterone were due to the classical genomic mechanism or to receptor activation at the cell membrane. Finasteride, an inhibitor of 5α-reductase, which inhibits the synthesis of neurosteroids that have agonistic activity at $GABA_A$ receptors (e.g., allopregnanolone, tetrahydrodeoxycorticosterone (THDOC), androsterone), did not affect the number of SWDs in a 24 h recording period. However, finasteride did antagonize the increase in number of SWDs induced by progesterone in this rat model. In contrast, injection of RU 38486, an antagonist of intracellular progesterone receptors, had no effect on SWDs and did not block the stimulatory effect of progesterone. These findings demonstrate that progesterone has two types of effects: The normally-occurring SWDs during baseline periods were not affected by finasteride and RU 384886, and are most likely controlled by genomically mediated mechanisms. However, the SWDs induced by progesterone were blocked by finasteride, suggesting that this increase was due to nongenomic effects of progesterone, most likely those of allopregnanolone or 3α-OH-dihydroprogesterone, both potent endogenous positive modulators of CNS $GABA_A$ receptor function. Similar outcomes and conclusions have been presented by Persad and coworkers in the atypical absence seizure model. Interestingly, ovariectomy has little effects on SWDs; therefore it seems that the circadian rhythm of SWDs is not controlled by swings in plasma concentration but by central effects. The increase in SWDs during proestrus, however, coincides with increased plasma levels; how SWDs are specifically enhanced during the proestrus hours remains to be established.

Two neurosteroids, which are synthesized in the brain from cholesterol (pregnenolone sulphate) or from progesterone (allopregnanolone), have been tested. Allopregnanolone potently increased SWDs dose-dependently in WAG/Rij rats. The action is most parsimoniously explained by its action in stimulating the $GABA_A$ receptor. In support of this hypothesis is the recent report that isopregnanolone, an isomer of allopregnanolone, which is devoid of $GABA_A$ agonistic activity, when injected into the brains of WAG/Rij rat, has no effect on SWDs. Ganaxolone is a synthetic analogue of allopregnanolone. It is, like allopregnanolone, a potent positive allosteric

modulator of $GABA_A$ receptors. Ganaxolone has a robust anticonvulsant profile in a variety of animal models of epilepsy, is orally active and lacks hormonal side effects. Unlike diazepam, tolerance to the anticonvulsive action does not develop to ganaxolone following chronic therapy. However, ganaxolone has been found to increase SWDs when injected into some thalamic nuclei. Although the systemic effect of ganaxolone has not yet been tested in genetic animal models, we predict that it will aggravate SWDs and that ganaxolone should not be given to patients with absence seizures.

An increase in SWDs was also found after the highest dose of pregnenolone sulphate. This effect was explained by its agonistic effects on the NMDA receptor, although other mechanisms could not be excluded, such as an action through its conversion via progesterone to allopregnanolone.

Pregnancy is an interesting hormonal condition and epidemiological studies show an increase in seizure frequency in up to one-third of the woman with epilepsy because of hormonal and pharmacokinetic changes. Progesterone is chronically enhanced during pregnancy, to a concentration much higher than 17β-estradiol. However, in our study in WAG/Rij rats, SWDs were reduced during the first 18 days of pregnancy. Two days before delivery, progesterone decreases and SWDs increase significantly. These data demonstrate a negative relationship between plasma levels of progesterone and SWD in pregnant females, while the outcomes of the 96 h recording during the ovarian cycle and the acute pharmacological study demonstrate a positive relationship between plasma or brain concentrations of progesterone and absence seizures. The fact that these diametrically opposite relationships between absence seizures and progesterone have been demonstrated is a beautiful illustration of the fact that the relation between hormones and seizures may be completely opposite during different hormonal states (in this case, pregnancy).

Local vs. Systemic Injection of Progesterone in WAG/Rij Rats

The diametrically opposite relationship between progesterone and SWDs found during pregnancy versus the outcomes of the dose–response study and 96 h continuous recordings in female WAG/Rij rats has been a challenge for our further research. A first clue about how to understand these relationships comes from pharmacological studies in GAERS. In these studies, the effects of GABA-mimetic and antagonist, locally injected into the reticular thalamic nucleus, were opposite to the effects seen with injections of these drugs into the thalamic relay nuclei. Muscimol injected into the thalamic relay nuclei increased SWD frequency (the same as after systemic injections), but local injections in the RTN reduced SWDs.

A second clue comes after the demonstration of a cortical focus in absence epilepsy. This finding showed that SWDs are elicited in part of the somatosensory cortex, and the investigators predicted that local focal injections of GABA-mimetics (such as tiagabine) and modulators of the $GABA_A$ receptor should suppress SWD – in contrast to the effects of systemic studies of GABA-mimetic drugs (muscimol, THIP, SKF 89976, vigabatrin, tiagabine), which enhance SWDs. It quickly became clear that focal injection into this zone in the somatosensory cortex suppresses SWDs (compared with the effects of systemic injections), not only for GABA-mimetic drugs but also for antiepileptic drugs such as phenytoin. These results have led De Sarro and coworkers to compare the effects of local injections of neurosteroids into various regions in the thalamo-cortical loop (including the peri-oral region of the somatosensory cortex) in WAG/Rij rats. Allopregnanolone and ganaxolone, both with a potent enhancement of the GABA-response, injected in the thalamic VPM or VPL, increased SWD, similar to the systemic effects of allopregnanolone. The neurosteroid with a GABA antagonistic profile, pregnenolone sulphate, decreased SWDs after injections into the VPM, was ineffective in the VPL, and increased SWDs in the RTN. These regional differences are currently not well understood. Most interesting is that allopregnanolone and ganaxolone suppressed SWDs dose-dependently when administered into the peri-oral region of the somatosensory cortex. Pregnenolone sulphate showed the opposite effects of allopregnanolone and ganaxolone when it was administered into this region of cortex.

In all, the data show that neurosteroids like allopregnanolone, which modulate the $GABA_A$ receptor, can have different effects, depending on the location of the injection. Local cortical administration in the cortex blocks SWDs, while injection into thalamic relay nuclei enhances SWDs. Another example illustrating such differences is seen with local injections of progesterone and tiagabine (a GABA-reuptake inhibitor). Local hippocampal injections were effective in suppressing SWDs, while systemic injections of these drugs enhanced SWDs. Moreover, cortical injections outside the peri-oral cortical regions were without any effect. These data demonstrate that the same neuroactive steroid may have different consequences on seizure activity, dependent on the location of where the neurosteroids are active. They also suggest that the hippocampus, although not involved in the initiation and spread of absence seizures, may modulate absences seizure activity in the corticothalamic circuits.

17β-Estradiol

A second important neuroactive steroid hormone is 17β-estradiol. Beside an action via classical intracellular receptors on gene transcription, estrogen interacts with

specific receptors at the plasma membrane of neurons, and activation of these receptors activates intracellular signal transduction pathways. Estrogen has been shown to regulate the activation of some G-protein-coupled receptors (such as opioid, $GABA_B$ and dopamine D_2) and ionotropic receptors (such as 5-HT_3 and nicotinic). Estrogens also activate the *N*-methyl-D-aspartate (NMDA) excitatory neurotransmitter receptors in the hippocampus. It also affects the synthesis of DA. Considering the multiple action of estrogens, its action on absence seizures is difficult to predict.

We observed no difference in the number of SWDs during the first few hours of the light period between proestrus and the three other days of the cycle in female WAG/Rij rats in a 96 h EEG study, suggesting that 17β-estradiol has no effect on SWDs. Outcomes of pharmacological studies confirmed that 17β-estradiol does not affect absence seizures; in addition, tamoxifen, an antagonist of 17β-estradiol intracellular receptor, did not affect SWDs. In many other seizure models (e.g., kainic acid, ethyl chloride, kindling, and pentylenetetrazole), estrogens increase the number of seizures and decrease the threshold for electroshock-induced seizures. Interestingly, 17β-estradiol reduced absence seizures in the atypical absence seizure model.

Testosterone

There are no documented papers on the relationship between testosterone and absence epilepsy. Testosterone may have anti- or proconvulsant effects, depending on the dose, the animal's age, and the seizure type. The variable actions of testosterone can be partly explained by the metabolism of testosterone. Testosterone is metabolized via aromatization to 17β-estradiol (which is not effective, see above), and testosterone can also be reduced to 5α-dihydrotestosterone which is then converted to 3α-androstanediol; the latter is a powerful and positive modulator of the $GABA_A$ receptor. Therefore a relatively late increase in SWDs after systemic administration of testosterone can be predicted based on the GABA-mimetic effects of this metabolite. The first metabolite, 5α-dihydrotestosterone, is known to mimic the action of NMDA antagonists, it may reduce absence seizures considering that all NMDA antagonists reduce absence seizures. The results of systemic injections of testosterone in 6-months-old male WAG/Rij rats are presented in Figure 2.

The solvent cyclodextrine (CD) increased SWDs in the first hour, but this increase was not found for testosterone. Testosterone did not show the increase in SWDs in comparison with the solvent at the first hour post-drug administration, at post 2 and 3 testosterone showed a clear increase in number of SWDs while CD was no longer active. Considering these outcomes, we propose that testosterone is active through its metabolites 5α-dihydrotestosterone and 3α-androstanediol, which have anti- and pro-absence action on SWDs, respectively. The increase by the solvent might be due to the small injection stress or by intrinsic effects through sponging neuroactive steroids from endogenous resources that alters neuronal excitability and accounts for the increase of SWDs.

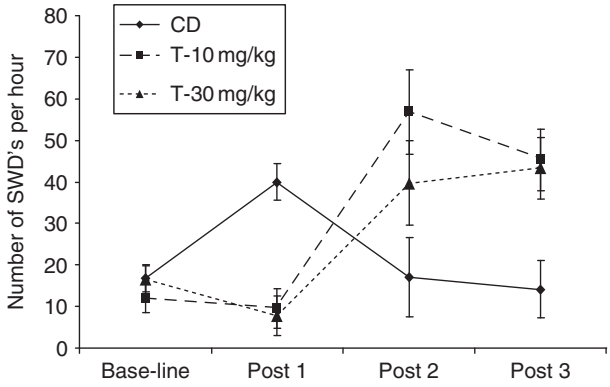


Figure 2 Effects of systemic injections of testosterone in WAG/Rij rats. Number and SEM of SWDs per hour during the baseline (preinjection), and in the first, second, and third hour following systemic injection with testosterone (10 or 30 mg kg^{-1}) of solvent CD (cyclodextrin). Note that in the first hour post-injection CD enhanced SWDs, and testosterone had less SWDs than CD alone, CD was no longer effective in the second and third hour post-administration, while both testosterone groups showed an increase in SWDs (courtesy of Alexandra Badura).

Hypothalamic–Pituitary–Adrenal Axis

Steroid hormones, secreted by the adrenal glands in response to stress, rapidly modulate brain function and behavior as well as cellular responses in general. The stress response in rats emerges within a few minutes after systemic injection of glucocorticoid. It modulates neurotransmission in the limbic system, e.g., the hippocampus, by a rapid and transient increase in the intracellular levels of inhibitory neurotransmitter GABA and the excitatory amino acids aspartate and glutamate. Systemic injections of corticosteroids in WAG/Rij rats produced a short decrease followed by an increase in SWDs. We have speculated that the short lasting decrease in SWDs is due to activation of peripheral adrenalin and to the enhancement of catecholamines in the central nervous system – known to accompany arousal and activation and suppress SWDs. We propose that the subsequent increase in SWDs might be explained by the increase of glutamate in the hippocampus, since opposite effects (reduced SWDs) were obtained after the administration of GABA-mimetics in the hippocampus. However, this possibility needs experimental verification. Interestingly, experiments with daily footshock presentation induced a similar

pattern – a short lasting suppression of SWDs was followed by a return to baseline or an increase. The data of our recent study are presented in Figure 3. The initial decrease in SWDs on day 1 after the footshock subsided over days, with a subsequent increase in SWDs that became larger over days. These data show that acute stress has a biphasic effects on SWDs and it is very likely that corticosteroids play a role in the increase of SWDs through their action in the hippocampus. These data show also that the increase in SWDs after repeated footshock exposure is elevated in ovariectomized rats. This observation has been attributed to the fact that ovarian and hypothalamic–pituitary–adrenal (HPA) axis hormones both contribute in the coping response to stress.

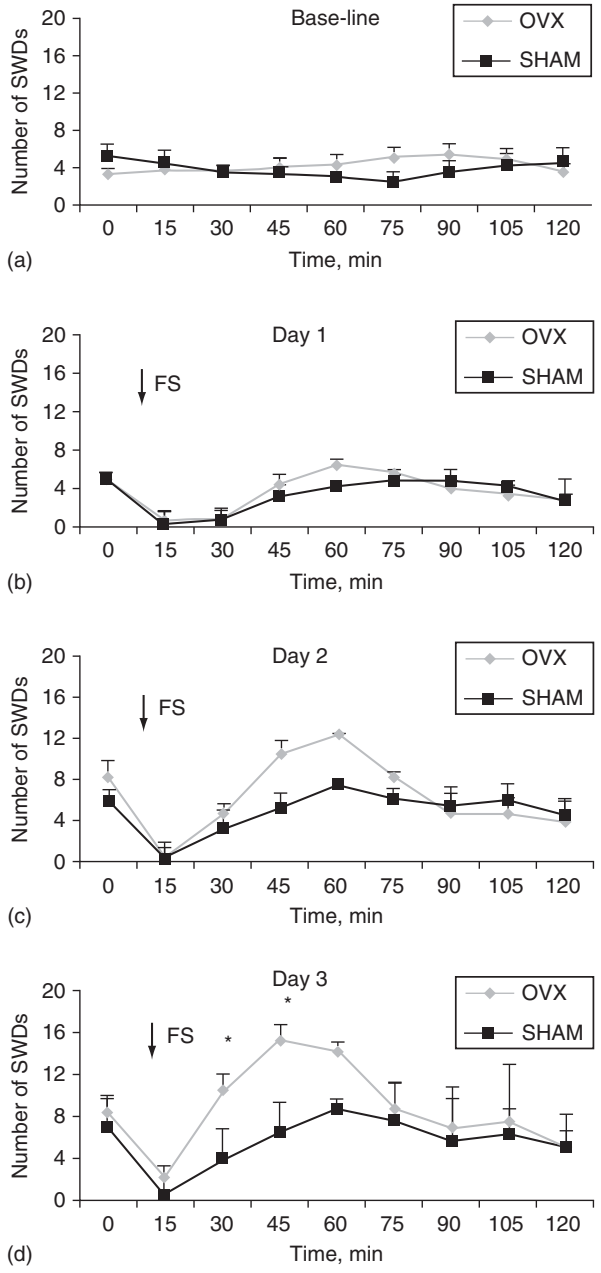


Figure 3 The effects of daily footshocks on the number of SWDs in a 2 h recording period. Note that the number of SWDs are immediately decreased after the shock and return to preshock levels, that the number of SWDs increase over days both pre and after footshocks, and that the increase over days (a)–(d) post-footshocks becomes larger over days. The latter effect is pronounced in ovariectomized females (OVX). Reproduced from Tolmacheva EA and van Luijtelaar G (2007) The role of ovarian steroid hormones in the regulation of basal and stress induced absence seizures. *Journal of Steroid Biochemistry and Molecular Biology* 104: 281–288, with permission from Elsevier.

Other Hormones, Peptides, and Absence Epilepsy

Although the thalamus is not the origin of absence seizures, it is definitely involved in the amplification and spread of these seizures. There are many neuropeptide hormones and peptides that can alter rhythmic thalamic activity, including somatostatin, cholecystokinin, neuropeptide Y, nociceptin/orphanin FQ, substance P, and vasoactive intestinal peptide. These peptides have all been localized within the thalamus, along with their respective receptors, and have been shown to alter the excitability of thalamic neurons by changing intrinsic properties of thalamic cells (such as resting membrane potential, input resistance, membrane conductance, action potential firing mode, and synaptic transmission). One peptide hormone, PrRP (prolactin-releasing peptide – which releases prolactin from pituitary cells), has been investigated in vivo and in vitro in experiments on GAERS. This peptide inhibits rhythmic activity in the RTN in the slice preparation, and it influences non-REM sleep and SWDs in intact animals. Both effects may emerge through action on receptors in the RTN; the mRNA for the receptor is expressed in the RTN and present on the GABAergic neurons in the RTN. Considering these effects on RTN neurons, one might predict that PrRP has anti-absence effects – and this effect has indeed been found. A second peptide hormone, TRH (thyrotropin-releasing hormone), administered i.c.v. decreased dose-dependently the number and mean duration of SWDs in WAG/Rij rats, and also has mild anti-absence effects.

Neuropeptide (NP) Y, a likely endogenous antiepileptic substance with possible antiepileptogenic effects, was shown to dose-dependently suppress SWDs in GAERS. Subsequent studies with specific NP Y receptor agonists showed that NP Y Y2 receptors are more important than Y1 and Y5 in mediating this effect, so that Y2 receptor agonists may represent targets for novel drugs against generalized epilepsies.

Future Challenges

In order to understand the relation between steroid hormones and absence seizures, it is necessary to find the

factors involved in the GABA hyperfunction responsible for SWDs as measured during baseline periods, and the changes that occur in SWDs during different hormonal states (such as pregnancy and various phases of the ovarian cycle). The hyperfunction and its changes can be due to elevated concentration of neuroactive steroid hormones in the cortex, thalamus and hippocampus, and/or to local concentrations of GABA-mimetic agents such as allopregnanolone, THDOC and 3α-androstanediol. These concentrations should also be determined and compared between WAG/Rij and control rats of different age, before the onset of seizures, and when the seizures are fully developed. Also the local concentration of enzymes that metabolize steroid hormones to the positive neurosteroid GABA modulators (e.g., 5α-reductase and 3α-hydroxysteroid dehydrogenase) should be compared in different hormonal conditions in WAG/Rij rats, and between WAG/Rij and control rats.

It would also be challenging to investigate whether the GABA hyperfunction is caused by action of GABA-mimetic neurosteroids (even at their unchanged level) on presynaptic, postsynaptic, or extrasynaptic $GABA_A$ receptors, the relevant receptor subunits, and the phosphorylation status of the receptors. These data should be compared between WAG/Rij and control rats. It is known that the ability of neurosteroids to affect GABA receptor function depends on the $GABA_A$ subunit expression. In some brain regions, extrasynaptic $GABA_A$ receptors, especially those containing delta subunits are more sensitive to neurosteroids than synaptic receptors. Also, phosphorylation of $GABA_A$ receptors regulates the interaction of neurosteroids with this receptor.

The changes that occur in childhood absence epilepsy, around puberty, are not understood. Therefore, it would be useful to develop a model for the transition from childhood absence epilepsy to other generalized (tonic–clonic) epilepsies.

It is challenging to study further the interaction between various hormones in the stress coping response. This response is relevant to our understanding of absence seizures, considering that the hormonal state changes dramatically in females – and that the way in which they cope with stress is highly dependent on these endocrinological changes. Moreover, acute stress increases the concentration of neuroactive steroids (allopregnanolone, THDOC) in plasma and brain, and neurosteroids in turn inhibit the release of both corticotropin-releasing hormone (CRH) and gonadotropin-releasing hormone (GnRH).

Allopregnanolone was shown to inhibit not only HPA axis activity but also glucocorticoid-mediated gene transcription. Interactions occurring among steroid hormones, neurosteroids, and glucocorticoids in response to stress (acute/chronic) should be compared between WAG/Rij and control rats.

Acknowledgement

The authors thank Prof. Dr. W. Lason (Institute of Pharmacology, Polish Academy of Sciences, Cracow, Poland) and Prof. Dr. M. Oitzl (Medical Pharmacology of Leiden University Medical Centre (LUMC) and Leiden/Amsterdam Center for Drug Research (LACDR), and University of Amsterdam) for their expertise help with experiments and discussions.

See also: **Absence Seizures**: Genetic Models of Absence Epilepsy: New Concepts and Insights; **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; Sex and Seizure Sensitivity; Sex Differences in Seizure Sensitivity.

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Hormones and Epilepsy

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Introduction

The interaction between hormones and seizures is complex. There is strong evidence that hormones influence seizures, particularly in women, and there is some evidence that seizures influence hormones in both men and women. This article will focus primarily on research investigating how the ovarian steroid hormones, estrogen and progesterone, influence seizures in women with epilepsy and in animal models of epilepsy. Understanding the mechanisms of hormone-sensitive seizures suggests targets for novel antiepilepsy therapies.

A relationship between seizures and the reproductive cycle in women with epilepsy has been recognized since the Greek era. In the mid to late nineteenth century, Locock and Gowers, separately, published the first clinical descriptions of seizures related to the menstrual cycle. In 1956, Laidlaw coined the term 'catamenial epilepsy' (from the Greek, *katamênios*, meaning monthly) to describe an exacerbation of seizures at various points in the menstrual cycle.

Typically, there are three times in the cycle in which increased seizure frequency is observed: around the time of menses, during ovulation, or in the second half of anovulatory cycles. Of these three, the perimenstrual seizure is the most common. A catamenial seizure pattern can occur in women with either generalized or focal epilepsy, and it has been reported that catamenial seizures are more resistant to control with antiepileptic drugs. For some women, the frequency or severity of their seizures also can be affected by reproductive life stage – for example, at puberty, during pregnancy, or after menopause. Seizure patterns related to these additional life stages have been less well studied than seizures related to the menstrual cycle.

The apparent prevalence of catamenial epilepsy depends heavily on the criteria used to define it. For example, as many as 78% of reproductive age women with epilepsy report a relationship between their menstrual cycle and their seizures. The number drops to about 30%, however, when a criterion of doubled average daily seizure frequency is applied, and to about 12% for a sixfold increase. At least a doubling of seizure frequency (during one of the three menstrual cycle points noted above) is generally accepted as the definition of catamenial epilepsy.

Although several nonhormonal causes of catamenial epilepsy have been suggested, there is strong evidence that catamenial seizure patterns are due, in large part, to fluctuating levels of ovarian steroid hormones across the menstrual cycle. Ovarian steroids do not cause seizures (except in very high doses), but they can make the neurons that support seizure activity more or less likely to fire action potentials, and particularly to fire synchronously. In this way, steroids alter susceptibility to the generation and/or propagation of seizure activity, and can influence seizure severity.

One characteristic that links the points in the menstrual cycle at which seizure frequency is increased in catamenial epilepsy is a high ratio of circulating estradiol to progesterone. The principal estrogen in reproductive age women who are not pregnant is estradiol; estriol dominates during pregnancy, and estrone after menopause. Laidlaw originally noted that elevated progesterone during the menstrual cycle is associated with reduced incidence of seizures, and so might be anticonvulsant. Subsequently, Bäckström observed that seizure frequency also can be increased during the preovulatory estradiol surge. In 1976, he modified Laidlaw's original hypothesis and suggested that the *ratio* of estradiol to progesterone is the most relevant factor in producing catamenial seizures. This view then developed into the relatively simple idea that estradiol is generally proconvulsant, whereas progesterone is generally anticonvulsant. Animal studies consistently support anticonvulsant effects of progesterone, primarily through modulation of inhibitory γ-aminobutyric acid $(GABA)_A$ receptors. There is also strong experimental evidence for proconvulsant effects of estradiol; however, the actions of estradiol are more diverse, and results of seizure studies with estradiol are more variable.

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Perimenstrual seizure exacerbation is likely due to the precipitous drop in progesterone that occurs just before menses, sometimes referred to as 'progesterone withdrawal.' Seizures that occur mid-cycle are most likely due to proconvulsant effects of estradiol, which surges just prior to ovulation. Seizures that increase during the second half of anovulatory cycles are also likely to be due to the actions of estradiol, together with a relative lack of progesterone. During ovulatory cycles, a corpus luteum forms from the follicle that releases its egg. The corpus luteum is the primary source of ovarian progesterone, which normally increases after ovulation – i.e., in the latter half of the cycle. In an anovulatory cycle, the corpus luteum fails to form (causing a condition sometimes referred to as 'inadequate luteal phase'). In these anovulatory cycles, progesterone levels do not rise and thus proconvulsant effects of estradiol are unopposed by anticonvulsant effects of progesterone. **Figure 1** depicts fluctuating estradiol and progesterone levels across the menstrual cycle, and the consequences for neuronal activity.

Background

Hormones and Seizures in Animal Models of Epilepsy

Animal research into the roles that progesterone and estradiol play in regulating seizure susceptibility began with the pioneering work of Selye in the case of progesterone, and D. E. Woolley, Teresawa, and Timiras in the case of estradiol. In 1942, 14 years before Laidlaw suggested that progesterone could be anticonvulsant during the menstrual cycle, Hans Seyle reported that progesterone had anticonvulsant effects in rats. Later, in the 1960s, Timiras *et al.* conducted the first animal studies investigating the effects of estradiol on seizures. Her group confirmed, again in rats, that progesterone is anticonvulsant. They further found that estradiol is proconvulsant in that seizure threshold is reduced both by exogenous estradiol treatment and during the high estradiol phase of the estrous cycle (analogous to the menstrual cycle in primates). These classic observations set the stage for the next six decades of research into the mechanisms by which ovarian steroids influence seizures through their effects on neuronal physiology. Given the importance of the hippocampus in temporal lobe epilepsy, the most common form of epilepsy in adulthood, the majority of these studies have focused on the hippocampus and related brain areas.

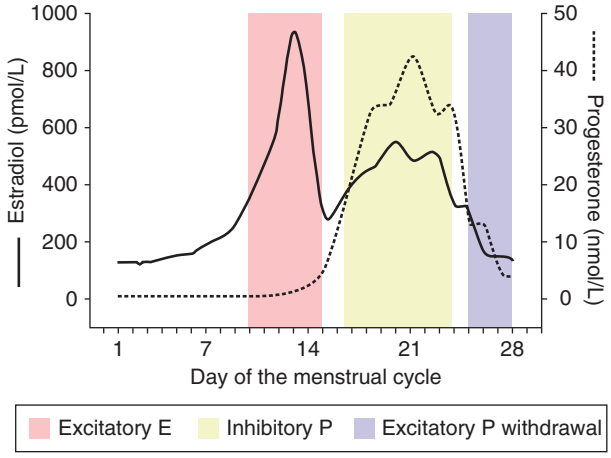


Figure 1 Median levels of estradiol (E) and progesterone (P) across the typical 28-day menstrual cycle in reproductive age women. Colored portions represent periods in the cycle during which estradiol and progesterone's effects on neuronal physiology in seizure-prone neural circuits alter seizure frequency. Ovulation typically occurs mid-cycle, and menses occurs at the end of the cycle. Estradiol and progesterone levels data are obtained from Table 1B in Stricker, R., Eberhart, R., Chevalilier M.C., *et al*. (2006) Establishment of detailed reference values for luteinizing hormone, follicle stimulating hormone, estradiol, and progesterone during different phases of the menstrual cycle on the Abbott ARCHITECT analyzer. *Journal of Clinical and Chemical Laboratory Medicine* 44, 883–887.

Progesterone and estradiol, like other steroid hormones, exert their effects in the brain through multiple mechanisms, with differing time courses. The classical (or genomic) mechanism of steroid action is through nuclear receptors. Steroid hormones secreted by peripheral endocrine glands are released into circulation, readily cross the blood–brain barrier, and diffuse into cells in the brain. In cells that contain the appropriate receptor(s), hormone–receptor complexes bind to hormone response elements in DNA and regulate gene expression. Owing to its dependence on gene expression, this mechanism produces effects with a delayed time course, on the order of hours to days.

In addition to this classical mechanism, steroid hormones also exert rapid, nonclassical effects that occur on a time scale of minutes to hours. That these rapid effects contribute to hormone regulation of seizures was predicted by the work of Selye. He found that the anticonvulsant effects of progesterone could occur within minutes. The nonclassical effects of steroids may be mediated by classical nuclear receptor proteins acting outside of the nucleus, by G-protein-coupled receptors and second messenger pathway activation, and/or by direct modulation of ion channels.

Methods

Most of the studies described here involve treating animals with estradiol and/or progesterone, and then either measuring some aspect of electrographic or behavioral seizures or making specific electrophysiological or anatomical measurements. Electrophysiological and anatomical studies give insight into the types of cellular or circuit

changes that parallel changes in behavioral seizures (and likely contribute to them). Most of the relevant measurements at the cellular and circuit level have been made in the hippocampus. In these experiments, typically, hormone levels first are normalized by removing an animal's ovaries; after a defined period, animals are then treated with known doses of estradiol and/or progesterone or vehicle, and analysis is performed after a known duration of hormone exposure.

Seizure studies typically involve measuring how animals treated with estradiol and/or progesterone respond (relative to controls) to a seizure-inducing stimulus. Responses are assessed to electrical stimulation of a specific pathway or brain area, or following treatment with a chemical convulsant (such as the glutamate analogue, kainic acid). Electrophysiological recordings have been carried out in vivo or, more typically, in vitro – in acute slices of the hippocampus or in dissociated hippocampal neurons. Some of these studies have involved comparing intrinsic or synaptic physiology in hippocampal slices from animals treated with hormones; other studies have focused on effects of direct application of hormones to slices, or to individual dissociated neurons, on intrinsic and synaptic physiology. Anatomical measurements have been made using light and/or electron microscopy in hippocampal tissue from animals treated with hormones. Electron microscopy has been particularly useful for studying hormone-induced structural plasticity of hippocampal circuitry, as this technique provides nanometer resolution of synaptic ultrastructure.

Results

Progesterone – Cellular Level

As noted earlier, progesterone appears to exert its anticonvulsant effects largely through regulation of the inhibitory GABAergic neurotransmitter system. There is ample evidence that alterations of GABAergic inhibition influence seizure susceptibility. Drugs that inhibit GABA synthesis or $GABA_A$ receptors (responsible for fast synaptic inhibition) cause seizures, whereas drugs that enhance GABAergic function are anticonvulsant. Some genetic forms of human epilepsy are associated with $GABA_A$ receptor mutations that impair receptor function. In animal models, mutation of the gene for the GABA synthesizing enzyme, glutamic acid decarboxylase (GAD), decreases GAD activity and GABA levels in the brain, and increases susceptibility to seizures.

Experimental studies have demonstrated two distinct and complementary mechanisms by which progesterone regulates GABAergic inhibition. Interestingly, in both cases, it appears that metabolites of progesterone, rather than progesterone itself, mediate the effects. In 1986, it was discovered that one metabolite of progesterone, allopregnanolone (3α-OH-5α-pregnan-one), is a powerful positive allosteric modulator of $GABA_A$ receptors. Allopregnanolone binds directly to the $GABA_A$ receptor, increases channel open time, and thus potentiates the inhibitory postsynaptic currents (IPSCs) mediated by this receptor.

Similar to other agents that increase GABAergic inhibition, allopregnanolone is anticonvulsant. Its immediate precursor, 5α-dihydroprogesterone, is anticonvulsant as well. Importantly, several groups have found that the anticonvulsant effects of progesterone depend upon its conversion to one or both of these metabolites. First, it was demonstrated that finasteride, an inhibitor of the enzyme that converts progesterone to 5α-dihydroprogesterone (5α-reductase), blocks progesterone's anticonvulsant effects on behavioral seizures. The importance of 5α-reductase was then confirmed in 5α-reductase knockout mice; progesterone is significantly less effective in inhibiting seizures in knock outs than in wildtype controls. The lack of involvement of the classical progesterone receptor was demonstrated using progesterone receptor knockout mice. Progesterone was similarly effective in inhibiting seizures in these mice as in wildtype mice, and finasteride similarly inhibited the effects of progesterone on seizures in these knockouts. Together, these studies make a very strong case that the progesterone metabolites, 5α-dihydroprogesterone and/or allopregnanolone, are critically important in mediating anticonvulsant effects of progesterone independently of the classical progesterone receptor.

A second mechanism of progesterone (or its metabolites) action was revealed using animal models designed to mimic the perimenstrual drop in progesterone levels during the menstrual cycle (i.e., progesterone withdrawal). As described earlier, this point in the cycle is associated with an increase in seizure frequency. In these studies, rats were treated chronically with progesterone or allopregnanolone and then the steroid was withdrawn. Shortly after withdrawal, expression of the α4 subunit of the $GABA_A$ increased markedly. This increased expression is relevant to seizures because $GABA_A$ receptor channels that contain α4 produce IPSCs with a relatively fast decay time, leading to less inhibition and greater neuronal excitability. As predicted, this progesterone withdrawal-induced increase in α4 was associated with greater hippocampal circuit excitability and increased behavioral seizure susceptibility/severity. The importance of α4 in progesterone withdrawal was confirmed by showing that suppression of α4 expression with antisense mRNA inhibits both the changes in IPSC decay time and in seizure behavior.

Given these two effects of progesterone metabolites on GABAergic inhibition, the perimenstrual phase of the menstrual cycle represents a 'double-hit' increase in neuronal excitability. At this point in the cycle, the direct GABA-potentiating effect of progesterone metabolites is lost and the GABA-suppressing progesterone withdrawal

effect is induced. Importantly, in the context of catamenial epilepsy, levels of allopregnanolone also rise in the second half of ovulatory menstrual cycles, in parallel with progesterone. Levels of allopregnanolone are also particularly high during pregnancy, which may explain why some pregnant women with epilepsy experience a decrease in their seizures during this time.

Estradiol – Cellular Level

In contrast to progesterone, which appears to have uniformly anticonvulsant effects mediated through the GABA system, estradiol's effects on seizures are considerably more complex. Estradiol regulates both the GABAergic system and the excitatory glutamatergic system, and its effects vary across brain regions. Additionally, estradiol's effects on inhibitory and excitatory neurotransmitter systems, even within the same brain region, occur with differing time courses. Thus, whether and how estradiol affects seizures depends on both duration of estradiol exposure and the brain regions involved in the type of seizure studied. This complexity was evident even in the earliest studies of estradiol's effects on seizures. For example, in their studies in the 1960s, Timiras' group found that estradiol decreased seizure threshold in the dorsal hippocampus and the medial amygdala, but at the same time, it increased seizure threshold in the lateral amygdala. The following discussion will focus on the hippocampus, in which most studies have been done.

Estradiol's effects on hippocampal physiology can be divided into three phases: potentiation of intrinsic excitability and excitatory synaptic transmission that occurs within minutes, transient suppression of GABAergic inhibitory synaptic transmission that occurs at ~24 h, and a secondary enhancement of excitatory synaptic transmission that occurs at 48–72 h. Each of these will be discussed separately below.

In 1980, Teyler *et al.* published the first paper demonstrating a rapid effect of estradiol to increase hippocampal excitability. Using hippocampal slices from rats, they found that 5–10 min of estradiol exposure potentiated the slope and amplitude of extracellularly recorded field excitatory postsynaptic potentials. The effects they observed could have been due to either an increase in intrinsic excitability and/or potentiation of excitatory synaptic transmission. Subsequently, both of these possibilities were shown to contribute. Several groups have reported that estradiol rapidly increases hippocampal excitability by modulating K^+ currents and suppressing the after-hyperpolarization (AHP) that follows an action potential. This effect is likely due to decreased Ca^{2+} influx through L-type Ca^{2+} channels. Because the AHP depends, in part, on Ca^{2+}-activated K^+ currents, suppression of Ca^{2+} influx suppresses the AHP. Estradiol also rapidly increases the amplitude of excitatory postsynaptic currents (EPSCs) in hippocampal neurons. As in many other brain regions, these EPSCs are mediated by glutamate. Reports in the 1990s indicated that the rapid increase in EPSC amplitude was due to greater postsynaptic responsiveness to glutamate (or glutamate analogues). More recent studies, however, point to increased probability of glutamate release as the mechanism for rapid EPSC potentiation.

These rapid excitatory effects of estradiol on hippocampal physiology lead to amplification of synaptic potentiation mechanisms, which likely contributes to increased seizure susceptibility or severity. Thus, if a high-frequency tetanus is delivered to a hippocampal slice in the presence of estradiol, the long-term potentiation (LTP) that results is significantly greater than the potentiation that occurs with the same tetanus given in the absence of estradiol. This enhanced excitatory mechanism can produce a vicious cycle of excitation in which intense excitatory synaptic activation – such as occurs with seizures – amplifies synaptic strength with a high gain, leading to more excitatory synaptic activation. Whether or how these rapid actions of estradiol, specifically, affect seizure susceptibility has not yet been investigated. However, a reasonable prediction is that rapid effects on intrinsic excitability and EPSC potentiation increase seizure susceptibility during the initial phase of the preovulatory estradiol surge.

With somewhat longer exposure, ~24 h in rats, estradiol suppresses GABAergic inhibition of hippocampal neurons. This effect is transient in that, by 72 h, inhibition recovers to control levels. In a series of studies, electrophysiology and electron microscopy have been used in my laboratory to show that estradiol suppresses inhibition by decreasing the probability of GABA release at inhibitory synapses. We found that estradiol decreases the amplitude of synaptically evoked IPSCs in hippocampal neurons, the frequency (but not amplitude) of miniature IPSCs, and paired-pulse depression of IPSCs. These changes are paralleled ultrastructurally by a decrease in the number of vesicles docked at inhibitory synapses, indicating fewer vesicles available for release. A decrease in vesicles available for release then translates into decreased probability of GABA release. Importantly, changes in GABAergic IPSCs occur in the absence of any effect on EPSCs; this effect leads to a disinhibition of hippocampal neurons, making them more likely to fire an action potential in response to synaptic input. As one would predict from this scenario, susceptibility to hippocampus-dependent behavioral seizures induced by kainic acid (KA) is increased with 24 h of estradiol treatment.

With an even longer exposure, 48–72 h, estradiol again enhances excitatory synaptic transmission in the hippocampus, but through a different mechanism than the rapid effects. This time range corresponds to the peak of estradiol levels during the estrous cycle in the rat and to the greatest hippocampal seizure susceptibility observed by

Timiras' group in the 1960s. Multiple studies indicate that this delayed enhancement of excitatory synaptic transmission is related, at least in part, to a physical increase in the number of dendritic spines and excitatory synapses on hippocampal neurons. Dendritic spines are small protrusions that cover the dendrites of many neuron types in the brain, and are the postsynaptic sites of excitatory synaptic input. In 1990s, Gould, C. S. Woolley, and McEwen published a series of papers demonstrating that the density and number of dendritic spines and excitatory synapses on hippocampal CA1 pyramidal cells is increased ~30% by 72 h of estradiol exposure. Progesterone given after estradiol counteracts the effect and decreases dendritic spine density. Importantly, spine and synapse density also fluctuate naturally as estradiol and progesterone levels rise and fall during the estrous cycle. Although these early studies were done in rats, subsequent work by other groups has replicated these studies in primates (both African Green monkeys and Rhesus macaques). Thus, it is highly likely that estradiol produces similar structural synaptic plasticity in women.

The estradiol-induced increase in hippocampal dendritic spine/synapse number is paralleled by increased sensitivity to excitatory synaptic input, specifically input mediated by the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor. This enhanced sensitivity suggests that estradiol-induced synapses may be functionally different from preexisting synapses, which typically contain both NMDA and non-NMDA receptors. The greater sensitivity to NMDA receptor-mediated input is due to an increased contribution of receptors containing the NR2 subunit – a finding that has implications for LTP, since higher expression of NR2 is associated with greater LTP. Indeed, several groups have confirmed that LTP is enhanced by 48–72 h of estradiol treatment, both in vitro and in vivo. Similar to the rapid effect(s), longer exposure to estradiol also produces a situation in which excessive excitatory synaptic activity increases synaptic strength with a higher gain than in animals not exposed to estradiol. As predicted by the original Timiras studies, and consistent with its effects on hippocampal synaptic structure and function, estradiol treatment for 48–72 h increases susceptibility to behavioral seizures induced by KA.

However, it should be noted that not all studies that have investigated estradiol's effects on seizures have found that it is proconvulsant. As should be clear from the description above, the various durations of estradiol exposure produces various cellular changes that could underlie differences in seizure behavior. For example, although 72 h exposure to estradiol increases susceptibility to KA-induced seizures, longer treatment with estradiol may decrease susceptibility to seizures induced the same way. In addition, different methods of seizure induction, which engage different brain areas and/or cellular mechanisms, are likely to produce different results. For example, the same 72 h estradiol treatment that increases KA-induced seizure susceptibility has no effect on flurothyl-induced seizures. Similarly, estradiol promotes seizures induced by kindling in the dorsal hippocampus, but has no effect on kindling in the ventral hippocampus. Finally, it is important to distinguish between effects on seizure susceptibility, seizure severity, and neuroprotection from seizure-induced damage. Some investigators use the degree of seizure-related brain damage as a post hoc indication of seizure severity. However, because estradiol has neuroprotective effects irrespective of seizures, this indication can lead to confusing results.

In addition to the generally proconvulsant actions of estradiol described above, recent studies indicate that estradiol may have an anticonvulsant influence as well. This suggestion stems from the observation that estradiol can increase levels of the anticonvulsant neuropeptide, neuropeptide Y (NPY), in the hippocampus. NPY is thought to be released from hippocampal GABAergic neurons during intense seizure activity and it has been shown in many in vitro and in vivo studies to help terminate seizures. The consequences of estradiol's effects on hippocampal NPY for behavioral seizures are still somewhat controversial. Some groups report that with relatively long estradiol exposure (4 days) seizure susceptibility is reduced. Others find that shorter estradiol exposure decreases seizure severity through NPY. Thus, more work must be done to understand this NPY-mediated effect fully. And, as with estradiol's effects on the GABA and glutamate systems, understanding how estradiol/NPY interactions influence seizures will require careful attention to the duration of estradiol exposure, type of seizure analyzed, etc. Nevertheless, finding that estradiol can have a seizure mitigating effect suggests estradiol regulation of NPY as a promising avenue for development of novel antiseizure therapies.

Estradiol and Progesterone – Circuit Level

Estradiol's ability to increase the density/number of dendritic spines and synapses in the hippocampus not only increases excitatory drive onto hippocampal neurons, but also alters the pattern of hippocampal synaptic connectivity in a way that could promote seizures. Any useful understanding of how estradiol and progesterone influence seizures requires integration of their cellular level effects with their circuit level effects.

The effects of estradiol and progesterone at a circuit level involve changes in the relative proportions of different types of presynaptic axonal boutons. The majority of excitatory boutons in the hippocampus are in synaptic contact with one dendritic spine; these are termed single synapse boutons (SSBs). The remaining boutons form more than one synapse with more than one spine, and are termed multiple synapse boutons (MSBs). The

multiple spines in contact with an MSB may arise from the same postsynaptic neuron (forming a same-cell MSB), or from different postsynaptic neurons (forming a different-cell MSB). A series of detailed serial-section electron microscopic/3D reconstruction analyses of dendritic spines and axonal boutons in rats treated for 72 h with estradiol (compared with low estradiol controls) revealed that the new dendritic spines induced by estradiol form their synapses with preexisting boutons, transforming boutons that previously were SSBs into MSBs. Most importantly, the MSBs formed in this way are of the different-cell type. That is, the spines in contact with these MSBs arise from different postsynaptic cells. The effect of estradiol-induced dendritic spines to transform SSBs into different-cell MSBs is depicted in Figure 2.

The estradiol-induced transformation from few to many different-cell MSBs serves to increase the coupling of neighboring postsynaptic neurons through shared presynaptic input. Because different-cell MSBs are sites at which a single presynaptic input can simultaneously activate two or more postsynaptic cells, the increased proportion of different-cell MSBs is predicted to promote synchronous synaptically driven neuronal firing. Since seizures depend, in part, on synchronous firing, this structural plasticity is likely to be an important component of estradiol's proconvulsant effects. Furthermore, progesterone counteracts the spine/synapse-inducing effects of estradiol, so that progesterone (or its metabolites) would tend to return the pattern of hippocampal synaptic connectivity to an SSB-dominated state, decreasing synchronization of neuronal firing. Although still speculative, this hypothesis describes a mechanism that may be an additional source of progesterone's anticonvulsant effects.

Future Directions

Implications for Treatment Strategies

Understanding how hormones influence seizures has led to some therapeutic strategies that are being used now, and suggests other avenues for the future. One therapy being used now, though to a limited extent, is treatment with progesterone as a supplement to typical antiepileptic drug therapy. Several case histories in the literature indicate that this adjunctive treatment can be effective, particularly with a catamenial seizure pattern. The

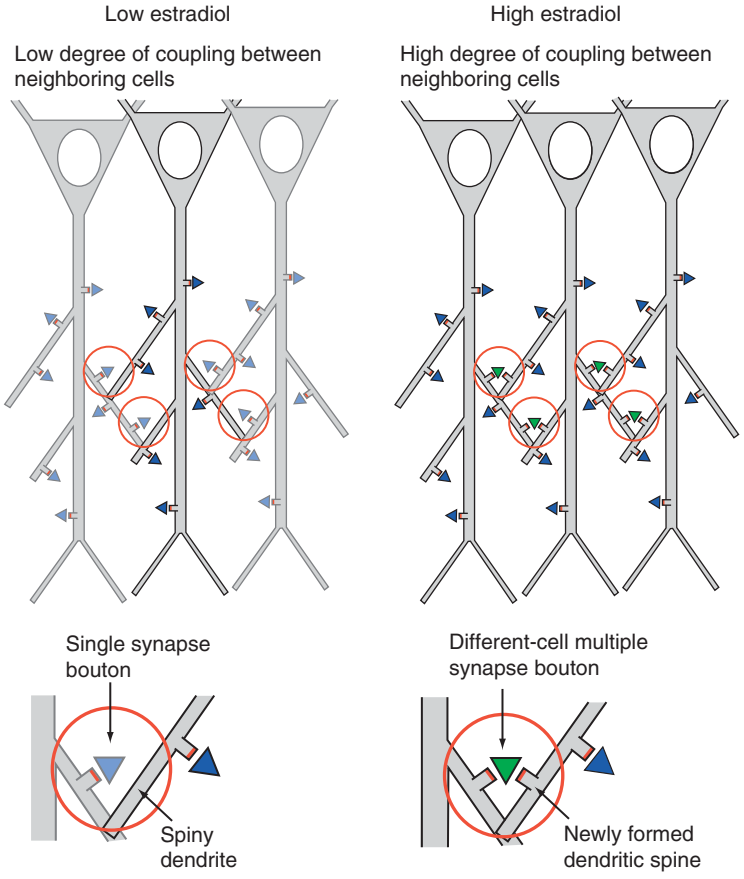


Figure 2 Schematic diagram of three hippocampal neurons showing how estradiol-induced dendritic spines transform single synapse boutons into different-cell multiple synapse boutons, increasing coupling between neighboring cells through shared presynaptic inputs. See text for details. Adapted from Yankova M, Hart SA, and Woolley CS (2001).

importance of progesterone metabolism in this treatment strategy is exemplified by the following case: A 34-year-old woman presented with intractable complex partial and secondary generalized seizures that increased in frequency and severity during the second half of each menstrual cycle. Hormone assays confirmed anovulatory cycles. The patient's seizures persisted despite trials with phenytoin, carbamazepine, valproate, and barbiturates. Adding progesterone to carbamazepine, however, reduced her seizure frequency from several per month to two per 6 months. Then, unbeknownst to her neurologist, a dermatologist prescribed finasteride for a different condition. Recall that finasteride inhibits conversion of progesterone to metabolites that positively modulate $GABA_A$ receptors. As predicted by animal studies, finasteride caused this patient's seizure frequency to increase dramatically (by 13-fold). As soon as her neurologist realized she was taking finasteride, it was discontinued, and her seizure frequency returned to two per 6 months. This case clearly demonstrates a therapeutic benefit of progesterone and, importantly, that its metabolism is essential to that benefit.

There are at least two avenues by which research on estradiol's effects on seizures may lead to new and better antiepilepsy therapies. One avenue is to study the molecular mechanisms by which cellular and circuit level effects of estradiol on the GABA and glutamate systems promote seizures. Greater understanding of the changes in seizure-prone circuits that lead to increased seizure occurrence, and particularly of how those changes come about, will point to ways of preventing those changes. A second avenue stems from the very recent discoveries that estradiol enhances the expression and/or actions of hippocampal NPY, which is anticonvulsant. With more study, knowledge of how estradiol regulates NPY could be used to target this anticonvulsant effect specifically, and avoid the proconvulsant effects of estradiol itself.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormonal Modulation of Neuronal Excitability; Hormones and Absence Epilepsy; Hormones and Seizures; Male Sex Steroids and Neuronal Excitability; Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; Sex and Seizure Sensitivity; Sex Differences in Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility; **Plasticity**: Dendritic Abnormalities in Epilepsy.

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Hormones and Seizures

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Introduction

It is widely accepted that nervous, immunological, and hormonal systems cooperate with each other. Furthermore, there is bidirectional interdependence in the relationship between hormones and seizures. That is, hormones affect seizure phenomena and seizures influence release of several hormones. Several types of seizures, for example in the course of temporal lobe epilepsy (TLE) or catamenial epilepsy, are positively correlated with the respective pattern of steroid hormones. Accurate knowledge about these relations may increase utility of hormones and their derivative neurosteroids in the treatment of epilepsy.

Background

Influence of Seizures on Hormone Release

A large number of studies of experimental seizures in rodents have revealed diverse hormonal changes. For instance, it is widely accepted that maximal electroshock, an animal model of generalized tonic–clonic convulsions, is associated with substantial and prolonged increases of thyrotrophin-releasing hormone (TRH) concentrations in such epilepsy-related brain regions as hippocampus, amygdala, piriform, and frontal cortices. Corticotrophin-releasing hormone (CRH), oxytocin (OXT), and vasopressin release is increased after kindled seizures (a model of complex partial seizures in humans), while significantly enhanced plasma concentrations of ACTH, glucagon, cortisol, vasopressin, and aldosterone are observed after pentylenetetrazole-evoked convulsions (an animal model of myoclonic epilepsy). Increased concentrations of OXT are also observed after kainate-induced convulsions.

An increased concentration of prolactin is a marker of generalized seizures, both in animals and humans, and may be viewed as part of an endogenous anticonvulsant system. Interestingly, this rise in prolactin is usually not seen after psychogenic seizures. Variability of baseline luteinizing hormone (LH) secretion, as well as in the frequency of transient hormonal 'pulses,' is observed in men with complex partial seizures. Acute seizures induce timing irregularity in LH secretion, whereas chronic epilepsy is associated with changes in LH pulse frequency and amplitude.

Increased concentrations of somatostatin (STS) may be found in the cerebrospinal fluid of epileptic patients and in children with febrile seizures. STS is considered to be a very early and long-term cerebrospinal seizure marker. There is a positive correlation between STS and CRH.

Influence of Hypothalamic Hormones on Seizures

Thyrotrophin-releasing hormone

In vitro studies have revealed that TRH inhibits pathological discharges in epilepsy-related brain structures. Acute treatment with TRH and its analogues (RGH 2202, DN-1417) results in an antiepileptic effect in several animal models of epilepsy, such as amygdala- or cortically-kindled seizures, maximal electroshock, audiogenic seizures, and glutamate-, kainate-, pentylenetetrazole-induced convulsions. A number of add-on human studies and case reports suggest an antiseizure effect of TRH in intractable epilepsies like infantile spasms, Lennox-Gastaut syndrome, myoclonic seizures, and other generalized and partial seizures. In Lennox-Gastaut syndrome, the hormone may benefit atypical absence seizures and electroconvulsive therapy-induced seizures more than tonic seizures. Undesired therapy side-effects during treatment for infantile spasms are significantly less in patients receiving TRH as compared with those treated with ACTH. The anticonvulsant action of TRH is probably mediated by two metabotropic receptors (TRH-R1, TRH-R2) activating phospholipase C. It has been hypothesized that hippocampal TRH inhibits seizure propagation, whereas striatal TRH reduces seizure susceptibility. The antiseizure action of TRH may be also due to attenuation of cholinergic and glutamatergic neurotransmission and simultaneous enhancement of the monoaminergic (noradrenaline, dopamine, serotonin) system. Decreased brain glutamatergic activity may result from decreased release of glutamate and aspartate into the synaptic cleft, as well as increased synthesis of kynurenine. Both processes are mediated by TRH.

Corticotrophin-releasing hormone

In the majority of neuronal circuits, CRH functions as an excitatory modulator. Amygdala and hippocampus, parts of the limbic system, are particularly rich in CRH receptors. Elevated brain concentrations of CRH may induce phenotypic 'limbic seizures,' resembling those produced by kainic acid or rapid amygdala-kindling, and eliminated by the administration of a selective CRH antagonist.

In vitro studies demonstrate that CRH amplifies excitatory stimuli in the network, and triggers neurodegeneration

process in areas involved in learning and memory. In animal studies, CRH augments the action of some proconvulsant substances like kainic acid. The hormone exacerbates, among others, cocaine-induced convulsions, as well as Shigella dysenteriae exposure-evoked seizures and lethality. Intracerebroventricular administration of CRH, at doses not affecting the plasma corticosterone concentrations, induces seizures in rodents. This demonstration excludes the possibility of glucocorticoid involvement in the initiation of CRH-induced seizures. The proconvulsant effect of CRH seems also to be independent of glutamatergic neurotransmission.

Proposed mechanisms of CRH action are widely attributed to two G-protein-coupled receptors for CRH (CRF1 and CRF2). Excitatory effects of CRH are mediated through activation of CRF1 receptors, leading to increased activity of GABA transporter 1 (GAT-1) in several strategic brain areas, including hippocampus, neocortex, piriform cortex, prepiriform cortex, and amygdala. Therapeutic effects of adrenocorticotrophin hormone (ACTH) and glucocorticoids in infantile spasms have been hypothesized to be because of a negative feedback system leading to suppressed CRH secretion.

Influence of Pituitary Hormones on the Course of Seizures

Adrenocorticotrophin

Both pro- and anticonvulsant action of ACTH has been observed in vitro. ACTH treatment reduces seizure susceptibility in immature rat brain slices and has no long-term deleterious effects on neuronal growth. In contrast, a proconvulsant effect of the hormone has been shown in neurons of adult rats.

In rodents, ACTH is entirely ineffective against the pentylenetetrazole-induced seizures or maximal electroshock. However, both acute and chronic treatment with ACTH slows the development of amygdala-kindled seizures. In contrast, proconvulsant effects of the hormone have been observed in seizures kindled from hippocampus.

The anticonvulsant effect of ACTH in humans is age dependent. The hormone is effective in children, but not in adults. ACTH has been successfully used for treating children with West or Lennox-Gastaut syndrome. Usefulness of adrenocorticotrophin for generalized seizures other than infantile spasms is limited by its transient efficacy. ACTH treatment is occasionally associated with serious adverse effects, including immunosuppression, body weight gain, and hypertension.

Most ACTH effects on the central nervous system have been attributed to increased glucocorticoid synthesis. However, ACTH also reduces CRH expression in amygdala neurons, which may be responsible for its anticonvulsant effect.

Gonadotrophin releasing hormone

Complex partial seizures may disrupt hypothalamic regulation of pituitary secretion in animals and in humans. There is a strong predominance of left-sided discharges in women with hypergonadotropic hypogonadism and right-sided discharges in those with hypogonadotropic hypogonadism. Gonadotrophin releasing hormone agonists and analogues (e.g., leuprolide acetate) may suppress catamenial seizures, and trigger atypical absence seizures in pediatric patients with diffuse brain damage.

Arginine vasopressin

Data on the influence of arginine vasopressin (AVP) on seizure phenomena are contradictory. The AVP-induced increment of ACTH release may contribute to a net anticonvulsant action. However, both intracerebroventricular and systemic administration of AVP can induce behavioral seizures in rodents. AVP also decreases the threshold of pentylenetetrazole-induced convulsions. However, the effect of AVP on the pilocarpine-induced seizures is far from clear; some reports show a proconvulsant action of AVP on pilocarpine-induced seizures, while other studies do not confirm such observations. AVP, through the activation of the V_2 receptors, may also be involved in the pathogenesis of febrile convulsions.

Oxytocin

OXT can influence seizure phenomena through its ability to stimulate ACTH release.

Somatostatin

Pharmacological studies show that STS affects electrophysiological properties of neurons, modulates classical neurotransmission, and has anticonvulsant activity in experimental models of seizures. The protective action of STS in hippocampus may depend on the activity of the inhibitory GABAergic neurons and STS_2 receptor activation, leading to the increment of hippocampal extracellular dopamine and serotonin concentrations. Activation of STS_2 receptors may play a role in the control of partial complex seizures and, therefore, it may be useful as a therapeutic target for TLE. STS may also exhibit antiepileptogenic effects in animal models of partial complex seizures. STS gene knockout animals have been shown to have a shorter average latency to experimental seizures of different types, higher average seizure score, and longer afterdischarge durations.

Influence of Thyroid Hormones on Seizures

On the level of cell membrane, thyroid hormones increase oxygen consumption and Na^+/K^+-ATPase activity (which controls the intracellular Na^+/K^+ ratio). In vitro, accumulation of triiodothyronine increases delayed excitability in several brain areas, for instance in the hippocampus and

visual cortex. In experimental studies, acute exposure to triiodothyronine has no effect on experimentally induced epileptiform activity; however, increased susceptibility to some convulsants (e.g., isoniazide, picrotoxin) has been observed after repeated administration of triiodothyronine. Thyroxin significantly lowers the seizure threshold in several animal models, including electroconvulsions and audiogenic seizures. Both pro- and anticonvulsant action of the hormone has been reported in lidocaine-induced convulsions. In clinical conditions, there have been two case reports of single tonic–clonic convulsions in a child after taking an overdose of levothyroxine.

Influence of Parathormone on Seizures

Hypocalcemia due to hypoparathyroidism may result in intractable seizures of nonepileptic origin, but there are no available data on the effect of parathormone on epileptic seizures.

Influence of Insulin on Seizures

In vivo, hypoglycemia is frequently associated with seizures. Nitric oxide may play a protective role in acute hypoglycemia-induced seizures. However, partial motor seizures are also common in severe nonketotic hyperglycemia. In vitro, decreases in extracellular glucose do not induce epileptiform discharges, and even attenuate preexisting low magnesium-induced epileptiform activity in the enthorinal cortex and hippocampal CA1 region.

Under normoglycemic conditions, insulin may directly inhibit the neural firing rate, increase resting membrane potential, and activate membrane ion transport. In normoglycemic animals (with additional glucose supplementation), insulin decreases incidence, intensity, and mortality rate of a variety of chemically-induced convulsions. It has been hypothesized that the antiseizure action of insulin may be realized via activation of the brain Na^+/K^+-ATPase.

Influence of Adrenal Hormones on Seizures

Glucocorticoids

Glucocorticoids can increase neuronal excitability and facilitate seizure development of kindled seizures. Moreover, when applied at supratherapeutic doses, glucocorticoids may aggravate electrically- and chemically-induced convulsions.

In human studies, ACTH-evoked hypercortisolemia is not associated with any interictal EEG changes, and cortisol therapy may be useful in the treatment of refractory tonic–clonic convulsions. In contrast, chronic hypercortisolemia, as a result of stress, anxiety, and depression, may facilitate the development and progression of epilepsy.

Corticosterone at physiologically relevant concentrations can aggravate absence seizures, possibly via a temporary increase of excitatory neurotransmitters. Simultaneously, the hormone also enhances the proconvulsant action of excitatory amino acids. Chronic exposure to high corticosterone concentrations accelerates experimental convulsions and increases spike amplitude in the CA1 hippocampal area. Corticosteroid receptors can modulate expression of some experimental seizures.

Deoxycorticosterone, an agonist of mineralocorticoid receptors and an antagonist of glucocorticoid receptors, plays a role in stress-related changes in seizure control. The hormone is metabolized by 5α-reductase to the inhibitory neurosteroid, allodeoxycorticosterone, a positive modulator of $GABA_A$ receptor. Deoxycorticosterone may exhibit anticonvulsant action against chemically- and electrically-induced convulsions.

Mineralocorticoids

Aldosterone, a selective agonist of mineralocorticoid receptors, may be anticonvulsant in developing rats, although they are rather proconvulsant in adult animals. In young rats, aldosterone has been shown to inhibit minimal and maximal pentylenetetrazole-induced convulsions, but appears to be ineffective against maximal electroshock- and hippocampal-kindled seizures.

Adrenal androgens

Dehydroepiandrosterone sulfate was reported to have both $GABA_A$-antagonistic and NMDA-agonistic properties. However, the net action of the hormone in experimental seizures is anticonvulsant.

Influence of Gonadal Hormones on Seizures

Estradiol/progesterone

Clinical and animal data show that estrogens may be ineffective or present both proconvulsant and anticonvulsant effects. The action of estrogens depends on various factors, such as the dose, treatment duration, latency prior to the seizure testing, hormonal status, the region/neurotransmitter system involved, the seizure type/model, and sex of subjects. Estradiol at physiological concentrations decreases the threshold, increases seizure activity, may trigger status epilepticus, and accelerates kindled seizures in female animals. However, the opposite (anticonvulsant) effects are observed after chronic treatment with estrogen. It has been hypothesized that estrogens may confer anticonvulsant and neuroprotective effects by enhancing formation of progesterone and allopregnanolone. According to another hypothesis, estrogens can be proconvulsant in adult animals, but ineffective in immature animals. It is interesting that in ovariectomized female rats, both estradiol and progesterone prolong seizure duration. In gonadectomized male rats, estrogen may have either

proconvulsant or anticonvulsant effects, depending on the dose of the chemoconvulsant used to induce seizures. Generally, hormonal changes during puberty, menopause, or hormonal contraception do not influence seizure frequency. However, estrogen may trigger catamenial seizures and contribute to pregnancy-related changes in seizures. Mid-cycle exacerbation of seizure activity is usually attributed to the preovulatory surge of estrogen that is unaccompanied by a significant rise in progesterone.

Progesterone is transformed by 5α-reductase to the neurosteroid, allopregnanolone, a potent positive modulator of $GABA_A$ receptors. Moreover, the hormone may potentiate the effect of endogenous anticonvulsant adenosine. In vitro studies have shown that the action of progesterone may depend on brain region; the hormone inhibits the development of epileptiform activity in the enthorinal cortex, but accelerated it in the CA1 hippocampal region.

In animal models, progesterone at physiologically relevant concentrations inhibits seizure susceptibility in various models of epilepsy, including focal cortical spikes, kindled and audiogenic seizures, as well as convulsions induced by kainic acid and pentylenetetrazole. Progesterone increases the threshold and reduces the magnitude of seizure activity in female, male, and gonadectomized animals. Withdrawal from chronic therapy with progesterone has proconvulsant effects. The hormone can also slow both amygdala and hippocampal seizure development, thus demonstrating antiepileptogenic activity.

In women, progesterone concentrations are significantly lower in patients with catamenial epilepsy. Premenstrual exacerbation of seizures is often attributed to withdrawal of the antiseizure effects of progesterone. In fact, progesterone administration can suppress epileptiform spikes in epileptic patients. In contrast to generalized seizures, progesterone exacerbates absence seizures in humans.

Androgens

Effects of testosterone on seizures depend on the metabolic route in which this androgen is involved. Testosterone is metabolized by 3α-reductase to 3α-androstanediol, a positive modulator of $GABA_A$ receptors. Aromatase is the enzyme for the conversion of testosterone to 17β-estradiol, a hormone with proconvulsant properties mediated via glutamate NMDA receptors. In animals, the proconvulsant action of both acute and chronic treatment with testosterone has been shown in the pentylenetetrazole-induced and electrically-kindled seizures models. It is interesting that this effect may be accompanied by increased plasma concentrations of 17β-estradiol. Also, androstanediol and dihydrotestosterone have proved to exhibit anticonvulsant and neuroprotective activity in chemically-induced convulsions in rodents. It worth mentioning that gonadectomy increases susceptibility of male rats to generalized tonic–clonic convulsions.

In humans, testosterone administration reduces seizures in the course of posttraumatic epilepsy. Furthermore, male epileptic patients usually have higher levels of dihydrotestosterone than do men without seizure disorders.

Methods

In our laboratory, we have studied the influence of steroid hormones on the basic experimental models of epilepsy: maximal electroshock- and pentylenetetrazole-induced convulsions in mice, as well as amygdala-kindled seizures in rats. The three models are required screening tests for potential anticonvulsant drugs. Maximal electroshock reflects generalized tonic–clonic seizures, pentylenetetrazole-evoked convulsions are an equivalent of myoclonic epilepsy, while amygdala-kindled seizures resemble complex partial seizures in humans.

Electroconvulsions are produced by an alternating current (50 Hz, 0.2 s, fixed current intensity of 25 mA, and maximum stimulation voltage of 500 V) delivered via ear-clip electrodes. Tonic hindlimb extension (the hind limbs of animals outstretched 180° to the plain of the body axis) is taken as the endpoint. Results are expressed as CS_{50} values for the hormones tested, where CS_{50} is current strength (in mA) necessary to induce tonic convulsions in 50% of animals.

In the pentylenetetrazole-induced convulsions model, the chemoconvulsant is injected at its CD_{50} dose, defined as a dose producing clonic convulsions in 50% of tested animals. Subsequently, the influence of steroid hormones on the CD_{50} value of pentylenetetrazole is determined and interpreted as an effect on the seizure threshold.

Kindled seizures are evoked electrically from basolateral amygdala. Each stimulus consists of a 1-s train of 50 Hz, 1 ms biphasic square-wave pulses, with pulse amplitude of 500 μA, delivered every 24 h. Stimuli are delivered until at least 10 sequential fully kindled seizures are generated. At that point, four parameters are measured: (1) the seizure threshold, (2) seizure severity, assessed according to a modified Racine's system, (3) duration of limbic seizures (stage 1–2) and motor seizures (stage 3–5), and (4) EEG afterdischarge duration defined as spikes with a frequency of at least 1 Hz and amplitude at least twice greater than the prestimulation baseline. The after-discharges from the amygdala are recorded prior to and after the stimulation. After-discharge threshold (ADT) is determined after administration of a test drug (or vehicle control) by administering a series of stimulations at intervals of 3 min, increasing stimulus amplitude in steps of about 20% over the previously applied current until an after-discharge (duration of at least 3 s) is evoked. At this ADT current, seizure severity, seizure duration, and after-discharge duration are

recorded and the data analyzed. Control readings are made 2 days before and 2 days after respective treatments.

Results

Steroid hormones administered intraperitoneally, but only at supratherapeutic doses, affect seizures in all three animal models of epilepsy. The revealed relationship is markedly dose-dependent. Testosterone (5–7.5 mg kg^{-1}) and progesterone (4.5–6 mg kg^{-1}) significantly increase the electroconvulsive and pentylenetetrazole threshold in mice. In contrast, estradiol (1.125–3.375 mg kg^{-1}) decreases the threshold of pentylenetetrazole-induced convulsions, but does not influence the electroconvulsive threshold. In amygdala-kindled rats, estradiol (3.375–4.5 mg kg^{-1}) decreases the ADT and prolongs the seizure duration. Seizure duration is shortened by progesterone and testosterone, but prolonged by estradiol. Progesterone (6–12 mg kg^{-1}) significantly decreases seizure severity and shortens the seizure and after-discharge durations. However, testosterone (5–10 mg kg^{-1}) does not affect any seizure parameter in this model. Cortisol (hydrocortisone; 25–50 mg kg^{-1}) decreases the threshold in pentylenetetrazole- and maximal electroshock-induced convulsions, but is ineffective in the amygdala-kindled seizures model. Finally, aldosterone (1–10 mg kg^{-1}) does not seem to affect seizures in animal models used in our studies.

These results support the hypothesis that among all steroid hormones, the gonadal hormones exert the most powerful influence on seizure phenomena. Estrogens are generally proconvulsant, while progestins and androgens present anticonvulsant activity.

Future Goals

A major challenge for this area of research is to determine accurate hormonal influences – i.e., at physiological, therapeutic, and supratherapeutic plasma concentrations – on seizure generation and propagation in primates, including humans. Exceptionally important in such an analysis is a focus on the synthesis of hormone derivatives that preserve, or even exceed, the anticonvulsant activity of their precursors. Ideally, such derivatives would not exhibit hormonal actions, the most frequently undesirable side-effects in hormonal treatments of epilepsy. To date, only few synthetic inhibitory neurosteroids meet these criteria – for instance, ganaxolon, which is presently in phase III clinical trials.

See also: **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Absence Epilepsy; Hormones and Epilepsy; Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; Sex and Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility.

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Male Sex Steroids and Neuronal Excitability

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Introduction

Gonadal steroid hormones have profound effects on the growth and differentiation of the central nervous system. Male-typical steroids, androgens, which are secreted from the testes into the circulatory system, have long been understood to maintain reproductive behavior. Emerging evidence indicates that androgens also exert trophic effects on the brain, which may influence the processing, storage, and retrieval of information, and may underlie the positive effects of androgens on cognitive performance and/or mood state. Changes in androgens may also influence the incidence and/or expression of certain types of neurodegenerative disorders, such as Alzheimer's disease, which are characterized by reduction in androgen levels. Seizure disorder is the most common neurological condition among children, and for adults only Alzheimer's disease and stroke are more prevalent. As such, the role of androgens in epilepsy is of great interest.

Research from our laboratory focuses on the effects and mechanisms by which steroids mediate variations in social, cognitive, and affective behavior in both normative and pathophysiological conditions. To examine neuroprotective effects and mechanisms of androgens, we have primarily used animal models of seizure disorders. Our findings to date suggest that androgens have robust antiseizure effects in a number of different animal models using chemoconvulsants. Seizures and/or antiepileptic drugs (AEDs) can also alter sexual, cognitive, or anxiety behaviors, as well as bioavailability of androgens. As such, whether androgen deficiencies in epilepsy contribute to, or are a consequence of, some seizure disorders and/or its treatments is of great interest.

One of the challenges to understanding androgens is the broad spectrum of activity they influence. Although a comprehensive discussion of androgens' mechanisms is beyond the scope of this article, it is generally accepted that androgens exert their effects through 'genomic' actions at intracellular androgen receptors (ARs) to modulate gene expression. Testosterone, the principal androgen, and its metabolite 5-dihydrotestosterone (DHT) produce some of their effects through binding to intracellular ARs, members of the nuclear receptor superfamily. ARs, and other nuclear steroid receptors, act as ligand-inducible transcription factors to modulate target genes. These actions via ARs involve protein synthesis and take hours or days to occur. However, there are also rapid effects of androgens, which occur too quickly to be explained by the classical AR mechanisms. These rapid androgen effects occur within seconds to minutes, significantly faster than androgen-enhanced protein synthesis. These 'nongenomic' effects of androgens can occur when androgens act at cell membranes or in systems lacking ARs. Further, they are not abolished by inhibition of transcription or translation. Nongenomic actions of hormones are now generally accepted to contribute to the physiological effects of steroids. Given that rapid actions of androgens may ultimately have downstream effects to modulate transcriptional activity of ARs or other factors, the term 'nonclassical,' rather than nongenomic, has been used more recently to describe these effects. This review summarizes some of the effects and mechanisms by which androgens, seizures, and AEDs influence reproductive, cognitive, or affective behaviors.

Background

Androgens and Reproductive Function

Testosterone, the primary androgen secreted by the testes, probably mediates male sexual development, maturation, and maintenance of male reproductive organs through both genomic and nonclassical actions. One well-investigated, androgen-mediated reproductive function is for testosterone to regulate spermatogenesis through its actions on Sertoli cells in seminiferous tubules. Using an organotypic approach to investigate testosterones effects on Sertoli cells, investigators have revealed novel actions of testosterone. Application of free testosterone or testosterone conjugated to bovine serum albumin (T-BSA), which does not permeate cell membranes, produces rapid and prolonged increases in intracellular Ca^{2+} inhibitors, indicating a plasma membrane action of testosterone. Testosterones membrane effects on Sertoli cells may involve rapid activation of Gq protein-mediated phospholipase C signaling to inhibit ATP-sensitive K^+ current to produce membrane depolarization, which in turn activates L-type Ca^{2+} channels and thereby Ca^{2+} influx. These increases in Ca^{2+} may influence cytoskeleton and/or cell–cell junctions to regulate metamorphosis, gene transcription, and/or secretions in Sertoli cells. In addition to these nonclassic actions of testosterone, AR-mediated actions of testosterone may also be involved in this system. For example, testosterone rapidly regulates transcription factors, such as cAMP response element binding protein (CREB), via mitogen-activated

protein kinase (MAPK) and ERK1/2 signaling pathways in Sertoli cells. However, the rapid effects of testosterone to induce ERK and CREB phosphorylation are not seen in testicular feminized (tfm) rats, which lack functional ARs. Furthermore, these effects are blocked by AR antagonists or interference RNAs. These insights from this system clearly demonstrate that nonclassical actions of testosterone on Sertoli cells may also involve ARs; whether these mechanisms are relevant for the etiology or the therapeutic treatment of androgen-relevant dysfunctions is of interest.

The neural control of reproductive behavior in laboratory rodents involves both genomic and nonclassical actions of androgens in an area of the hypothalamus referred to as the medial preoptic area (MPOA). Stimulation of the MPOA can elicit sexual behavior, and ablation can abolish reproductive behavior. There are many ARs in the MPOA. Castration inhibits male sexual behavior, but application of testosterone or T-BSA to the MPOA is sufficient to reinstate reproductive responses. Also, application of testosterone to brain regions such as the central tegmental field or medial amygdala (that project to the MPOA) can reinstate androgen-mediated sexual behavior. The precise downstream substrates through which androgens mediate reproductive behavior remain to be elucidated, but likely targets to be investigated are those identified above for peripheral actions of androgens.

Androgens and the Hippocampus

Androgens can also have profound effects on nonreproductive behaviors that are mediated in part through nonclassic actions in the hippocampus. The hippocampus is an important site of androgen action. Tritiated testosterone is taken up in the hippocampus of rats. Excitability of hippocampal tissue of male mice is altered following castration and in vivo or in vitro androgen administration restores it to levels of intact males. Similarly, castration of rats or vervet monkeys reduces the density of synaptic contacts on dendritic spines of CA1 pyramidal neurons. Administration of androgens that are potent modulators of ARs, such as testosterone or the nonaromatizable androgen DHT, and androgens that are only weakly active at ARs similarly reverse the effect of castration. These effects of androgens are not blocked by coadministration of an AR antagonist. The androgen-stimulated increase of hippocampal spine synapse density does not require ARs. Interestingly, the effects of androgens to enhance cognitive function and mood state, processes that are mediated in part by the hippocampus, also do not seem to require actions through ARs. Thus, it has been proposed that androgens' mnemonic and/or antianxiety effects may result, at least in part, from androgens' hippocampal neurotrophic responses.

Actions of androgens on the hippocampus are of particular interest because maintenance of androgen levels in this region may minimize the risks of neurodegeneration. Patients with Alzheimer's disease, characterized by damage in the hippocampus, have demonstrated a reduction in circulating concentrations of androgens in comparison to normal controls. Androgens also have protective effects in other models of neurodegeneration. Androgen administration reduces cell death in the granule cell layer of the hippocampus of male rats following adrenalectomy. Notably, the decrease in cell death following androgen administration is associated with functional effects as well. Androgen-administered rats that had less cell death in the hippocampus performed better in a hippocampally mediated memory task than did their vehicle-administered counterparts with more cell death. Thus, the hippocampus, a brain area involved frequently in partial and predominantly in generalized seizures, may be an important target of androgens' antiseizure actions.

Androgens and Seizures

Evidence from clinical and basic research studies also suggests that androgens can have antiseizure effects. In support of this view, alcohol withdrawal-induced seizures are less likely in men who have higher endogenous levels of testosterone. Androgens also reduce trauma-induced or epileptic seizures among men. In animal models, male (compared with female) rodents are less susceptible to allylglycine-, flumazenil-, or picrotoxin-induced seizures. There are developmental changes in seizure susceptibility which are related to androgen secretion. Intact, adult male rats, with high endogenous androgen levels, have fewer seizures than do either intact juvenile (30 days old) or middle-aged (13 months old) male rats, both of which have lower endogenous androgen levels. In adult rats, castration increases ictal activity and abrogates sex differences in seizure susceptibility, but androgen administration ameliorates the enhanced sensitivity to chemoconvulsants caused by castration.

AR-Mediated Antiseizure Effects

Temporal lobe epilepsy, one of the most prevalent classes of epilepsy, also involves the hippocampus. Actions of androgens in this region are antiepileptic. Administration of androgens directly to the hippocampus of castrated rats reduces pentylenetetrazol (PTZ)-induced seizures. The antiseizure effects of androgens delivered directly into the hippocampus are similar to the effects of systemic testosterone administration (**Figure 1**, top).

Notably, blocking androgen's actions in this region by administering flutamide, an AR antagonist, has proconvulsant effects; this effect can be produced with androgen injection either systemically or directly to the hippocampus of gonadally-intact male rats (**Figure 1**, bottom). Lifelong deletion of ARs, as occurs among tfm mice, is also

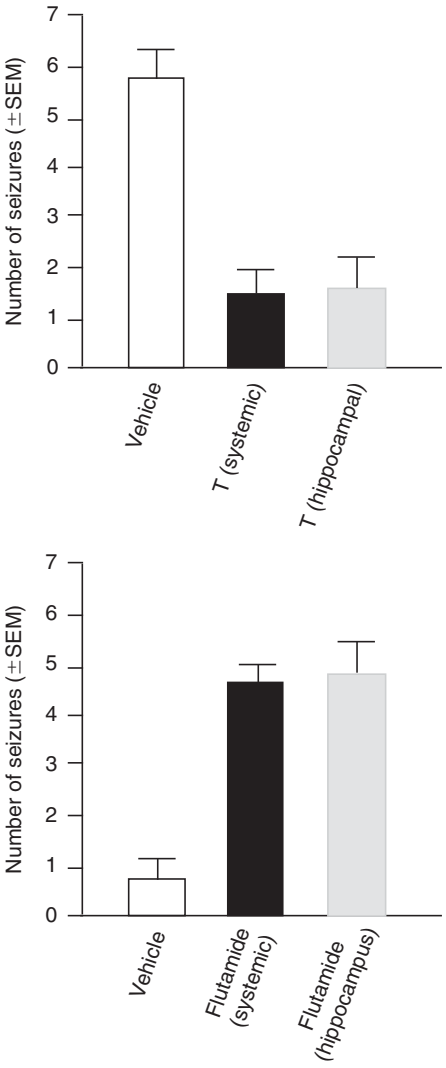


Figure 1 Top panel represents average (±SEM) seizure incidence of gonadectomized rats administered vehicle (white bar), systemic testosterone (black bar), or intrahippocampal testosterone (gray bar). Bottom panel represents average (±SEM) seizure incidence of intact rats administered vehicle (white bar), systemic flutamide (black bar), or intrahippocampal flutamide (gray bar).

deleterious. Tfm mice do not demonstrate the antiseizure effects of androgens that are produced in their wildtype counterparts when androgen is administered (**Figure 2**).

Thus, some antiseizure effects of androgens may occur through actions at ARs.

AR-Independent Antiseizure Effects

In addition to AR-mediated antiseizure effects, nonclassical actions of androgens may also underlie some anticonvulsant effects of androgens. Indeed, a large body of research suggests that some of testosterones antiseizure effects may be due, in part, to its metabolism to DHT, and

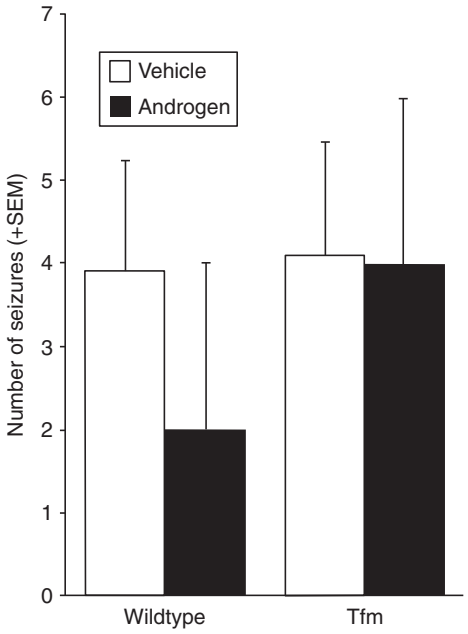


Figure 2 Average (±SEM) seizure incidence of gonadectomized wildtype (left) or tfm (right) mice administered vehicle (white bars) or systemic testosterone (black bars).

its subsequent conversion to 3α-androstanediol (3α-diol) by 3α-hydroxysteroid dehydrogenase. In support, testosterone, DHT, and 3α-diol all similarly reduce seizures in male rodents. Blocking formation of 3α-diol from DHT or testosterone, either systemically or in the hippocampus, attenuates antiseizure effects (**Figure 3**, top).

Administration of testosterone to mice that are genetically unable to metabolize testosterone to 3α-diol is not effective at reducing seizures. Further, concentrations of 3α-diol, but not testosterone, are reduced among aged male rats that have more seizures compared to their younger counterparts. These data, together with those presented earlier, suggest that androgens have antiseizure effects and that these effects are due, at least in part, to actions of testosterones metabolite, 3α-diol.

Testosterones and its metabolites, DHT and 3α-diol, may have multiple mechanisms in the hippocampus for antiseizure effects. Notably, testosterone and DHT bind with a very high affinity for ARs; however, at physiological concentrations, 3α-diol does not bind ARs. Instead, 3α-diol typically has its actions through membrane ionotropic neurotransmitter receptors, such as $GABA_A$ and *N*-methyl-D-aspartate (NMDA) receptors. Both $GABA_A$ and NMDA receptors occur at high levels in the hippocampus. Blocking $GABA_A$ receptors in the hippocampus attenuates antiseizure effects of 3α-diol (**Figure 3**, bottom). Blocking NMDA receptors also inhibits the anticonvulsant effects of androgens. Thus, antiseizure effects of testosterone metabolites, such as 3α-diol, may involve nonclassical actions of androgens in the hippocampus.

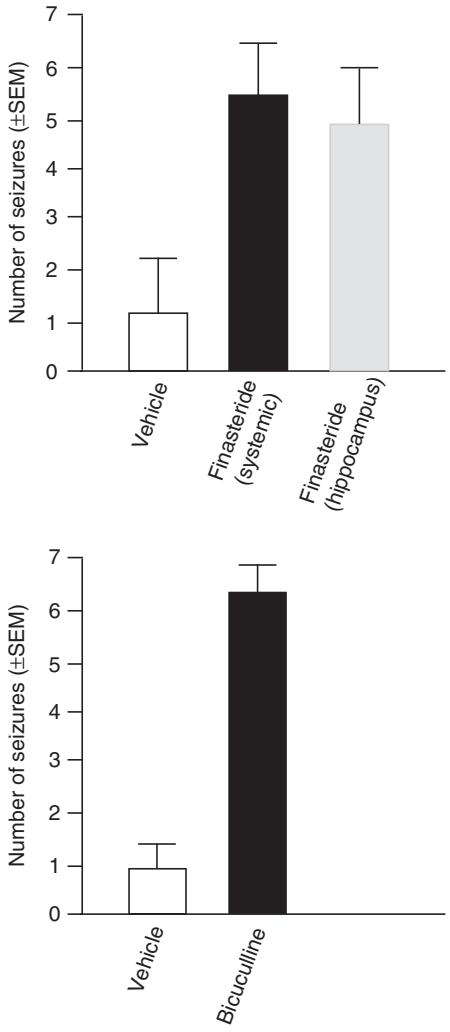


Figure 3 Top panel represents average (±SEM) seizure incidence of intact rats administered vehicle (white bar), systemic finasteride (black bar), or intrahippocampal finasteride (gray bar). Bottom panel represents average (±SEM) seizure incidence of intact rats administered vehicle (white bar) or bicuculline (black bar).

Epilepsy and Reproductive Function

People with seizure disorders, particularly those with temporal lobe epilepsy, have an increased likelihood of reproductive dysfunction relative to the general population. Sexual dysfunctions, such as reduced potency or fertility, erectile dysfunction, hyposexuality, impotency, and reduced libido, are more prevalent among men with epilepsy. These effects may be due in some cases to hypogonadotropic hypogonadism, which is associated with reductions in the secretion of gonadal hormones because of dampened feedback from the hypothalamus and/or pituitary. However, neuroendocrine dysfunction could occur at multiple levels of the hypothalamic–pituitary gonadal axis.

Some AEDs may influence the neuroendocrine system and thereby alter the production, secretion, and bioavailability of steroid hormones. Some AEDs, such as phenytoin, carbamazapine, and barbiturates, can induce cyctochrome P450 enzymes; this action can enhance production of sex hormone-binding globulin and increase the metabolism of androgens, thereby reducing bioavailable androgens. In contrast, valproate inhibits steroid hormone metabolism, and can thereby increase androgen levels (due to reduced hepatic metabolism) and produce associated sexual dysfunction. Comparison of newer and classical AEDs, with respect to their effects on sexual function, suggests promising advantages of the newer generation of drugs. Gabapentin is associated with reversible sexual dysfunction. Men with epilepsy using lamotrigine had sexual function and androgen levels that were comparable to those of untreated males and control men without epilepsy.

Methods

The majority of the earlier data from our laboratory was collected using the PTZ model of generalized seizures. The seizure progression produced with our PTZ regimen ($70\,mg\,kg^{-1}$ for rats, $85\,mg\,kg^{-1}$ for mice) is consistent, and reliably observed and quantified, using a modification of the scale devised by Racine (mouth and facial movements, head nodding, forelimb clonus, rearing, and rearing and falling). Further, we observe similar effects of androgens on each stage of seizure progression (myoclonus, tonic clonic seizures, and survival). As evidenced by the studies described earlier, this PTZ protocol allowed us to characterize androgens' effects and mechanisms with respect to seizures. To address the acute effects of seizures on androgen-mediated behavior, the chronic effects of epilepsy on these behaviors, and the use of AEDs on androgen-mediated behavior, we have recently employed a chronic pilocarpine model of temporal lobe epilepsy. Brain damage associated with pilocarpine-induced status epilepticus involves the hippocampus and amygdala, areas that are typically damaged in patients with temporal lobe epilepsy. As discussed earlier, there is some evidence that seizures can alter cognitive, affective, reproductive, and neuroendocrine processes. Hence, we have begun to extend our prior studies by using this model to examine effects of chronic seizures on these endpoints. In this pilocarpine model, rats are administered pilocarpine ($60\,mg\,kg^{-1}$, i.p.) on postnatal day 10 and observed for the occurrence of status epilepticus. Rats are then allowed to grow-up and are tested for affective behavior and reproductive function. After completing these behavioral tests, rats are readministered pilocarpine ($100\,mg\,kg^{-1}$) and scored for seizure severity. In the subsequent weeks, differences in androgen-dependent behaviors and hormones levels are examined.

Table 1 Reproductive, cognitive, and affective behaviors and 3α-diol levels of control rats and rats exposed to pilocarpine-induced seizures perinatally, in adulthood, or both

	Control	*Perinatal seizures*	*Adult seizures*	*Both*
Number of sexual contacts	15 ± 1	9 ± 1	12 ± 1	7 ± 1
Latency to cross-over	298 ± 15	222 ± 33	252 ± 21	214 ± 34
Central square entries	28 ± 8	15 ± 3	10 ± 2	6 ± 2
Hypothalamic 3α-diol (ng g^{-1})	3.9 ± 0.1	2.6 ± 0.2	3.1 ± 0.3	2.3 ± 0.2
Hippocampal 3α-diol (ng g^{-1})	4.0 ± 0.2	2.1 ± 0.4	2.5 ± 0.4	2.3 ± 0.2

Recent Results

Hypothalamic- and hippocampally mediated androgen-dependent behaviors are altered by pilocarpine when administered perinatally or in adulthood. Pilocarpine-induced seizures produce deficits in reproductive, cognitive, and affective function. Rats that experienced pilocarpine-induced seizures, either early in development or during adulthood, engaged in significantly fewer sexual contacts than did rats that did not have seizures (Table 1). Rats exposed to pilocarpine, either perinatally or in adulthood or at both times, had shorter latencies to shock-avoidance (indicating poorer cognitive performance) and entered fewer central squares in the open field (more anxiety behavior) than did rats that were never exposed to pilocarpine (Table 1). Levels of 3α-diol, but neither testosterone nor DHT, in the hypothalamus and hippocampus, were reduced among pilocarpine-treated rats compared to controls (Table 1). These data suggest that pilocarpine-induced seizures can produce decrements in androgen-mediated social, cognitive, and affective behavior that may be due, in part, to reductions in 3α-diol in the hypothalamus and hippocampus.

We have begun to investigate effects of different classes of AEDs on reproductive, cognitive, affective behaviors, as well as androgen levels of male rats. Adult intact male rats were administered valproate (325 mg kg^{-1}, i.p., b.i.d.) (a nonenzyme-inducing AED), phenytoin (50 mg kg^{-1}, i.p., b.i.d.) (an enzyme-inducing AED), or vehicle (saline, b.i.d.) for 30 days. On the last day of drug administration, rats were tested for sexual behavior (with a stimulus female), anxiety (open field), or cognitive behavior (object recognition), and observed for ictal activity. Phenytoin administration, compared to vehicle or valproate, significantly decreased the incidence of sexual contacts, reduced the amount of time investigating a novel object, and decreased central entries in the open field (Table 2). Concomitant with these behavioral changes, phenytoin-administered rats had significantly-decreased levels of 3α-diol (but not estradiol or testosterone levels), in the hippocampus and hypothalamus. We next examined whether coadministration of 3α-diol could attenuate these effects of phenytoin. Rats with silastic implants of 3α-diol or vehicle were administered phenytoin or vehicle for 30 days and then tested as above. Rats that had empty silastic implants in conjunction with phenytoin showed deficits in sexual, cognitive, and affective behavior,

Table 2 Reproductive, cognitive, and affective behaviors and 3α-diol levels of rats administered vehicle, valproate, and phenytoin

	Vehicle	*Valproate*	*Phenytoin*
Number of sexual contacts	24 ± 6	27 ± 9	11 ± 11
Time with novel object	71 ± 11	69 ± 9	49 ± 6
Central entries	38 ± 6	37 ± 7	23 ± 9
Number of seizures	11 ± 1	5 ± 2	4 ± 1
Hypothalamic 3α-diol (ng g^{-1})	3.9 ± 0.6	4.0 ± 0.9	1.8 ± 0.7
Hippocampal 3α-diol (ng g^{-1})	2.8 ± 1.1	3.1 ± 1.3	0.9 ± 0.8

and lower 3α-diol concentrations in the hippocampus and hypothalamus, as described earlier. These effects were abolished in the rats that were administered phenytoin but had silastic implants of 3α-diol (Table 3). Together, these data suggest that enzyme-inducing AEDs can have negative effects on reproductive, cognitive, and affective processes, and that these effects may be due in part to 3α-diol.

Summary and Future Goals

Research described and discussed here supports a relationship between androgens, androgen-mediated behaviors, and ictal activity. First, testosterone and 3α-diol reduce seizures, in part through actions at ARs and $GABA_A$ receptors in the hippocampus, respectively. Second, ictal activity has reciprocal effects on androgen levels in the brain. Seizures, either early in development or in adulthood or at both time points, reduce 3α-diol levels in the hypothalamus and hippocampus and produce decrements in sexual, cognitive, and affective behaviors. Third, enzyme-inducing AEDs decrease 3α-diol levels in the hippocampus and hypothalamus concomitant with deficits in reproductive, cognitive, and affective processes; these decrements are ameliorated by 3α-diol administration. Together, these data suggest a unique relationship between ictal activity and androgens (Figure 4). Further investigation of this relationship is necessary to capitalize on the putative therapeutic effects of androgens while reducing negative side effects of seizures and AEDs.

Table 3 Reproductive, cognitive, and affective behaviors and 3α-diol levels of rats administered vehicle or phenytoin with or without 3α-diol silastic implants

	Vehicle		*Phenytoin*	
	Empty silastic	*3α-Diol silastic*	*Empty silastic*	*3α-Diol silastic*
Number of sexual contacts	21 ± 2	25 ± 3	6 ± 3	20 ± 5
Time with novel object	78 ± 10	75 ± 7	46 ± 13	79 ± 11
Number of seizures	21 ± 2	25 ± 3	6 ± 3	20 ± 5
Hypothalamic 3α-diol (ng g^{-1})	3.6 ± 0.7	3.9 ± 0.9	1.9 ± 0.9	4.1 ± 1.1
Hippocampal 3α-diol (ng g^{-1})	3.2 ± 1.0	3.1 ± 0.9	0.8 ± 0.7	3.0 ± 0.8

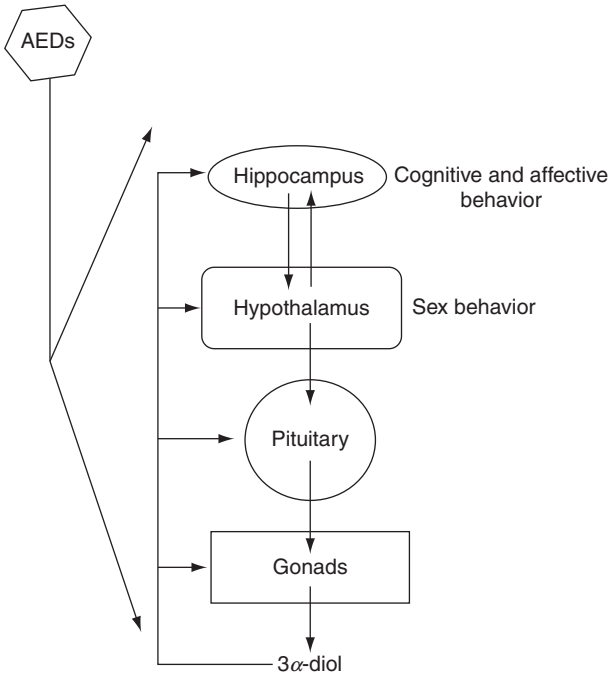


Figure 4 Schematic representation of putative effects of antiepileptic drugs on neuroendocrine and behavioral processes.

There are two major goals for future research in this area. First, it is critical to further identify membrane targets through which androgens may exert nonclassical effects to decrease seizures. Although testosterone can bind to plasma membrane in different models, a specific membrane protein through which testosterone has nonclassical actions has not yet been identified. There are, however, several possibilities. Testosterone bound to sex hormone binding globulin (SHBG) can act through a G-protein coupled SHBG receptor that has yet to be cloned. Testosterone may also bind a membrane-associated classical AR, which may anchor to caveolae or lipid rafts that are enriched with signaling proteins. Alternatively, a membrane AR distinct from the classic AR may exist; however, a specific membrane protein through which testosterone has nonclassical actions has not yet been cloned. Despite the lack of identification of discrete target sites to date, there is evidence that dynamic minute-to-minute changes in local androgen concentrations in various brain areas may explain some of the rapid physiological responses via membrane associated receptors in target cells. Second, the relationship between androgens, ictal activity, and AEDs needs to be better understood. Reductions in androgen levels of men with epilepsy, on some AED therapies, are associated with decreased sexual functioning and may also alter other androgen-dependent behaviors. Our initial data are intriguing and suggest androgens, ictal activity, and AED therapies interact with, and influence, one another. Given the challenge of addressing the relative contributions of each of these factors in a clinical population, we will continue to develop this line of research using animal models. It is vital to understand each of the putative mechanisms through which androgens may have their effects, and the relationship between androgens, seizures, and AEDs, in order to enhance the therapeutic options for those with seizure disorders.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Sex and Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility.

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Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy

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Introduction and Background

Temporal lobe epilepsy (TLE) is a common form of epilepsy in adults; in many patients, it is medically refractory. Patients with TLE have a characteristic neuropathology, which most significantly affects the hippocampus. Removal of the hippocampus and amygdala leads to the remission of seizures in over 70% of patients. Women with temporal lobe epilepsy frequently suffer from seizure exacerbations during menstruation – a condition often referred to as catamenial epilepsy. In 1957, Sir Charles Locock provided the first documented evidence for seizures in the 'catamenial period' (Gk. *kata*, by; *men*, month), suggesting a hormonal modulation of seizures. Subsequent studies have demonstrated that steroid hormones are precursors to the generation of neuroactive steroids that critically modulate seizure activity. The term *neurosteroids* refers to a group of compounds synthesized in the brain (largely by glia) from circulating steroid hormones and de novo from cholesterol, which exert their actions in the CNS via actions on neurotransmitter receptors.

Studies over the past few decades have identified that the $GABA_A$ receptor-mediated inhibitory system is a critical target for neurosteroids. Physiological concentrations of neurosteroids are known to modulate $GABA_A$ receptors and inhibitory neurotransmission, and reduction in endogenous neurosteroid levels is associated with increased seizure frequency.

Hippocampal seizures consist of synchronized tonic–clonic bursts of action potentials riding on a prolonged wave of depolarization in a network of neurons. Within the normal hippocampus, pyramidal CA1 and CA3 neurons can generate bursts, but the dentate gyrus resists their spread into other regions. Thus, the dentate gyrus has been proposed to act as a 'gate' that filters the flow of information from the entorhinal cortex to the hippocampus proper. A breakdown of this gating function of the dentate gyrus is considered crucial for the occurrence of seizures. Many investigators have proposed – and tested – the hypothesis that this breakdown of the filtering function of the dentate gyrus is due to reduced $GABA_A$ receptor-mediated inhibition. Both pre- and postsynaptic mechanisms of plasticity have been proposed. The presynaptic changes include (1) loss of GABAergic interneurons, which would lead to decreased frequency of inhibitory postsynaptic currents received by DGCs, (2) increased recurrent connectivity among granule cells due to mossy fiber sprouting, and increased neurogenesis and aberrant connectivity of newly born neurons.

Changes in the postsynaptic properties of the $GABA_A$ receptors may also contribute to the breakdown of the gating function of the DGCs. The plasticity of $GABA_A$ receptors on DGCs of epileptic animals was first reported in the kindling model, and subsequently confirmed using the lithium/pilocarpine model of TLE. In the DGCs from epileptic animals, there was a diminished expression of the $\alpha 1$ and δ subunit of the $GABA_A$ receptor, together with an increase in the expression of $\alpha 4$ subunit mRNA and protein. These electrophysiological, mRNA, and protein expression studies demonstrated clearly that $GABA_A$ receptors on DGCs were altered in animal models of epilepsy.

The neurosteroid sensitivity of $GABA_A$ receptors depends in large part on their subunit composition. It has been suggested that while the δ subunit confers neurosteroid sensitivity, an increase in the expression of one of its partnering subunits, the $\alpha 4$ subunit, diminishes

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Introduction and Background

Temporal lobe epilepsy (TLE) is a common form of epilepsy in adults; in many patients, it is medically refractory. Patients with TLE have a characteristic neuropathology, which most significantly affects the hippocampus. Removal of the hippocampus and amygdala leads to the remission of seizures in over 70% of patients. Women with temporal lobe epilepsy frequently suffer from seizure exacerbations during menstruation – a condition often referred to as catamenial epilepsy. In 1957, Sir Charles Locock provided the first documented evidence for seizures in the 'catamenial period' (Gk. *kata*, by; *men*, month), suggesting a hormonal modulation of seizures. Subsequent studies have demonstrated that steroid hormones are precursors to the generation of neuroactive steroids that critically modulate seizure activity. The term *neurosteroids* refers to a group of compounds synthesized in the brain (largely by glia) from circulating steroid hormones and de novo from cholesterol, which exert their actions in the CNS via actions on neurotransmitter receptors.

Studies over the past few decades have identified that the $GABA_A$ receptor-mediated inhibitory system is a critical target for neurosteroids. Physiological concentrations of neurosteroids are known to modulate $GABA_A$ receptors and inhibitory neurotransmission, and reduction in endogenous neurosteroid levels is associated with increased seizure frequency.

Hippocampal seizures consist of synchronized tonic–clonic bursts of action potentials riding on a prolonged wave of depolarization in a network of neurons. Within the normal hippocampus, pyramidal CA1 and CA3 neurons can generate bursts, but the dentate gyrus resists their spread into other regions. Thus, the dentate gyrus has been proposed to act as a 'gate' that filters the flow of information from the entorhinal cortex to the hippocampus proper. A breakdown of this gating function of the dentate gyrus is considered crucial for the occurrence of seizures. Many investigators have proposed – and tested – the hypothesis that this breakdown of the filtering function of the dentate gyrus is due to reduced $GABA_A$ receptor-mediated inhibition. Both pre- and postsynaptic mechanisms of plasticity have been proposed. The presynaptic changes include (1) loss of GABAergic interneurons, which would lead to decreased frequency of inhibitory postsynaptic currents received by DGCs, (2) increased recurrent connectivity among granule cells due to mossy fiber sprouting, and increased neurogenesis and aberrant connectivity of newly born neurons.

Changes in the postsynaptic properties of the $GABA_A$ receptors may also contribute to the breakdown of the gating function of the DGCs. The plasticity of $GABA_A$ receptors on DGCs of epileptic animals was first reported in the kindling model, and subsequently confirmed using the lithium/pilocarpine model of TLE. In the DGCs from epileptic animals, there was a diminished expression of the $\alpha 1$ and δ subunit of the $GABA_A$ receptor, together with an increase in the expression of $\alpha 4$ subunit mRNA and protein. These electrophysiological, mRNA, and protein expression studies demonstrated clearly that $GABA_A$ receptors on DGCs were altered in animal models of epilepsy.

The neurosteroid sensitivity of $GABA_A$ receptors depends in large part on their subunit composition. It has been suggested that while the δ subunit confers neurosteroid sensitivity, an increase in the expression of one of its partnering subunits, the $\alpha 4$ subunit, diminishes

neurosteroid sensitivity of $GABA_A$ receptors. In our studies, we tested whether mechanisms mediating neurosteroid sensitivity of synaptic $GABA_A$ receptors on DGCs are altered in a rat model of temporal lobe epilepsy.

Methods

Our experiments were performed on DGCs from animals made epileptic using the continuous hippocampal stimulation protocol. Briefly, rats were anesthetized with ketamine ($50\,mg\,kg^{-1}$) and xylazine ($40\,mg\,kg^{-1}$) and implanted with a pair of bipolar stimulating electrodes in the left posterior ventral hippocampus (AP 3.6, ML 4.0, DV 5.0 from the dura, incisor bar at +5.0). After 1 week of recovery, the left hippocampus was stimulated with 10-sec trains of 50 Hz, 1 ms, 400-mA biphasic square wave current pulses, delivered every 13 s for 90 min, to induce status epilepticus (SE). Approximately 4–6 weeks after the stimulation, the rats developed spontaneous limbic seizures with the typical tonic–clonic motor component. The seizures were documented either by continuous EEG recording or by direct observation of the spontaneous seizures. For all experiments, age-matched control animals were used.

Whole-cell patch clamp recordings of $GABA_A$ receptor-mediated currents were performed on acutely isolated DGCs. Rats were decapitated under halothane anesthesia, and the brains were dissected free and chilled to 4 °C for 1 min in a piperazine-*N*,*N*′-bis(2-ethanesulphonic acid) (Pipes) buffer solution composed of (mM): NaCl 120, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, D-glucose 25, Pipes 20 (pH 7.0) (all chemicals from Sigma, St. Louis, MO, USA, unless noted otherwise). The region containing the hippocampus was blocked. The brain was blot-dried, mounted on a vibratome (Camden Instruments, UK) stage with the caudal part facing up, and 400–500 µm coronal slices containing hippocampus were cut. The slices were allowed to recover for at least 1 h in Pipes solution before being transferred to an enzyme solution. The slices were incubated in continuously oxygenated Pipes solution, containing $3\,mg\,ml^{-1}$ type XXIII protease enzyme from *Aspergillus oryzae*, at 32 °C for 45 min. The enzyme incubation was terminated by transferring the slice into enzyme-free Pipes solution; after a minimum of 30 min of recovery, DGCs were isolated from caudal parts of both left and right ventral hippocampi. The dentate gyrus was dissected from the rest of the slice under a microscope and cut into $0.5\text{-}mm^3$ chunks. These chunks were triturated through 0.5- and 0.3-mm diameter fire-polished glass pipettes, and the isolated neurons were plated on 35-mm plastic Petri dishes. DGCs were identified by their typical oval shape and single process.

Horizontal hippocampal sections of 300-µm thickness were used for patch clamp recordings of $GABA_A$ receptor-mediated currents in brain slices. The ACSF consisted of the following (in mM): 127 NaCl, 2 KCl, 1.5 $CaCl_2$, 1.5 $MgSO_4$, 25.7 $NaHCO_3$, 1.1 KH_2PO_4, and 10 glucose (osmolarity, 300–305 mOsm), and was continuously oxygenated with 95% O_2–5% CO_2. The internal pipette recording solution consisted of the following (in mM): 153.3 CsCl, 1.0 $MgCl_2$, 10.0 HEPES, and 5.0 EGTA, pH 7.2 (with CsOH) (osmolarity, 285–295 mOsm). The electrode shank contained the following (in mM): 3 ATP Mg^{2+} salt and 0.1 GTP Na^+ salt. Visualized patch clamp recordings of $GABA_A$ receptor-mediated chloride currents were performed at room temperature in a symmetrical chloride environment. The neurons were voltage clamped to −65 mV. Currents reversed at 0 mV and were blocked by bicuculline, indicating that they are $GABA_A$-receptor mediated. The recordings were analyzed with Mini Analysis software. In parallel, anatomical studies, including light, confocal, and electron microscopy, were performed.

Results

We first studied neurosteroid modulation of $GABA_A$ receptor-mediated whole cell currents in acutely dissociated DGCs. DGCs obtained from epileptic rats will be hereafter referred to as 'epileptic DGCs' and those isolated from control animals will be hereafter referred to as 'control DGCs.'

We observed that $GABA_A$ receptors on epileptic DGCs were less sensitive to the neurosteroid allopregnanolone than those of controls. Allopregnanolone (10 and 30 nM) enhancement of 10 µM GABA-evoked currents in epileptic DGCs was significantly diminished compared to that in control DGCs. While the efficacy remained unaltered, the potency (EC_{50}) of allopregnanolone enhancement of GABA-evoked currents was markedly diminished in epileptic DGCs compared to that in control DGCs. In epileptic DGCs, there was a significant right shift of the allopregnanolone concentration-response curve (**Figure 1**).

In addition, we found that in epileptic DGCs, there was a loss in the sensitivity of diazepam, whereas the sensitivity to Zn^{2+} was enhanced. Since whole-cell $GABA_A$ receptor currents comprise currents from synaptic and extrasynaptic receptors, and because synaptic and extrasynaptic currents have distinct properties, we then studied neurosteroid modulation of synaptic inhibition in acute hippocampal slices.

The neurosteroid sensitivity of synaptic $GABA_A$ receptors in control and epileptic DGCs was compared by bath application of allopregnanolone (10 nM) in slices from control and epileptic animals. Allopregnanolone (10 and 30 nM) prolongation of mIPSC decay time constant (**Figure 2(a)** and **2(c)**) was lost in epileptic DGCs (**Figure 2(b)** and **2(b)**). This loss of neurosteroid sensitivity of synaptic currents raised the possibility

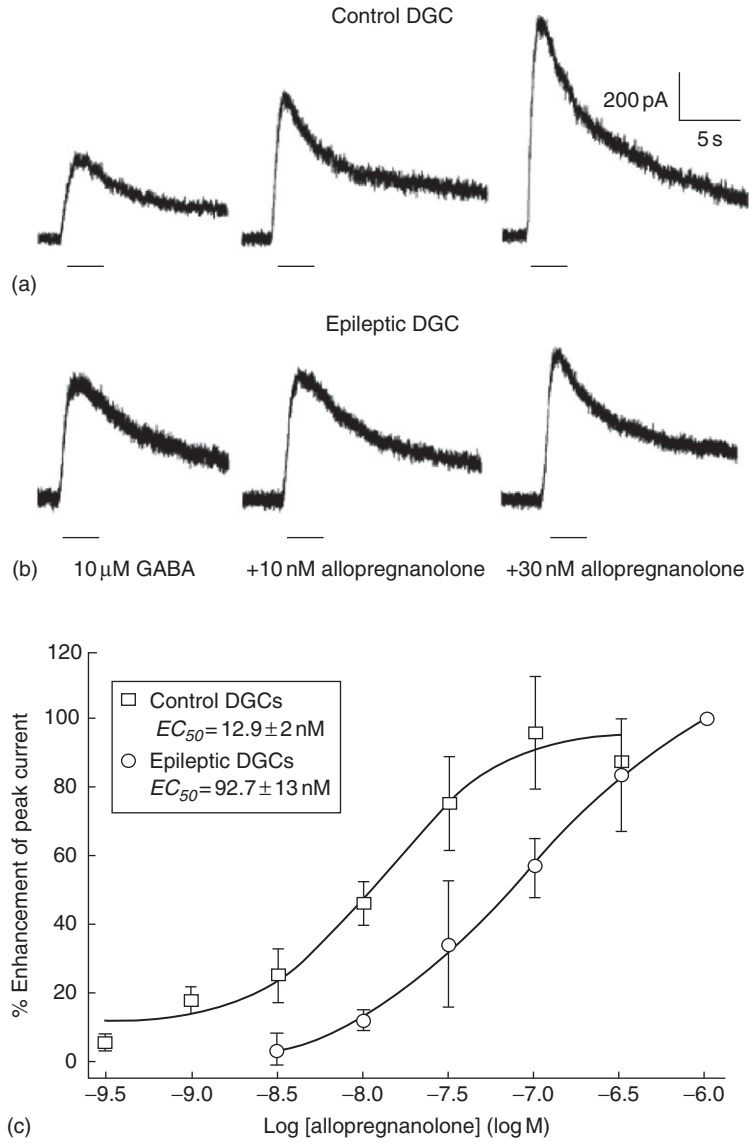


Figure 1 The peak current elicited by GABA was robustly enhanced by 10 and 30 nM allopregnanolone (a). In contrast, the same concentrations of allopregnanolone poorly enhanced $GABA_A$ receptor currents in an epileptic DGC (b). There was a right shift in the allopregnanolone concentration-response curve of epileptic DGCs than in the controls (c). Reproduced from Mtchedlishvili Z, Bertram EH, and Kapur J (2001) Diminished allopregnanolone enhancement of GABA(A) receptor currents in a rat model of chronic temporal lobe epilepsy. *Journal of Physiology* 537: 453–465, with permission from Wiley-Blackwell.

that α4 subunit-containing $GABA_A$ receptors were present on postsynaptic membrane of epileptic but not control DGCs. The α4 subunit-containing receptors have unique benzodiazepine pharmacology. They are insensitive to the classical, broadly active benzodiazepine, diazepam. However, the imidazobenzodiazepine, RO 15-4513, is a partial agonist at the benzodiazepine insensitive, α4 and γ2 subunit-containing receptors and acts as an inverse agonist at receptors with classical benzodiazepine pharmacology (α1βxγ2, α2βxγ2, and α5βxγ2). Therefore, we compared the actions of diazepam and RO 15-4513 on mIPSCs recorded from control and epileptic animals.

We found that the sensitivity of synaptic currents to diazepam was lost in epileptic DGCs. Diazepam (30 nM) prolonged the mIPSC decay time constants, and enhanced the amplitude of mIPSCs in control (**Figure 3(a)** and **3(c)**), but not epileptic DGCs (**Figure 3(b)** and **3(d)**). We then tested whether the response to RO 15-4513 had also changed. RO 15-4513 (300 nM) inhibited mIPSCs amplitude but had no effect on the decay time constant in control DGCs (**Figure 4(a)**); however, it potentiated the amplitude and decay of mIPSCs in epileptic DGCs (**Figure 4(b)**).

These observations suggested the presence of synaptic $GABA_A$ receptors containing the α4 and γ2 subunit of

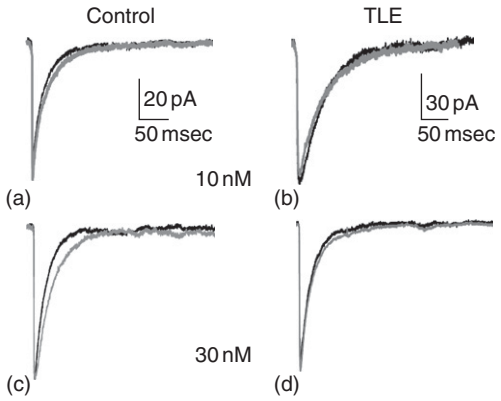


Figure 2 Allopregnanolone (10 and 30 nM) prolonged decay of mIPSCs recorded from control DGCs (a, c) but did not alter decay of mIPSCs recorded from epileptic DGCs (b, d). In this and subsequent figures, the black trace of averaged mIPSCs represent recordings in ACSF, and gray trace represents recordings after bath application of drug. Reproduced from Sun C, Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of $GABA_A$ receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

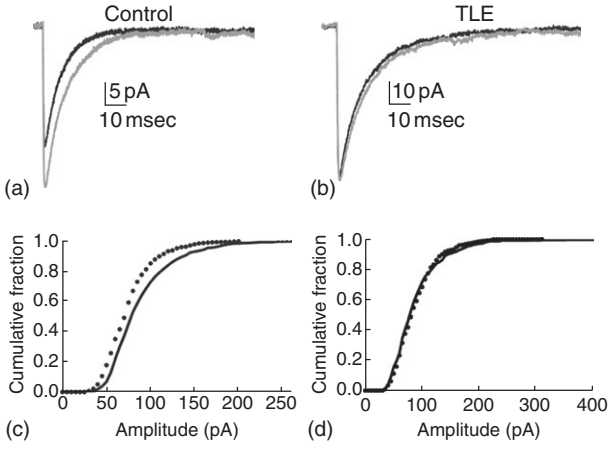


Figure 3 Diazepam (30 nM) strongly potentiated amplitude and decay of mIPSCs recorded from control DGC (a), whereas it had no effect on mIPSCs recorded from an epileptic DGC (b). Cumulative fraction plots of distribution of mIPSC amplitudes derived from six control and six epileptic DGCs before (black line) and after (dotted lines) application of drug indicate that diazepam caused a leftward shift in control (c) but not in epileptic (d) DGCs. Reproduced from Sun C, Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of $GABA_A$ receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

the receptor. We further confirmed the presence of the α4 subunit, by studying the sensitivity of the synaptic currents to furosemide, which inhibits α4 subunit-mediated $GABA_A$ currents with a high affinity. In control

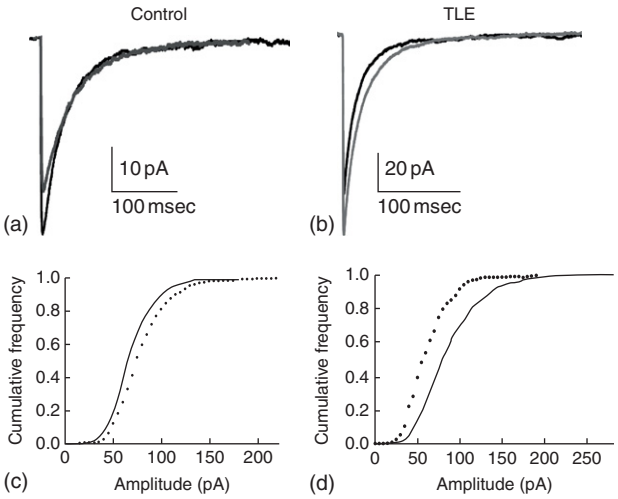


Figure 4 Averaged trace of mIPSCs recorded from a control (a) and an epileptic (b) DGC before and after bath application of 300-nM RO 15-4513. RO 15-4513 did not alter decay time constants, and the median amplitude decreased slightly in control DGC (a), whereas in an epileptic DGC (b), it significantly prolonged slow decay time constant and increased peak amplitude. Cumulative fraction plots of mIPSC amplitude data from seven control DGCs (c) and eight epileptic DGCs (d) before (black line) and after (dotted line) application of 300-nM RO 15-4513; the drug reduced mIPSC amplitude in control DGCs but increased it in epileptic DGCs. Reproduced from Sun C,Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of $GABA_A$ receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

DGCs, furosemide (100 μM) did not have any inhibitory effect on mIPSCs. There was a slight, but statistically insignificant, increase in the amplitude of mIPSCs; decay of mIPSCs was not affected (**Figure 5(a)** and **5(c)**). However, in epileptic DGCs, furosemide inhibited mIPSCs as demonstrated by a faster decay (**Figure 5(b)**) and a significant reduction in amplitude (**Figure 5(d)**).

The results of these pharmacological studies suggested that the loss of neurosteroid sensitivity in epileptic DGCs may be due to the presence of α4 subunit-containing $GABA_A$ receptors on the postsynaptic membrane. Indeed, it has been previously reported that there is an increase in α4 subunit-containing $GABA_A$ receptors following progesterone withdrawal, and that this change predisposes to an increase in seizure susceptibility. We employed anatomical methods, therefore, to determine the presence of α4 subunit containing receptors at the inhibitory synapses of the epileptic DGCs. Using double-label immunohistochemistry and confocal laser microscopy, we found that in epileptic DGCs, there was an increased colocalization of the α4 subunit with GAD65, the presynaptic marker of GABAergic synapses (**Figure 6**). GAD65 labels synapses; hence, the association of α4 colocalization with GAD65 suggested the presence of the α4 subunit in the synapse.

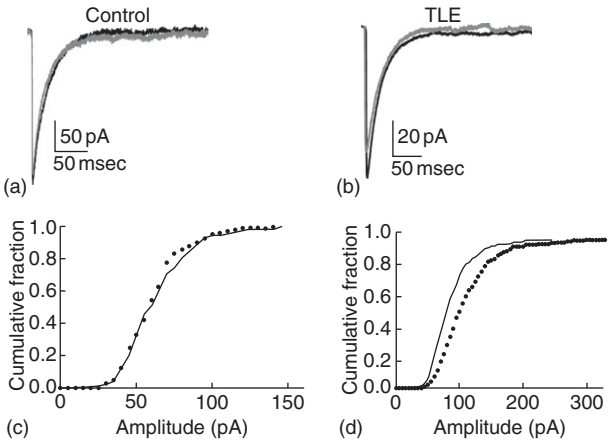


Figure 5 Averaged mIPSC traces demonstrate that furosemide (100 μM) had no effect on mIPSCs recorded from control DGCs (a), but it inhibited mIPSCs recorded from epileptic DGCs (b). Cumulative fraction plot of mIPSC amplitudes recorded from eight control (c) and seven epileptic (d) DGCs before (black line) and after (dotted line) drug application. Reproduced from Sun C, Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of $GABA_A$ receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

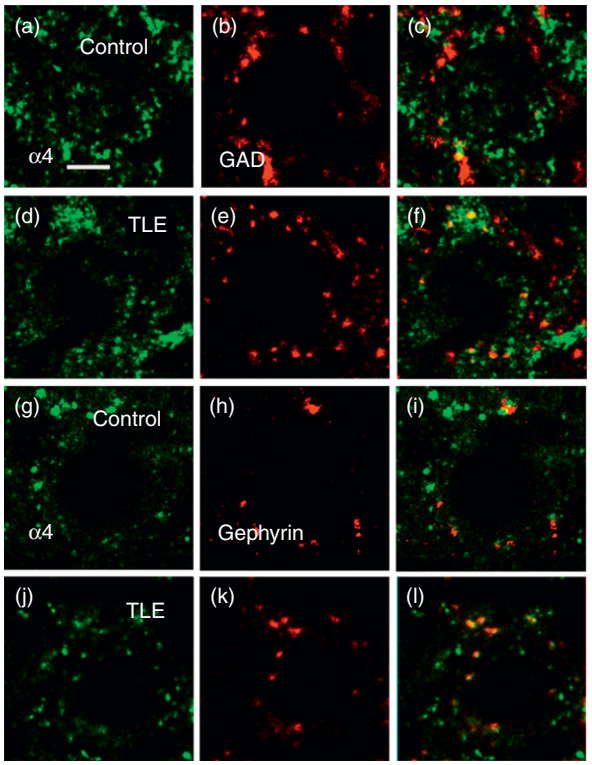


Figure 6 There were more clusters of the α4 subunit of the $GABA_A$ receptor colocalized with synaptic makers (GAD65 and gephyrin) in epileptic DGCs than in controls. The DGCs were double labeled with the α4 subunit of the $GABA_A$ receptor (a, d, g, j) and the presynaptic marker GAD65 (b, e) or the postsynaptic marker gephyrin (h, k). The subunit was visualized by secondary antibody conjugated with Alexa Fluor 488 (green), and synaptic markers were visualized with Alexa Fluor 594 (red). Parts (c), (f), (i), and (l) are merged images of the subunit and synaptic markers. Scale bar, 5 μm. Reproduced from Sun C, Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of $GABA_A$ receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

To further confirm the location of the α4 subunit of the $GABA_A$ receptor, we used postembedding immunogold electron microscopy. Quantitative analysis was performed on randomly selected grids of α4 subunit-labeled synaptic profiles within the granule cell layer, and the synapses were considered labeled with α4 immune particles only if two or more gold particles were present at or around a synapse. The synapse was defined as the area where the pre- and postsynaptic membranes remained parallel to each other and were separated by a synaptic cleft. The distribution of α4 immunogold particles was classified as one of the following: (1) within the central third of the synapse (**Figure 7**, label 1), (2) within the outer third of the synapse (**Figure 7**, label 2), (3) within 40 nm of the ends of synapses (considered to be perisynaptic) (**Figure 7**, label 3), and (4) extrasynaptic, located 40–100 nm from the end of synapses along the adjacent membrane (**Figure 7**, label 4). Gold particles within the center third and outer third were classified as synaptic labeling, whereas those within perisynaptic and extrasynaptic were classified as extrasynaptic labeling.

In epileptic DGCs, the α4 subunit immunogold particles redistributed from the extrasynaptic membrane to the synapses as shown in **Figure 7**. The number of gold particles per synapse in control and epileptic animals was similar (control 3.38 ± 0.27 vs. epileptic 4.04 ± 0.31, unpaired t-test $p > 0.05$). However, in epileptic DGCs, the fraction of gold particles in the center of the synapse was increased from 12.9% in controls to 23.5% in epileptic DGCs, and from 16.9% in controls to 32.8% in the outer third synaptic membrane. Also, there was a clear reduction in the number of gold particles in the extrasynaptic region in epileptic DGCs (25.5%) than in controls (45.5%). Thus, compared with controls, a larger faction of α4 subunit labeling was found within the perisomatic symmetric synapses on epileptic DGCs. Further analysis of the symmetric synapses on proximal dendrites of DGCs revealed that compared with controls, epileptic DGCs had a larger faction of α4 subunits within synapses, and a smaller fraction in the extrasynaptic and perisynaptic regions of the synapses.

Our physiological and anatomical studies thus demonstrated a loss of neurosteroid sensitivity of synaptic currents in epileptic DGCs. Other studies have suggested the presence of the γ2 subunit in the peri- and extrasynaptic sites. Together, these results suggest that a potential

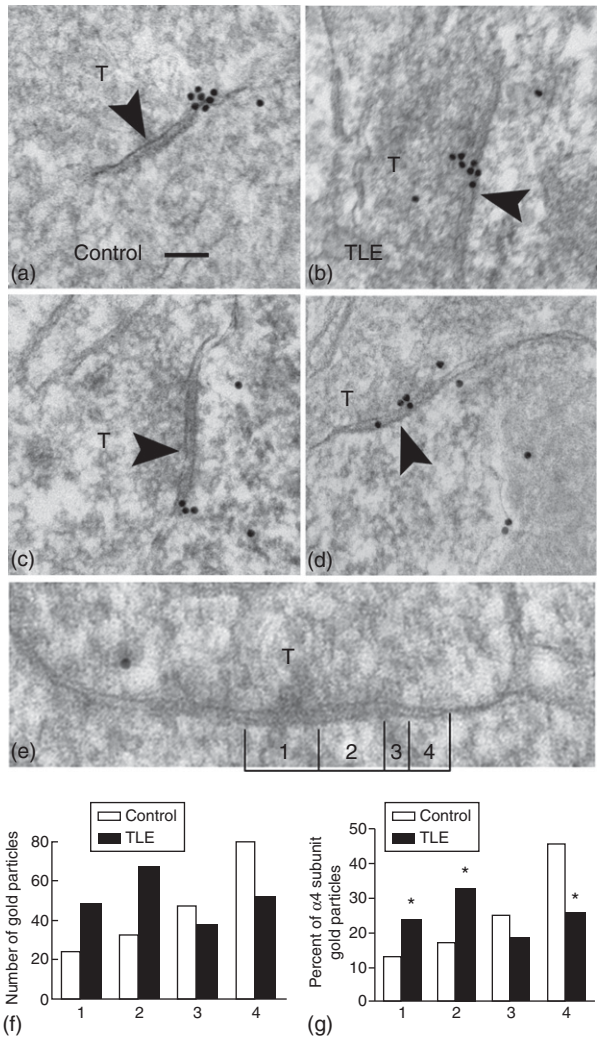


Figure 7 While α4-immunoreactive gold particles were found close to or at the end of symmetric axosomatic synapses in the hippocampal DGC layer from control animals (a, c), they were also found at the symmetric synapses in hippocampal DGC layer from epileptic animals (b, d). Terminals are indicated by T, and the arrowheads in (a–d) indicate the center of synapses. Scale bar: (a–d), 100 nm. (e) A representation of the classification of subdivisions of the synaptic and extrasynaptic regions. (f) More number of immunogold particles were found in center and outer third of synapses, and less in the peri and extrasynaptic regions in epileptic DGCs. (g) Immunogold particles in regions 1–4 expressed as a percentage fraction of total number of gold particles in symmetric synapses in control and epileptic DGCs. *$p < 0.05$, χ^2 test. Reproduced from Sun C, Mtchedlishvili Z, Erisir A, Kapur J (2007) Diminished neurosteroid sensitivity of synaptic inhibition and altered location of the a4 subunit of GABA-A receptors in an animal model of epilepsy. *Journal of Neuroscience* 27(46): 12641–12650, with permission from Society for Neuroscience.

mechanism underlying the loss of neurosteroid sensitivity may be the presence of novel $GABA_A$ receptors with pharmacological properties similar to those containing the α4 and γ2 subunits in the postsynaptic membrane.

Future Goals

Altered neurosteroid modulation of $GABA_A$ receptors on DGCs in temporal lobe epilepsy may have consequences in the precipitation of seizures or catamenial exacerbation of seizures. It is possible that this diminished sensitivity results in increased vulnerability to seizures. Further studies are underway to confirm if similar changes in neurosteroid sensitivity and $GABA_A$ receptor assembly occur in an animal model of catamenial epilepsy. An important question that remains to be answered is whether the loss of neurosteroid sensitivity occurs after the establishment of epilepsy, or during the latent period (epileptogenesis) after *status epilepticus*. The loss of sensitivity in the latent period would suggest that loss of neurosteroid sensitivity may predispose to the development of epilepsy.

See also: **GABA**: Molecular Pathology of Genetic Epilepsy Associated with $GABA_A$ Receptor Subunit Gene Mutations; Pathophysiology of Status Epilepticus; **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Hormones and Seizures; Steroid Hormones and Sex Differences in Seizure Susceptibility.

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Sex and Seizure Sensitivity

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Introduction

Animal studies have revealed that subtle sex differences in basal seizure susceptibility exist between males and females. It has been hypothesized that the distal portion of chromosome 1 in mice harbors a gene that has a fundamental role in regulating seizure susceptibility (Ferraro *et al.*, 2001). During early ontogenetic development, gonadal hormones regulate the process of sexual differentiation and are involved in the maturation of many brain structures such as the hypothalamus, hippocampus, and substantia nigra. Sex differences in seizure susceptibility are not restricted to hormonal modulation of receptor activity. Alterations in the concentration of neurotransmitters, especially glutamate and γ-aminobutyric acid (GABA), may contribute to the variations in the susceptibility to seizures. It should also be emphasized that hormones and their neurosteroid derivatives modulate a variety of ionotropic receptors, including $GABA_A$, and *N*-methyl-D-aspartate (NMDA) receptor complexes.

Hippocampus and substantia nigra are two main subcortical structures essential for seizure generation and propagation. Substantia nigra is, furthermore, involved in the expression of movement disorders. Within this area, sex-specific differences in functionality of the GABAergic system have been determined in a variety of animal studies. In mature male rats, there are two distinct regions mediating opposite effects on seizure phenomena. In females, in contrast, only one region that can affect seizures has been confirmed. Testosterone seems to be responsible for the development of 'proconvulsant' region of substantia nigra in male rats. In infantile rats, neural depolarization and intracellular Ca^{2+} increases are participants in the pathways that regulate gene expression differently in male and female substantia nigra, thus promoting their sexual differentiation. Moreover, sex differences in the expression of cation-chloride cotransporters may underlie the GABA-mediated sexual differentiation of certain brain structures.

The pattern of gonadal steroids contributes to seizure susceptibility in adult males and females. Testosterone, which is predominant in males, is generally metabolized by 5α-reductase to 5α-dihydrotestosterone, which is converted to 3α-androstanediol, a powerful positive modulator of $GABA_A$ receptors, thus yielding anticonvulsant properties. Nevertheless, testosterone in the presence of aromatase is converted to 17β-estradiol, exhibiting some proconvulsant action, probably via glutamatergic NMDA receptors. Therefore, testosterone may increase seizure susceptibility in females and decrease it in males. In females, estrogen enhances, but progesterone and its metabolite allopregnanolone diminish, the convulsive threshold. Allopregnanolone, as a very potent positive modulator of $GABA_A$ receptor, decreases sensitivity to most seizures, with the exception of absence epilepsy. In a model of absence epilepsy (rats treated during development with a cholesterol synthesis inhibitor, AY9944), progesterone and allopregnanolone exacerbated, while 17β-estradiol attenuated seizure severity.

Steroid hormones and other factors may differentially affect the response of $GABA_A$ receptors to various ligands. For instance, acute restraint stress selectively decreases the potency of GABA to modulate $GABA_A$ receptors. Chronic ethanol consumption decreases α1 subunit expression in male rat, but not female rat cortex.

The effect of sex and gonadal hormones on seizure susceptibility is observed also in humans. One obvious example is the influence of the menstrual cycle on catamenial seizures in women. Moreover, childhood absence epilepsy affects more girls than boys, in contrast to myoclonic epilepsy, which affects more boys than girls. The existing data suggest that sex differences in seizure

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Introduction

Animal studies have revealed that subtle sex differences in basal seizure susceptibility exist between males and females. It has been hypothesized that the distal portion of chromosome 1 in mice harbors a gene that has a fundamental role in regulating seizure susceptibility (Ferraro *et al.*, 2001). During early ontogenetic development, gonadal hormones regulate the process of sexual differentiation and are involved in the maturation of many brain structures such as the hypothalamus, hippocampus, and substantia nigra. Sex differences in seizure susceptibility are not restricted to hormonal modulation of receptor activity. Alterations in the concentration of neurotransmitters, especially glutamate and γ-aminobutyric acid (GABA), may contribute to the variations in the susceptibility to seizures. It should also be emphasized that hormones and their neurosteroid derivatives modulate a variety of ionotropic receptors, including $GABA_A$, and *N*-methyl-D-aspartate (NMDA) receptor complexes.

Hippocampus and substantia nigra are two main subcortical structures essential for seizure generation and propagation. Substantia nigra is, furthermore, involved in the expression of movement disorders. Within this area, sex-specific differences in functionality of the GABAergic system have been determined in a variety of animal studies. In mature male rats, there are two distinct regions mediating opposite effects on seizure phenomena. In females, in contrast, only one region that can affect seizures has been confirmed. Testosterone seems to be responsible for the development of 'proconvulsant' region of substantia nigra in male rats. In infantile rats, neural depolarization and intracellular Ca^{2+} increases are participants in the pathways that regulate gene expression differently in male and female substantia nigra, thus promoting their sexual differentiation. Moreover, sex differences in the expression of cation-chloride cotransporters may underlie the GABA-mediated sexual differentiation of certain brain structures.

The pattern of gonadal steroids contributes to seizure susceptibility in adult males and females. Testosterone, which is predominant in males, is generally metabolized by 5α-reductase to 5α-dihydrotestosterone, which is converted to 3α-androstanediol, a powerful positive modulator of $GABA_A$ receptors, thus yielding anticonvulsant properties. Nevertheless, testosterone in the presence of aromatase is converted to 17β-estradiol, exhibiting some proconvulsant action, probably via glutamatergic NMDA receptors. Therefore, testosterone may increase seizure susceptibility in females and decrease it in males. In females, estrogen enhances, but progesterone and its metabolite allopregnanolone diminish, the convulsive threshold. Allopregnanolone, as a very potent positive modulator of $GABA_A$ receptor, decreases sensitivity to most seizures, with the exception of absence epilepsy. In a model of absence epilepsy (rats treated during development with a cholesterol synthesis inhibitor, AY9944), progesterone and allopregnanolone exacerbated, while 17β-estradiol attenuated seizure severity.

Steroid hormones and other factors may differentially affect the response of $GABA_A$ receptors to various ligands. For instance, acute restraint stress selectively decreases the potency of GABA to modulate $GABA_A$ receptors. Chronic ethanol consumption decreases α1 subunit expression in male rat, but not female rat cortex.

The effect of sex and gonadal hormones on seizure susceptibility is observed also in humans. One obvious example is the influence of the menstrual cycle on catamenial seizures in women. Moreover, childhood absence epilepsy affects more girls than boys, in contrast to myoclonic epilepsy, which affects more boys than girls. The existing data suggest that sex differences in seizure

susceptibility are intimately related to the modulation of the activity of the $GABA_A$ receptor complex by sexual hormones.

Background

Pentylenetetrazole-Induced Seizures

Female rats, having higher threshold than males, are less susceptible to seizures induced by pentylenetetrazole. Since ovariectomy reduces the threshold of females to approximately that of the males, it is believed that progesterone and allopregnanolone are responsible for this discrepancy between intact female and male rats. In contrast, female mice seem to be more susceptible to pentylenetetrazole than do males; however, this effect appears only when the chemoconvulsant is applied at a dose range of 50–60 mg kg^{-1} Qualitative differences of convulsion expression are also observed. Females most frequently display running clonus, while males often present with myoclonus.

It is interesting that L-NAME, an inhibitor of nitric oxide synthase, completely prevents the pentylenetetrazole-induced convulsions in male rats, but increases the seizure severity, frequency, and duration, as well as shortens the latency in female rats. A nitric oxide precursor, sodium nitroprusside, increases these seizure parameters in male rats, while decreases them in female rats. Given these data, the effect of nitric oxide on the seizure susceptibility appears to be clearly sex-dependent. It is also interesting that morphine, administered at relatively low doses (0.5–3 mg kg^{-1}), exhibits anticonvulsant effects against pentylenetetrazole-evoked convulsions in males and estrus females, but not in diestrus females. However, at higher doses (30 and 60 mg kg^{-1}), morphine decreases the seizure threshold in males and diestrus females, with relatively little effect in estrus mice. This result suggests that the net effect of morphine on seizure susceptibility in female mice depends on the administered dose of the opioid.

Bicuculline-Induced Convulsions

Bicuculline is a widely known antagonist of the $GABA_A$ receptor. In female rats, a higher concentration of progesterone is correlated with a heightened seizure threshold to bicuculline-induced convulsions (when compared with intact males and gonadectomized animals of both sexes). However, there are also reports that rats of both sexes are equally sensitive to the convulsant. Bicuculline has been used to investigate seizure susceptibility in pre- and peripubertal rats of both sexes, exposed prenatally to morphine. Males show increased susceptibility at the prepubertal ages and decreased susceptibility at peripubertal ages.

Picrotoxin-Induced Convulsions

In the model of picrotoxin-induced seizures, there is no significant sex difference in mean latencies to first convulsions. However, this effect may depend on the dose of the convulsant. Female rats treated with picrotoxin at low doses (4 mg kg^{-1}) have significantly shorter latencies to myoclonic seizures, and higher incidence of generalized tonic-clonic seizures than males do. Higher doses of picrotoxin (8 and 10 mg kg^{-1}) shorten relative latencies to myoclonic seizures in male individuals. No sex differences have been observed in the incidence of or latency to picrotoxin-induced focal or akinetic seizures.

There is evidence showing that sex differences in the sensitivity to picrotoxin are age-dependent. Picrotoxin is equipotent in producing convulsions in male and female 20-day-old rats. In contrast, adult females tend to be more, while adult males are considerably less sensitive to picrotoxin than young individuals. These observations suggest that gonadal hormones affect the seizure threshold for picrotoxin during maturation processes. Moreover, lower rates of synthesis of GABA in substantia nigra and, in consequence, the less effective GABA system in female rats may be responsible for their greater susceptibility to picrotoxin-induced convulsions. Nevertheless, the prior treatment of octreotide (a somatostatin analogue with $GABA_A$ receptor antagonistic activity) increases susceptibility for picrotoxin-induced seizures in male, but not female rats.

Ethanol Withdrawal-Induced Convulsions

Ethanol withdrawn female, but not male rats are more responsive to the anticonvulsant effects of the neurosteroid 3α-hydroxy-5α-pregnan-20-one. These findings show that sex has an effect on receptor adaptations elicited by ethanol. Proposed mechanisms for the development of ethanol dependence involve alterations in $GABA_A$ and NMDA receptors.

Pilocarpine-Induced Convulsions

Male rats appear to be more susceptible to the proconvulsant effects of pilocarpine, presenting a greater degree of full limbic convulsions when compared with female individuals. Rats of both sexes have similar latencies to onset of pilocarpine-evoked status epilepticus, but the status epilepticus-induced mortality is markedly higher in male rats. Ovariectomy shortens the latency to pilocarpine-induced status epilepticus, and increases the status epilepticus-related mortality.

Excitatory Amino Acid-Induced Convulsions

Male rats are more prone to kainate-induced seizures than females. Testosterone enhances the occurrence and

severity of seizures, and there is a reciprocal relationship between kainate-induced seizures and plasma testosterone level. Octreotide, acting as a $GABA_A$ receptor antagonist, decreases the number of kainate-evoked convulsions in animals of both sexes, but this effect is more pronounced in males. Onset of kainate-induced seizures is quicker in females than males; however, there is no effect of octreotide on the seizure latency.

NMDA-induced seizure activity is more severe in males when compared to female rats. Anticonvulsant effects of magnesium sulfate on the NMDA seizures are greater in males than females.

Maximal Electroshock

According to available data, female rats have a lower seizure threshold for electroshock-induced seizures than males.

Kindled Seizures

In the in vitro slice kindling model of epileptogenesis, some investigators have shown that the hippocampal CA1 shows larger excitatory postsynaptic potential amplitudes in males than in females. However, other authors have found that the natural changes in sex hormone levels during the estrous cycle in rats do not affect seizure susceptibility, at least in the amygdala kindling model; no sex differences were observed either in seizure parameters or in effectiveness of conventional antiepileptic drugs.

Methods

In our laboratory, we have studied the influence of steroid hormones on the basic experimental models of epilepsy: maximal electroshock- and pentylenetetrazole-induced convulsions in mice, as well as amygdala-kindled seizures in rats. The three models are required screening tests for potential anticonvulsant drugs. Maximal electroshock reflects generalized tonic–clonic seizures, pentylenetetrazole-evoked convulsions are an equivalent of myoclonic epilepsy, while amygdala-kindled seizures resemble complex partial seizures in humans.

Electroconvulsions are produced by an alternating current (50 Hz, 0.2 s, fixed current intensity of 25 mA, and maximum stimulation voltage of 500 V) delivered via ear-clip electrodes. Tonic hindlimb extension (the hind limbs of animals outstretched 180° to the plain of the body axis) is taken as the endpoint. Results are expressed as CS_{50} values for the hormones tested, where CS_{50} is current strength (in mA) necessary to induce tonic convulsions in 50% of animals.

In the pentylenetetrazole-induced convulsions model, the chemoconvulsant is injected at its CD_{50} dose, defined as a dose producing clonic convulsions in 50% of tested animals. Subsequently, the influence of steroid hormones on the CD_{50} value of pentylenetetrazole is determined and interpreted as an effect on the seizure threshold.

Kindled seizures are evoked electrically from basolateral amygdala. Each stimulus consists of a 1-s train of 50 Hz, 1 ms biphasic square-wave pulses, with pulse amplitude of 500 μA, delivered every 24 h. Stimuli are delivered until at least 10 sequential fully kindled seizures are generated. At that point, four parameters are measured: (1) the seizure threshold, (2) seizure severity, assessed according to a modified Racine's system, (3) duration of limbic seizures (stage 1–2) and motor seizures (stage 3–5), and (4) EEG after-discharge duration defined as spikes with a frequency of at least 1 Hz and amplitude at least twice greater than the pre-stimulation baseline. The after-discharges from the amygdala are recorded prior to and after the stimulation. After-discharge threshold (ADT) is determined after administration of a test drug (or vehicle control) by administering a series of stimulations at intervals of 3 min, increasing stimulus amplitude in steps of about 20% over the previously applied current until an after-discharge (duration of at least 3 s) is evoked. At this ADT current, seizure severity, seizure duration, and after-discharge duration are recorded and the data analyzed. Control readings are made 2 days before and 2 days after respective treatments.

Results

Our results demonstrate that male and female animals do not differ in susceptibility to seizures evoked in three basic models of experimental epilepsy described above. Estradiol administered intraperitoneally at the dose range of 1.125–3.375 mg kg^{-1} does not change the electroconvulsive threshold, but significantly decreases the threshold for the pentylenetetrazole-induced convulsions in mice of

Table 1 Effect of steroid hormones on the threshold of experimental seizures

	ECT		*PTZ*		*AKS*	
Hormone	*F*	*M*	*F*	*M*	*F*	*M*
Estradiol	–	–	↓↓	↓	↓	↓
Testosterone	↑	↑↑	↑	↑↑	–	–
Progesterone	↑↑	↑	↑↑	↑	–	–
Cortisol	↓	↓	↓	↓	–	–
Aldosterone	–	–	–	–	–	–

ECT, the electroconvulsive threshold; PTZ, the threshold for pentylenetetrazole-induced convulsions; AKS, the after-discharge threshold for amygdala-kindled seizures; –, lack of effect; F, females; M, males; ↓, proconvulsant effect; ↓↓, more intensified proconvulsant effect; ↑, anticonvulsant effect; ↑↑, more intensified anticonvulsant effect.

both sexes. However, the antiseizure effect is more distinct in females. Testosterone ($5\,mg\,kg^{-1}$) increases the electroconvulsive threshold only in males, but when applied at $10\,mg\,kg^{-1}$, it raises the threshold in animals of both sexes. Progesterone ($4.5\,mg\,kg^{-1}$) raises this seizure parameter only in females, while at $6\,mg\,kg^{-1}$, the hormone increases the threshold in female and male mice. In the pentylenetetrazole model of epilepsy, testosterone ($5–10\,mg\,kg^{-1}$) and progesterone ($4.5–6\,mg\,kg^{-1}$) decrease the 50% convulsive dose of pentylenetetrazole – testosterone more dramatically in males, while progesterone more dramatically in female individuals. Cortisol (hydrocortisone; $25–50\,mg\,kg^{-1}$) decreases the electroconvulsive and pentylenetetrazole threshold similarly in mice of both sexes. Aldosterone does not influence the seizure susceptibility in the two epilepsy models.

Among all applied steroid hormones, only estradiol (3.375–4.5 mg) decreases the ADT, and does so similarly in rats of both sexes. Progesterone ($6–12\,mg\,kg^{-1}$) reduces the seizure severity and duration, but does not affect the ADT.

In conclusion, steroid hormones certainly modulate seizure susceptibility, and this effect is particularly distinct in the case of gonadal hormones in animals of the respective sex. However, it should be emphasized that effective doses of hormones used in these studies significantly exceed the therapeutic range (for review see **Table 1**).

Future Goals

The most critical immediate goal is to determine the influence of these hormones on seizure susceptibility in humans. Relatively little is known about the role of hypothalamic and pituitary hormones in epileptogenesis, seizure generation, and seizure propagation.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormones and Epilepsy; Hormones and Seizures; Male Sex Steroids and Neuronal Excitability; Sex Differences in Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility.

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Sex Differences in Seizure Sensitivity

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Introduction

Similar to other neurological diseases, epilepsy shows sex differences in incidence, progression and severity, as well as in responsiveness to therapy. Clinical data suggest that incidence of seizures and epilepsy is slightly higher in males than in females. These data are supported by animal studies, which also demonstrate higher seizure susceptibility in males than in females, using several seizure models. Many factors are involved in determining sex differences in seizure susceptibility, including the presence of sexual dimorphism in brain structures involved in seizure generation and control, in regional connectivity, sensitivity of neurotransmitter systems, receptor distribution, dependence on hormonal milieu and on changes in sex hormone levels during the life span. For example, some epileptic syndromes may either remit (benign rolandic epilepsy, childhood absence) or have their onset (juvenile myoclonic epilepsy) in adolescence, indicating the likely influence of hormonal changes occurring around puberty. Sex differences have been described in patients with temporal lobe epilepsy, with respect to distinct regional distribution of brain dysfunction during interictal periods as well as to the extent of neuronal damage. Women tend to have less structural atrophy than men, regardless of the seizure rate.

Background

Hormones influence neuronal development and neuronal circuit formation in the brain, and modulate its activity either through excitatory or inhibitory mechanisms. During embryonic and postnatal development, steroid hormones induce differentiation of discrete brain regions by modulation of specific neuronal and glial components directly involved in synaptogenesis and myelinogenesis. At the cellular level, sex differences in the nervous system include the size of nucleus and nucleolus in neurons, size of synaptic vesicles and terminals, and dendritic branching pattern – all leading to differences in gross structural volume, connectivity, and neurotransmitter distribution. These steroid hormone-dependent sex differences include many areas in the CNS, such as preoptic area, amygdala, hippocampus, hypothalamus, cortex, substantia nigra, striatum and lumbar spinal cord.

Many of these sexual differences develop during the 'critical period' for sexual differentiation. During this developmental period, the CNS is more sensitive to the permanent effects of gonadal hormones. There are several generally accepted assumptions in the current theory about the development of sexual dimorphism:

1. Every individual, whatever its 'chromosomal sex' (XX or XY), is programmed to develop complete feminine features;
2. Testosterone or its metabolites, estradiol and dihydrotestosterone, determine the male-type of brain organization, irrespective of the genetic sex; and
3. The organizing effect that occurs during the 'critical period' is irreversible ('imprinting effect').

In the male developing rat, the surge of testosterone from the fetal testes starts in the last part of gestation (embryonic day 18–22) and starts again at the early neonatal period (postnatal day: PN 0–6). These periods of secretion are responsible for the masculinization and defeminization of the developing rat brain. Recently, however, some exceptions to these postulates have been discovered. New studies show that some sex differences exist prior to the surge in sex hormones, presumably due to the genetic influence. Finally, some sex differences are maintained, but depend on the continued presence of sex hormones.

Our interest in sex differences in the substantia nigra pars reticulata (SNR) has led to extensive studies. The SNR contains mainly GABAergic neurons and serves as a main output of the basal ganglia. The SNR belongs to an endogenous seizure-controlling system, and especially activation of its $GABA_A$ receptor system seems to play an important role in seizure control. In adult rats, pharmacological manipulations of the $GABA_A$ergic system revealed that the role of the SNR in control of flurothyl seizures is region-specific. In contrast, in rat pups there is no regional compartmentalization within the SNR with respect to seizure control.

Methods

Male and female Sprague Dawley rats have been used at different developmental stages.

Development of Sex Differences in SNR-Mediated Seizure Control

Male and female pups were kept with their dams. The day of birth was considered postnatal day (PN) 0. To determine

development of sexual differentiation of the SNR seizure control system, we used following groups of rats: (1) Naïve males; (2) Naïve females; (3) Neonatally castrated males (castration at PN 0); (4) Females injected with testosterone or its metabolites (estrogen and dihydrotestosterone) at PN 0–2; (5) Neonatally castrated males injected with testosterone or its metabolites (estrogen and dihydrotestosterone) at PN 0–2; (6) Males castrated at PN 3.

Neonatal castration (at PN 0) prevents the postnatal testosterone surge, which starts several hours after the birth. This testosterone surge leads to development of sex differences in many brain regions. Although the critical period extends till PN 6, the sensitivity of brain regions to testosterone or its metabolites decreases with age. Thus, the most pronounced differences in the majority of the brain structures develop within the first two days after the birth.

The hormones were injected daily subcutaneously in the following doses: testosterone propionate – 0.1 mg per 0.02 ml; diethylstilbestrol (a synthetic estrogen, which in contrast to β-estradiol does not bind to α-fetoprotein and thus can reach the brain tissue)-2 μg per 0.02 ml; 5α-androstan-17b-ol-3-one (5α-dihydrotestosterone)-100 μg per 0.02 ml.

At PN 13, rats were placed in a stereotaxic apparatus and equipped with bilateral cannulae (Plastics One, Roanoke, VA, USA) in the SNR under ketamine (70 mg kg^{-1}) – xylazine (10 mg kg^{-1}) anesthesia. The incisor bar was positioned 3.5 mm below the interaural bar. The cannulae were inclined at an angle of 15° from the sagittal plane. The stereotaxic coordinates for the SNR were: anterior-posterior = 5.3 mm from bregma, medial-lateral = 3.5 mm from midline, dorsal-ventral = 6.0 mm below the skull. Pups were allowed to recover after the surgery for 2 days with their dams.

At PN 15, muscimol, an agonist to $GABA_A$ receptors (100 ng per 0.25 μl saline), was injected in the SNR bilaterally 30 min prior to seizure testing. Controls were infused with saline. The volume injected into each SNR was 0.25 μl (using a Hamilton syringe) over a 2-min period. After the infusion was completed, the infusion cannulae remained inserted in the guide cannulae for an additional minute.

Permanent Presence of Sex Differences in the SNR-Mediated Seizure Control

Adult (PN 60) male and female rats were used. Rats were divided into groups of animals with symmetrical cannula placements in the anterior part of the SNR (SNRanterior) and animals with symmetrical cannula placements in the posterior part of the SNR (SNRposterior). Cannulae were stereotactically implanted bilaterally in the SNR as described above. The stereotaxic coordinates for the SNRanterior were: anterior-posterior = 5.2 mm from bregma, medial-lateral = 4.0 mm from midline, dorsal-ventral = 7.0 mm below the skull. The stereotaxic coordinates for the SNRposterior were: anterior-posterior = 6.0 mm from bregma, medial-lateral = 4.0 mm from midline, dorsal-ventral = 7.0 mm below the skull. Rats were allowed to recover after the surgery for 2 days and muscimol (100 ng per 0.25 μl per SNR) was infused the same way as in the pups. Controls received SNR saline infusions (0.25 μl per SNR).

Flurothyl-induced seizures were used to determine seizure susceptibility. The rat was placed in a chamber where flurothyl was infused by a pump at a constant rate. Inhalation of flurothyl vapors induces generalized clonic seizures followed by tonic-clonic seizures. In these studies, we monitored the onset of first clonic seizure following muscimol SNR infusions. Flurothyl seizure threshold was calculated as the amount of flurothyl (in μl) necessary to elicit the seizure. Clonic seizures were characterized by facial and forelimb clonus with preserved righting reflex.

At the end of the experiment, the rats were sacrificed, and their brains were collected and snap frozen in 2-methylbutane at −35° C. Coronal sections were cut and stained with thionin for histological examination of the cannulae placements. Only rats with bilateral symmetrical SNR placements of both cannulae entered statistical analysis. In this experiment, effects of SNR muscimol infusions were compared to saline-infused controls within each experimental group using Student's t-test.

Results

Development of Sex Differences in SNR-Mediated Seizure Control (Table 1)

Activation of $GABA_A$ receptors in the SNR by microinfusion of muscimol had sex-specific effects on flurothyl-induced seizure threshold. At PN 15, muscimol infusions in males had a proconvulsant effect on the

Table 1 Development of sex differences in the SNR-mediated seizure control in response to muscimol infusions

	Male type	*Female type*
Naïve rats	Proconvulsant	No effect
Males castrated PN 0		No effect
Males castrated PN 3	Proconvulsant	
Males castrated PN 0 + TP	Proconvulsant	
Males castrated PN 0 + E	Proconvulsant	
Males castrated PN 0 + DHT	Proconvulsant	
Females + TP	Proconvulsant	
Females + E	Proconvulsant	
Females + DHT	Proconvulsant	

TP – Testosterone propionate; E – Estrogen; DHT – dihydrotestosterone.

onset of clonic and tonic-clonic seizures compared to saline-infused controls ('male phenotype' of SNR responses to muscimol infusions). In contrast, in female pups, muscimol had no effect on the seizure threshold compared to saline-infused controls ('female phenotype' of SNR responses to muscimol infusions). The role of the postnatal testosterone surge in the proconvulsant muscimol effects in the SNR on flurothyl seizures in PN 15 males was confirmed using males castrated at PN 0. In neonatally castrated males, SNR muscimol infusions at PN 15 had no effect on flurothyl seizure threshold compared to saline-infused controls; a similar absence of effect was found in female rat pups.

We further explored the role of the early postnatal testosterone surge in the 'male phenotype' of SNR responses to muscimol infusions by injecting testosterone during the early postnatal period (PN 0–2) in females. This manipulation resulted in the loss of the 'female phenotype' of SNR responses; that is, muscimol infusions into the SNR in females treated with testosterone had proconvulsant effects on flurothyl seizure threshold compared to saline controls. Similarly, the males castrated at PN 0 regained the 'male phenotype' of SNR responses (i.e., muscimol infusions had proconvulsant effects compared to saline-infused controls) when injected with testosterone. In males castrated at PN 3, and therefore exposed to endogenous testosterone at the early neonatal period (PN 0–2), muscimol infusions had proconvulsant effects compared to saline controls, further demonstrating the importance of early postnatal testosterone in the development of the 'male phenotype' of SNR responses to muscimol infusions.

Finally, administration of either testosterone metabolite (estradiol or dihydrotestosterone) at PN 0–2 to females or males castrated at PN 0 leads to the 'male type' proconvulsant SNR muscimol responses when tested at PN 15. Thus, testosterone or its metabolites organize the SNR seizure-controlling network during the early postnatal period into the 'male type' effects of $GABA_A$ receptor system activation on seizures.

Permanent Presence of Sex Differences in SNR-Mediated Seizure Control (Table 2)

In adult female rats, flurothyl-induced seizure threshold did not depend on a stage of the estrous cycle, so we combined the data from all females regardless of estrous cycle stage. In adult rats, muscimol infusions in the SNR had region- and sex-specific effects. Muscimol infusions in the SNRanterior had an anticonvulsant effect on flurothyl seizure threshold compared to saline-infused rats in both sexes. However, infusions of muscimol in the SNRposterior had a proconvulsant effect in males – but no effect on flurothyl-induced seizure threshold in females compared to saline-infused rats. These data show that the sex-specific features of SNR-mediated seizure control remains into adulthood.

Future Goals

Many epileptic syndromes show gender-related prevalence. Here we illustrate that point using the example of the SNR, where the presence of sexual dimorphism in the brain influences how seizures are controlled. More studies need to be designed to understand the underlying mechanisms of the sex differences in the SNR seizure-controlling network, and to determine whether the seizure-controlling properties of the SNR are changing in animals with previous history of seizures or in a previously compromised brain (e.g., as a result of pre/postnatal stress, in models of migrational disorders, traumatic injury, etc.). At present, there are no similar studies of sexually dimorphic effects in other regions known to be involved in seizure initiation or suppression (i.e., amygdala, hippocampus, cortex). It would be important to initiate such studies of sex-specific roles of these structures in seizures and epilepsy.

The main goal for the future is to increase the awareness regarding sex differences in the brain. Sex differences may influence epileptic syndrome outcome, responsiveness to treatment, and also adverse effects of therapy. Thus, it is important to study the brain functionality separately in males and females. Sex differences are present from early development, so data from males and females in any age group should not be combined – at least not without determination that sex differences do not exist in the system studied.

Better knowledge of how sex differences influence brain function and neuronal activity may bring new insights in the mechanisms of sex-dependent seizure disorders and can help to design sex-specific treatments.

Table 2 Permanent presence of sex differences in the SNR-mediated seizure control in response to muscimol infusions

Male type		*Female type*	
SNRanterior	*SNRposterior*	*SNRanterior*	*SNRposterior*
Anticonvulsant	Proconvulsant	Anticonvulsant	No effect

Acknowledgements

Supported in part by grants NS-36238 and NS-20253 from NINDS.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Male Sex Steroids and Neuronal Excitability; Sex and Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility; **Networks**: The Substantia Nigra in the Control of Seizures.

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Steroid Hormones and Sex Differences in Seizure Susceptibility

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Introduction

Sex difference in seizure susceptibility is one of the long-standing issues in epilepsy research, at both the basic and clinical levels. Clinical evidence shows gender- and age-related expression in many seizure syndromes. The incidence of epilepsy is generally higher in men than in women. More women rather than men are diagnosed with idiopathic generalized epilepsy, but localization-related symptomatic epilepsies are more frequent in men, and cryptogenic localization-related epilepsies are more frequent in women. Overall, considerable evidences indicate that men are highly susceptibile to seizures than women. Many women exhibit greater fluctuations in susceptibility to seizures, including ovarian cycle-related changes in seizure activity.

During brain development, sex hormones have organizational effects leading to permanent differences between males and females in distinct brain regions. The presence or absence of testosterone determines the male or female brain phenotype. However, the precise mechanisms underlying the sex-dependent differentiation of the specific neuronal circuits, particularly brain regions involved in seizure control, are not clear. Sex-specific differences in the development of seizure suppressing neuronal networks may partly account for sex related susceptibility to seizures.

Sex-based differences in seizure sensitivity may arise from variations between men and women in factors such as body weight, steroid hormones, cytochrome P450 activity and biologic differences in neuronal networks in the brain. The intricate relationship between reproductive hormones and epilepsy suggests that seizures affect hormones and vice versa. Age-related gender differences in seizure sensitivity could be due to developmentally specific changes in neurotransmitters and receptor systems in the brain. For example, changes in seizure sensitivity are

Acknowledgements

Supported in part by grants NS-36238 and NS-20253 from NINDS.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Male Sex Steroids and Neuronal Excitability; Sex and Seizure Sensitivity; Steroid Hormones and Sex Differences in Seizure Susceptibility; **Networks**: The Substantia Nigra in the Control of Seizures.

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Sex-based differences in seizure sensitivity may arise from variations between men and women in factors such as body weight, steroid hormones, cytochrome P450 activity and biologic differences in neuronal networks in the brain. The intricate relationship between reproductive hormones and epilepsy suggests that seizures affect hormones and vice versa. Age-related gender differences in seizure sensitivity could be due to developmentally specific changes in neurotransmitters and receptor systems in the brain. For example, changes in seizure sensitivity are

evident at puberty, at which marked changes in reproductive hormones and behavioural patterns occurs. Steroid hormones and endogenous neurosteroids, including those derived from adrenal steroids and androgens, substantially influence seizure susceptibility (Figure 1). This article examines the biological basis of sex-based differences in seizure susceptibility and summarizes studies that have focused on steroid hormones and sexually dimorphic brain circuits.

Background

Sex Differences in Seizure Susceptibility

In general, men are more susceptible to injury-induced seizures than women. Higher frequency of infantile spasm, an age-specific epileptic syndrome in infants and young children, is more frequently observed in boys than girls. Recent findings have shown that in early-onset temporal lobe epilepsy, women show greater functional plasticity for verbal memory than men. The relationship between menstrual cycle and seizure sensitivity in women is well known and is greatly influenced by hormonal fluctuations associated with menstrual cycle phases. Considering the first day of menstrual bleeding as the first day of the cycle, we can divide the menstrual cycle into four phases: (a) *menstrual phase*, days −3 to +3; (b) *follicular phase*, days +4 to +9; (c) *ovulatory phase*, days +10 to +16; and (d) *luteal phase*, days +17 to −4. Analysis of seizure diaries shows that seizure frequency is greater during perimenstrual and periovulatory phases than at luteal phase. Women with epilepsy are at increased risk for polycystic ovary syndrome. Sexual dysfunction is common among patients with epilepsy, and studies have found reduced fertility rates among women with epilepsy.

Numerous studies on gender-related sensitivity in animal models of epilepsy characterizes male/female differences in severity of response to seizure stimuli and drug protection. In most animal models of induced seizures, male mice or rats are more seizure-prone than females. For example, our experimental results show male adult mice to have a lower seizure threshold than females in the pentylenetetrazol (PTZ) test (Figure 2). Sex differences in the occurrence or severity of seizures are found for both GABAergic and glutamatergic compounds. The most marked differences are observed with ovariectomy, gonadectemy, and pharmacological manipulations of reproductive milieu. In experimental animals, estrous cycle-related changes in seizure susceptibility are widely reported in the literature. As is seen clinically, the hormonal milieu present during estrus or proestrous phase reduces seizure susceptibility, but animals are more vulnerable to seizures during the metestrous or diestrous phase. In contrast, the chronic absence of an estrous cycle, such as that induced by ovariectomy, leads to a greater chronic susceptibility to seizures. In a recent study, late diestrous phase has been shown to be associated with enhanced expression of $GABA_A$ receptor δ subunit (a molecular signal for increased tonic inhibition) and thereby reduces seizure susceptibility.

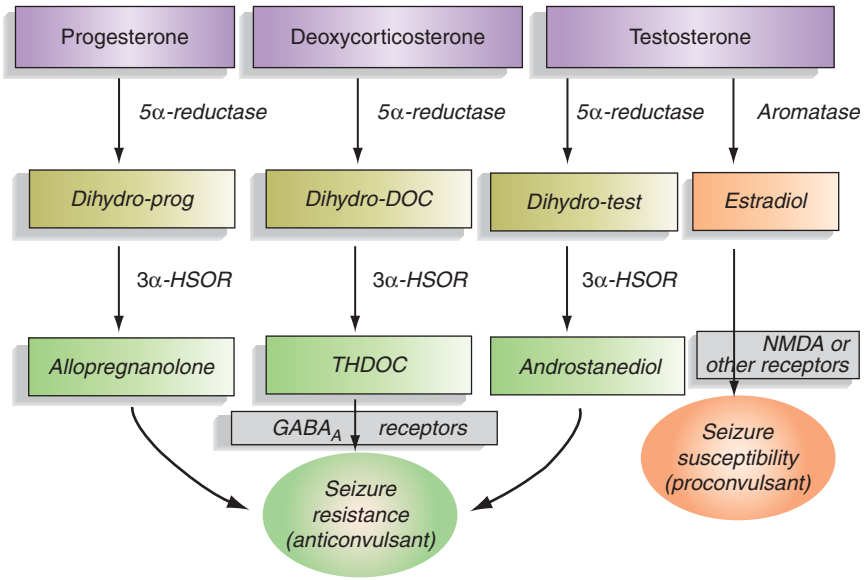


Figure 1 Biosynthetic pathways of steroid hormone metabolism and their influence on seizure susceptibility. 5α-Reductase converts progesterone, testosterone and deoxycorticosterone (DOC) into 5α-dihydro reduced steroids, which are then reduced further to 3α-hydroxylated neurosteroids by 3α-HSOR. Testosterone is converted into 17β-estradiol by the aromatase enzyme. The three enzymes are present in the brain.

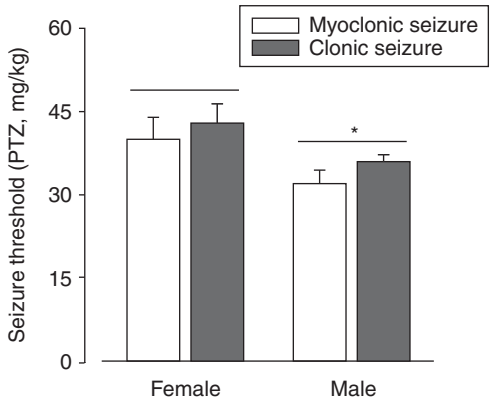


Figure 2 Seizure threshold in adult male and female mice. Seizure-threshold determinations were made by constant intravenous infusion of pentylenetetrazol (PTZ, 5 mg ml^{-1}). The times between the start of infusion and onset to myoclonic seizure and forelimb clonic seizure were recorded in seconds and subsequently converted to threshold convulsant dose in milligrams of PTZ per kilogram of body weight. Data represent mean ± SEM (n = 6–8 per group). *$p < 0.05$ vs. female group (Students' t-test).

Sexual Dimorphism in Brain Circuits and Seizure Susceptibility

The substantia nigra pars reticulata (SNR), a midbrain structure populated largely by GABAergic neurons, plays an important role in seizure control, in an age- and sex-dependent manner. In particular, GABAergic activation of SNR at P15 has sex-specific features on seizure control. Bilateral SNR microinjections of the $GABA_A$ receptor agonist muscimol have been associated with proconvulsant effects on flurothyl-induced seizures in male animals but not in female animals. Further studies have revealed that the postnatal testosterone is responsible for the male proconvulsant phenotype of SNR muscimol effects on seizures. In many neuronal types, GABAergic synaptic transmission is excitatory early in development and later switches to the mature hyperpolarizing type. This switching occurs in males around postnatal day (PN) 17 and in females around PN10. This sexual dimorphism may play a role in several other recognized sex differences in the development of SNR and in its regulatory role in seizures. Because the substantia nigra is an important brain nucleus involved in the expression of movement disorders, the two most common movement disorders affecting the substantia nigra – Parkinson's disease and Tourette syndrome – also show gender differences and age-related onset.

The central nervous system is an important target for steroid hormones such as progesterone and testosterone. These steroid acts in discrete regions of the female brain to regulate sexual behavior and other aspects of reproduction through classical genomic actions mediated by steroid receptors. Many of the biological actions of steroid hormones are mediated through intracellular receptors, which act as nuclear transcription factors. In steroid hormone-responsive target cells, steroid hormone binds to specific cytoplasmic receptors and the hormone-nuclear receptor complexes translocate to the cell nucleus where they activate or silence the transcription of downstream gene networks – thus affecting the physiological response of the target cell. The differential distribution of steroid hormones could account for sex differences in seizure susceptibility. It is well known that female mice and rats are more sensitive to the central nervous system actions of progesterone than are males. For example, our recent finding that wild-type, heterozygous and progesterone receptor knockout (PR-KO) female mice are more sensitive to the anticonvulsant effects of progesterone than males is consistent with the above viewpoint. Moreover, the fact that female PR-KO animals exhibit greater progesterone sensitivity demonstrates that known differences in the density or distribution of the progesterone receptors in the female and male brain are not responsible for the sexual dimorphism. Overall, the emerging evidence clearly supports the notion that although the hormonal milieu influences seizures, innate differences in brain structure between the sexes also contribute to sex differences in seizure sensitivity.

Methods

Animal Models of Epilepsy and Seizures

In our laboratory, a number of animal models are used to assess seizure susceptibility, seizure threshold, and anticonvulsant drug effects in rodents. Seizure susceptibility is determined by measuring the animal's response to a chemoconvulsant such as PTZ or other GABAergic antagonists (such as biculline or picrotoxin). The seizures induced by these chemoconvulsants are thought to resemble seizures in human partial epilepsies. PTZ (85 mg kg^{-1}, s.c.) is administered 15 to 30 min after administration of test drugs. Animals failing to show clinical seizure behavior lasting >5 s are scored as 'protected' by the drug of interest. Because the s.c. PTZ test provides only an all-or-none measure of seizure presence (at a given dose of PTZ), we have also adopted the intravenous PTZ test for a quantitative determination of changes in seizure threshold. In this test, 20 mg ml^{-1} PTZ solution (in sterile saline) is infused via a tail vein at a rate of 0.5 ml min^{-1} using a syringe pump. Three convulsion signs (myoclonic twitch, forelimb clonus, and tonic hindlimb extension), which are typically observed in sequence, are used as end points in the PTZ threshold test. This test allows us to evaluate agents or conditions that alter seizure threshold. In addition, maximal electroshock (MES), which stimulates large portions of the brain and is a suitable model for generalized tonic-clonic seizures, is also used to evaluate drug efficacy. In the MES test, animals are usually subjected to a 50 mA, 0.2 s, 60 Hz electrical

stimulus via corneal electrodes. Animals failing to show tonic hindlimb extension are scored as protected.

Kindling is a widely used model for epileptogenesis and seizure expression, and has been used to model complex partial seizures. Brief electrical stimulus trains (1-ms sine- or square-wave pulses, at a frequency of 60 Hz, for one second) are typically delivered via bipolar electrodes to a limbic structure (e.g., hippocampus or amygdala), to trigger after-discharge. With repeated stimulations, there is a progressive intensification of electrographic and behavioural seizures. In kindling studies, we measures the rate of kindling (number of stimuli required to reach a stage-5 seizure), after-discharge durations, and seizure stage. The kindled state is permanent, but may be altered by test compounds or experimental conditions.

Animal Models of Gender-Specific Epilepsy

There are few gender-specific models of epilepsy. Overiectomized or gonadectomized animals are commonly used to study the influence of gonadal hormones. Conventional models based on acute seizure induction are not suitable for studies on gender-specific forms of epilepsy such as catamenial seizures. There are two new types of models systems that partially represent catamenial epilepsy. The first simulates the rise and fall of estrogen and progesterone at the point in the estrous cycle associated with increased seizure susceptibility. Some examples of this type of model are: pseudopregnancy, chronic progesterone treatment, and a progesterone (or neurosteroid) withdrawal. The second category is based on the naturally-occurring estrous cycle or administration of exogenous hormones that simulate the specific stages of the estrous cycle in rats whose ovaries have been removed. We are using a rat model of perimenstrual catamenial epilepsy that was developed based on the premise that perimenstrual seizures are due to withdrawal of neurosteroids such as allopregnanolone. To assess the potential of drugs for treating catamenial epilepsy, a state of persistently high serum progesterone (pseudopregnancy) is induced in female rats with gonadotropins, and neurosteroids are withdrawn after 10 days with finasteride, a 5α-reductase inhibitor that blocks the conversion of progesterone to allopregnanolone. Finasteride treatment results in a reduction in the seizure threshold, and hence mimics perimenstrual seizure exacerbation. Plasma progesterone and neurosteroid levels are measured by liquid or gas chromatography and mass spectrometry.

Results

Hormonal Basis of Sex Differences in Seizure Susceptibility

Steroid hormones play vital roles in the neuroendocrine control of brain function. Adrenal-derived steroid hormones (glucocorticoids and mineralocorticoids), ovarian hormones (progesterone and estrogen), and hormones synthesized in the testis (testosterone and androgens) are essential for life. Steroid hormones play a key role in the gender-related differences in susceptibility to seizures. However, the precise mechanism underlying such sexual dimorphism is obscure. Studies have suggested that these sex differences in seizure sensitivity are due to gender-specific distribution of steroid hormones or other sexually dimorphic characteristics in specific brain areas relevant to epilepsy. Cyclical changes of ovarian hormones – estrogens and progesterone – are now widely believed to be important factors in fluctuations in seizure susceptibility observed in female cycling animal (**Figure 1**). Generally, progesterone has antiseizure effects, while estrogens have been generally shown to cause proconvulsant effects. There is emerging evidence that circulating steroid hormones serve as precursors for the synthesis of 'neurosteroids' – a term (coined by the French physiologist Etienne Baulieu) that refers to steroids that are synthesized de novo in the brain. Neurosteroids are endogenous modulators of neuronal excitability, and thus could play a vital role in gender-related differences in seizure susceptibility. Current experimental evidence indicates that progesterone- and testosterone-derived neurosteroids could be involved in sexual dimorphism in neural excitability and seizure susceptibility. For example, the progesterone-derived neurosteroid allopregnanolone is a powerful $GABA_A$ receptor-modulating agent with anticonvulsant properties. This neurosteroid shows a dose-dependent protection against PTZ seizures in both male and female mice lacking progesterone receptors. However, female mice exhibit significantly enhanced sensitivity to the protective activity of allopregnanolone as compared to males. In the pilocarpine seizure test, 3α-androstanediol has similar increased potency in female mice. Significant sex differences in responses to neurosteroids are also evident during alcohol withdrawal. These results underscore the possible role of GABAergic neurosteroids (such as allopregnanolone and 3α-androstanediol) in gender-related differences in seizure susceptibility and protection.

Progesterone, Neurosteroids and Catamenial Epilepsy

Progesterone plays a critical role in gender-related differences in seizure susceptibility. Progesterone is an anticonvulsant hormone. It has long been known to have antiseizure activity in animal models, and in clinical studies has also been found to reduce seizures. In many women with epilepsy, natural variations in progesterone levels during the menstrual cycle may influence seizure susceptibility. Seizures decrease in the mid-luteal phase when serum progesterone levels are high, and increase

premenstrually when progesterone levels fall (and decrease the serum progesterone to estrogen ratio). There is strong evidence that 'withdrawal' of progesterone is at least partly responsible for seizure exacerbation in the perimenstrual period.

There are two mechanisms by which progesterone affects reproduction and seizure susceptibility: binding to progesterone receptors and metabolism to the neurosteroid allopregnanolone. Neurosteroids such as allopregnanolone are synthesized from progesterone by two sequential A-ring reductions (**Figure 1**). Allopregnanolone is a potent, broad-spectrum anticonvulsant neurosteroid – a 'neurosteroid' that rapidly modulates neural excitability. Recently it has been discovered that allopregnanolone is a potent positive modulator of $GABA_A$ receptors. Structurally, $GABA_A$ receptors are believed to be pentameric, with five protein subunits that form the chloride ion channel pore. Allopregnanolone has distinct binding sites on $GABA_A$ receptors that are separate from those for benzodiazepines and barbiturates. Allopregnanolone binding to 'neurosteroid' sites on the $GABA_A$ receptor causes massive influx of chloride ions into the neuron, leading to postsynaptic hyperpolarization. This inhibitory mechanism almost certainly underlies allopregnanolone's protective actions against seizures.

To understand the gender-related antiseizure effects of neurosteroids, we examined the ability of progesterone and neurosteroids to protect against PTZ-induced seizures in male and female mice. In both male and female mice, progesterone and allopregnanolone protected against PTZ-induced seizures in a dose-dependent fashion (see **Table 1** for ED_{50} values). However, in male mice, the antiseizure ED_{50} value of progesterone as well as allopregnanolone was significantly higher than that of female mice, indicating an increase in the antiseizure potency of progesterone in female mice. These findings are consistent with previous reports that female animals are more sensitive to the central nervous system actions of progesterone than are males. The gender-related difference in antiseizure activity of neurosteroids is not due to known differences in the density or distribution of the PR in the female and male brain. However, it is not clear if sex differences in bioavailability or pharmacokinetics of drugs could contribute to such gender differences in neurosteroid actions.

Table 1 The antiseizure ED_{50} values (mg kg^{-1}) of progesterone and allopregnanolone in the PTZ test in male and female mice

Drug	*Male*	*Female*
Progesterone	106 (84–133)	70 (55–89)*
Allopregnanolone	15 (6–37)	7 (4–12)**

*$p < 0.05$ vs. male group (Litchfield and Wilcoxon X^2 test).
**$p < 0.05$ vs. male group (Fisher's exact test).
ED_{50} value (the dose at which 50% of tested mice are protected from seizures) with 95% confidence limits (CL) were determined by Litchfield and Wilcoxon procedure (N = 6–8 mice per group at each dose level).

A hallmark of epilepsy is the unpredictable occurrence of seizures. However, in many women with epilepsy, seizures do not occur randomly but cluster in association with the menstrual cycle, most frequently during perimenstrual and periovulatory phases. Recent studies indicate that 'catamenial' epilepsy affects from 39 to 60% of the women with epilepsy. Diagnosis of catamenial epilepsy is mainly based on the assessment of menstruation and seizure records for at least two cycles. The number of seizures in each phase is counted and a two-fold or greater increase in frequency is used as the criterion for diagnosis. There appear to be three patterns of catamenial epilepsy: perimenstrual, periovulatory and inadequate luteal-phase catamenial seizures, with the perimenstrual pattern most commonly observed. Currently, there is no specific drug therapy for catamenial epilepsy; a detailed understanding of the patterns and pathophysiology is essential for the development of rational approaches for the prevention and treatment of catamenial epilepsy.

Catamenial epilepsy is observed in women with ovulatory or anovulatory cycles. A variety of mechanisms, such as fluctuations in antiepileptic drug (AED) levels, changes in water and electrolyte balance, and physiological variation in ovarian hormone secretion are thought to contribute to catamenial epilepsy, with hormonal changes (in the circulating levels of estrogens and progesterone) during the menstrual cycle certainly playing a large role in the increased seizure susceptibility. There is emerging evidence that endogenous neurosteroids, including those derived from adrenal steroid hormones and circulating androgens, substantially influence seizure susceptibility. Because progesterone is a powerful anticonvulsant hormone, decreasing levels of progesterone (or neurosteroids) during the perimenstrual period could result in catamenial seizure exacerbation. Withdrawal from chronic progesterone or allopregnanolone has been shown to be associated with enhanced expression of $GABA_A$ receptor $\alpha 4$ subunit, a molecular signal for increased neuronal excitability and seizure susceptibility.

Several conventional AEDs are used in catamenial epilepsy therapy. However, catamenial seizures are often not successfully treated with these AEDs. Hormonal agents, such as progesterone and neurosteroids, may provide rational therapy for catamenial epilepsy. Progesterone supplementation has been shown to reduce catamenial seizure frequency in women with epilepsy. Using the catamenial epilepsy model, we evaluated the hypothesis that neurosteroid 'replacement' is an effective and rational therapy for catamenial epilepsy. Our results show that neurosteroids that positively modulate $GABA_A$ receptors have significant anticonvulsant potency in our

model, providing support for a neurosteroid 'replacement' approach to the treatment of catamenial epilepsy. Pilot clinical studies indicated beneficial effect with ganaxolone, but it is not yet evaluated in controlled clinical trials.

Estrogens, Aromatase and Seizure Susceptibility

Estrogens play an important role in seizure susceptibility. There are three biologically active forms of estrogen: 17β-estradiol, dominant in pre-menopausal women; estriol, the major form of estrogen during pregnancy; and estrone, which is prevalent after menopause. Estradiol has been shown in many studies to have significant proconvulsant effects. It facilitates various forms of induced seizures, and has been shown to worsen seizures in women with epilepsy. On a cellular level, estradiol, aside from its normal reproductive effects, enhances neural excitation and suppresses inhibition. It also leads to changes in the physical properties of some neurons – such as increase in excitatory dendritic spine density in the hippocampus – which are consistent with an increased potential for seizures. Therefore, the periovulatory catamenial exacerbation has been attributed to the midcycle surge of estrogen that is relatively unopposed by progesterone until early luteal phase. An increase in the ratio of estrogen-to-progesterone levels during perimenstrual period might at least partly contribute to the development of perimenstrual seizure exacerbation.

The effect of estrogens on cortical excitability and on seizure frequency, however, is controversial. There are also studies that support an inhibitory effect of estrogens on cortical excitability and an estrogen protection from cortical or hippocampal injury. Estradiol regulation of the hippocampal expression of glutamic acid decarboxylase (GAD), the principal enzyme for the synthesis of inhibitory neurotransmitter GABA, could lead to a decrease in seizure susceptibility. Moreover, estradiol reduces seizure-induced hippocampal injury in ovariectomized female but not in male rats, suggesting that the effects of estradiol on seizure threshold and damage may be determined by sex-related differences in the hormonal environment.

Aromatase is the key enzyme for the conversion of testosterone to 17β-estradiol, a neuroactive steroid that promotes seizures (**Figure 1**). The aromatase enzyme is expressed in discrete areas in the brain, such as hippocampus and neocortex, that are involved in epileptogenesis. Aromatase inhibitors could decrease brain excitability by decreasing local estradiol levels and, therefore, could be beneficial for the treatment of epilepsy. Consequently, three different aromatase inhibitors have been tested in men with epilepsy: testolactone, letrozole and anastrazole; preliminary data suggests a beneficial effect.

Testosterone, Androgenic Neurosteroids and Seizure Susceptibility

Testosterone is the primary circulating androgen, and it produces rapid modulation of neuronal excitability. Experimental studies with testosterone have shown both pro- and anticonvulsant effects, depending on the animal model and the seizure type. Epidemiological data indicate that the occurrence of epileptic seizures is ~50% higher in intact than in castrated animals. The incidence of epilepsy is ~15% higher in men than in women, and men with epilepsy exhibit testosterone deficiency. Consistent with this notion of testosterone involvement in seizure propensity, experimental studies with kainic acid and pilocarpine – two widely used convulsants for induction of chronic temporal lobe-like epilepsy – indicate that the males are more susceptible to the convulsant effects than females. The presence of testosterone, in intact and in gonadectomized males with testosterone replacement, increased the susceptibility to seizures. In contrast, testosterone and related androgens appear to have protective effects against seizures induced by chemoconvulsants. Moreover, studies in orchidectomized or castrated animals have shown that decreased testosterone is associated with higher incidence of seizures, and that replacement of testosterone in these animals attenuates seizures. Alterations in testosterone levels, therefore, may possibly contribute to exacerbation of seizures. However, the cellular mechanisms by which testosterone causes such bimodal effects on seizure susceptibility are unclear.

There are two potential mechanisms by which testosterone exert neuroendocrine control of brain function: binding to intracellular androgen receptors (genomic) and metabolism to neurosteroids (non-genomic). Testosterone-derived 'androgenic neurosteroids' could be involved in mediating the testosterone effects on neural excitability (**Figure 1**). However, very little is known about the potential role of androgenic neurosteroids in gender-related differences in seizure sensitivity. Testosterone is metabolized to neurosteroids via two distinct pathways, an 'androgen pathway' and an 'estrogen pathway' (**Figure 1**). In the androgen pathway, 3α-androstanediol is synthesized from testosterone by two sequential A-ring reductions. 5α-reductase enzyme first converts testosterone to the intermediate 5α-dihydrotestosterone, which is then further reduced by 3α-HSOR to form 3α-androstanediol. 3α-Androstanediol has been shown to be a positive modulator of $GABA_A$ receptors, with powerful anticonvulsant and protective effects. Thus, 3α-androstanediol could play a key physiological role in mediating the effects of testosterone on cortical excitability, seizure activity, and neuroprotection. In the estrogen pathway, testosterone is converted into 17β-estradiol by the aromatase enzyme (see above).

Testosterone modulation of seizure susceptibility is thought to occur through its conversion to neurosteroids

with 'anticonvulsant' and 'proconvulsant' actions, and hence the net effect of testosterone on neural excitability and seizure activity depends on the levels of distinct testosterone metabolites. Changes in brain androgenic neurosteroid biosynthesis could affect not only seizure susceptibility, but also conditions such as cognitive dysfunction. Since aging is associated with low levels of testosterone, the loss of testosterone may contribute to increased seizure propensity in the elderly. However, despite the antiseizure effects of testosterone-derived 3α-androstanediol (as seen in animal models), testosterone itself has not been reported to improve seizures clinically. One possible explanation is that AEDs that induce enzyme synthesis may enhance the conversion of testosterone to 17β-estradiol, and presumably reduce the net availability of testosterone for the synthesis of 3α-androstanediol. This conjecture is supported by the improved seizure control achieved with testosterone therapy when testosterone was used along with an aromatase inhibitor (testolactone) that inhibits 17β-estradiol synthesis.

Deoxycorticosterone, Stress and Seizure Susceptibility

Stress affects seizure susceptibility in both genders. Acute stress causes increased production of hypothalamic corticotrophin-releasing hormone (CRH), which increases both adrenocorticotropic hormone (ACTH) synthesis and secretion. Major physiological effects of ACTH result from its action on the adrenal cortex to increase the circulating levels of corticosteroids, principally cortisol. The adrenal cortex contains two anatomically and functionally distinct compartments: the outer zona glomerulosa, which secretes the mineralocorticoid aldosterone; and the inner zonae fasciculata/reticularis, which secrete the glucocorticoids cortisol as well as the adrenal androgens. Deoxycorticosterone (DOC), a mineralocorticoid precursor with anesthetic and antiseizure properties, is also produced in the adrenal zona fasciculata. Recent studies indicate that the antiseizure activity of DOC requires its enzymatic conversion to 3α,5α-allotetrahydrodeoxycorticosterone (THDOC), a neuroactive steroid that lacks classical hormonal properties but acts as a powerful positive allosteric modulator of the $GABA_A$ receptors (**Figure 2**).

It has been proposed that THDOC play a role in mediating DOC function in stress and related neuroendocrine conditions in males and females. Stress increases plasma and brain concentrations of corticosteroids and neuroactive steroids. Cortisol is the most important stress hormone in the hypothalamic pituitary adrenocortical system. However, a significant amount of DOC is also released during stress (which subsequently undergoes biotransformation to THDOC). Although the role of THDOC within the brain is undefined, recent studies indicate that stress induces THDOC to levels that can activate $GABA_A$ receptors. These findings have obvious implications for stress-sensitive conditions such as epilepsy and post-traumatic stress disorder. Moreover, it is widely believed that emotional factors can affect seizure control. In general, stressful events are associated with more frequent epileptiform discharges and seizures. However, acute stress has anticonvulsant-like effects in animals. Therefore, the exact pathophysiology of possible seizure facilitation by stress is unclear, but there are certainly many neural and endocrine pathways through which stress can alter excitability and thereby affect seizure susceptibility. The extent of seizure susceptibility during stress, in both males and females, is likely to reflect the balance between endogenous anticonvulsants (such as neurosteroids) and proconvulsant factors (such as glucocorticoids). Gender differences in stress-induced seizures may arise due to differences in the secretion of anticonvulsant or proconvulsant steroid hormones.

Directions for Future Research

Gender-related differences in epileptogenesis and AED therapy are not well studied. The major goal of epilepsy research is to characterize the biological basis of sex differences in susceptibility to seizure disorders and to develop rational therapeutic strategies for gender-specific epilepsies. Although steroid hormones and sexually dimorphic brain structures play an important role in gender-related seizure susceptibility, the precise neuroendocrine mechanisms underlying such sex differences in seizure occurrence and antiseizure drug actions remain unclear. Understanding the fluctuations in steroid hormones in men and women, and their influence on seizure susceptibility, is fundamental for developing gender-specific strategies for epilepsy therapy.

The effects of steroid hormones on neuronal plasticity are complex. Estrogens and testosterone have largely excitatory effects, while progesterone, DOC and neurosteroids are powerful anticonvulsants. There is emerging evidence on the role of neurosteroids in the regulation of GABAergic inhibition. Neurosteroid-based treatments are being developed for gender-specific conditions such as catamenial epilepsy. An NIH-funded study is evaluating the effects of progesterone in women with epilepsy. Future studies should focus on uncovering short- and long-term molecular changes in GABAergic and glutamatergic receptor systems in relation to natural fluctuations in steroid hormones. A major challenge in the field of neurosteroids is to develop specific antagonists for neurosteroid sites on GABA-A receptors. Further studies are, therefore, clearly warranted to uncover the molecular mechanisms of neurosteroid actions and the role of neurosteroids in men and women with epilepsy.

Acknowledgements

This research work was supported in part by the National Institute of Neurological Disorders and Stroke, NIH Grants NS051398 and NS052158. The author thank Philip Schwartzkroin for his editorial comments.

See also: **Hormones and Gender**: Female Sex Steroids and Neuronal Excitability; Hormonal Modulation of Neuronal Excitability; Hormones and Epilepsy; Hormones and Seizures; Male Sex Steroids and Neuronal Excitability; Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; Sex and Seizure Sensitivity; Sex Differences in Seizure Sensitivity.

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IMAGING

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Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In Vitro

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Introduction

Epilepsy is a paroxysmal neurological disorder characterized by synchronous high voltage discharges arising from a population of hyperexcitable neurons. Approximately 0.5–3% of the world population suffers from epilepsy. In most patients with epilepsy, the prognosis for seizure control is good. However, 20–30% of epileptic patients develop intractable epilepsies that are unresponsive to treatment, despite the advent of new antiepileptic drugs and development of intensive monitoring. Advances in brain imaging and seizure mapping techniques have made surgery an option for patients in whom a clearly defined, surgically approachable epileptic focus can be identified. The interest in surgical treatment for epilepsy over the past decades has been accompanied by an increasing opportunity for basic neuroscientists to perform research on epileptic patients (and tissue resected from these patients), not only to validate observations made in animal investigations but also to characterize specific aberrant phenomena in human epileptic brain. Surgical resection provides tissue that can be used for electrophysiological, morphological, biochemical, and molecular biological investigations. The availability of human brain tissue resected from patients undergoing neurosurgical treatment for refractory epilepsy has provided investigators with the opportunity to identify abnormalities promoting hyperexcitability in epileptic tissues, and to develop hypotheses regarding the underlying pathophysiological mechanisms of epilepsy. Such phenomena can then also be modeled in animals, and studied at a mechanistic level in order address specific novel hypotheses.

Background

Revealing the three-dimensional functional organization of a given cortical area is a key step towards understanding the mechanisms of information processing in that area. Thus, experimental methods that allow the visualization of functional organization across large areas of cortex are of special importance. Voltage-sensitive dye imaging provides the possibility for recording neuronal activity with adequate temporal and spatial resolution. Optical techniques, using a multiple-element photodiode array, have facilitated simultaneous multiple site recordings of

electrical activity in a given preparation. Optical imaging with voltage-sensitive dyes permits the visualization of cortical activity with a sub-millisecond time resolution and a spatial resolution of 50–100 μm. Other advantages of this method are: (i) the direct recording of the summed intracellular activity of neuronal populations, including fine dendritic and axonal processes; (ii) the possibility of repeated measurements from the same cortical region under different experimental and/or stimulus conditions over an extended period of time; and (iii) selective visualization of neuronal assemblies (**Figure 1**).

Methods

Our studies were carried out on slices of human neocortical tissue, excised for treatment of pharmacoresistant temporal lobe epilepsy. From a 1-cm^3 block of the inferior temporal gyrus, 450-μm coronal sections were made with a vibratome within 5 min after resection. These slices were placed in a portable incubation chamber with oxygenated (95% O_2–5% CO_2) and warmed (28 °C) artificial cerebrospinal fluid (ACSF) containing (in mM): 124 NaCl, 4 KCl, 2 $CaCl_2$, 1.24 NaH_2PO_4, 1.3 $MgSO_4$, 26 $NaHCO_3$, and 10 glucose (pH 7.35–7.45). Slices were stained with 12.5 μg ml^{-1} of the voltage-sensitive dye RH795 1 h prior to the experiment, and washed for 1 h before they were transferred to a submerged-type recording chamber (32 °C) mounted on an inverted microscope. Field potentials were recorded from cortical layers II/III and V using conventional glass micropipettes filled with ACSF. Slices were scanned electrophysiologically for spontaneous rhythmic epileptiform sharp waves (SRSW), by repositioning field potential electrodes to at least six different sites, distributed roughly equally across the slice.

Dye-related fluorescence signals were detected by a 464-element honey-comb-shaped photodiode array, at a rate of 785 frames per second; images were obtained via 10× and 20× objective lenses, yielding total fields of view of 1500 and 750 μm diameter, respectively. Xenon lamp illumination was allowed for a maximum of 4 s per single detection period, and detection periods were separated by at least 5 min each. Optical data were assessed as fluorescence changes relative to resting light intensity (dI/I) using NeuroPlex software (RedShirt Imaging, LLC, Fairfield, CT), precluding signal variability due to inhomogeneous illumination or differences in lens properties. For trace illustrations, optical and field potential signals were low-pass-filtered (Butterworth filter, 30 or 50 Hz). Unfiltered fluorescence changes were converted to pseudocolor images scaled to the center diode. The fluorescence change dI/I for typical event was 0.05–0.3% of resting light intensity. The correlation of the dye signal and bioelectrical neuronal activity is explained in **Figure 1**.

Results

Voltage-Sensitive Dye Imaging

In previous studies, spontaneous rhythmic epileptiform sharp waves (SRSW) were found to occur spontaneously in 20–30% of human epileptic neocortical slices. Such spontaneous potentials are characteristic of chronically epileptic human brain tissue, as these potentials cannot be observed in animal tissue, nor in human brain slices obtained from tumor resections from patients without clinical signs of seizures. Although synchronous rhythmic waves do not resemble typical ictal activity (as elicited in different in vitro models), these potentials reflect a heightened propensity of network synchronization. Spontaneous rhythmic potentials are accompanied by extended focal neuronal hyperpolarization and depolarization. As these potentials reflect a state of increased neuronal synchronization, it was suggested that spontaneous sharp waves might facilitate initiation or spread of seizures under certain conditions.

The epileptiform activity pattern in human tissue differed considerably across tissue samples. Localized and spatially inhomogeneous neuronal depolarizations, as determined by voltage-sensitive dye recordings, were found to underlie these sharp waves. Hot spots of neuronal depolarizations were spatially dynamic. With gradual build-up of the SRSW, an increasing number of photodiodes detected depolarizations, reaching a maximum at ~14 ms after the peak of the SRSW. Depolarizations at individual diodes could be distinguished at all times during the recording, creating dynamic activation patterns of activity: only rarely, at the peak of the sharp wave event, did some diodes with maximal activity converge to cover larger fields. Other diodes showed a decrease of noise instead of a clear-cut signal, possibly a sign of disfacilitation. Although maximal neuronal depolarizations were thus inhomogeneously distributed, SRSW-associated depolarizations were a population phenomenon. Before the SRSW, only 2% of the total area of the optical recording field showed maximal depolarization changes, possibly reflecting asynchronous spontaneous action potentials of single neurons or small neuronal clusters.

In experiments on brain slices from human epileptic cortex, we attempted to increase tissue excitability by superfusion of low Mg^{2+} solution. This treatment had no significant effect on the spatiotemporal distribution of epileptiform discharge in 70% of tissue samples. However, in 30% of these slices, the activity pattern appeared atypical with respect to the extent of spread of activity (both vertically and horizontally). Maximal stimulation in white matter evoked depolarizations of high amplitude, both in infragranular and in supragranular layers of the temporal neocortex – a pattern not normally seen in nonsensory neocortex. In addition, stimulation in layer

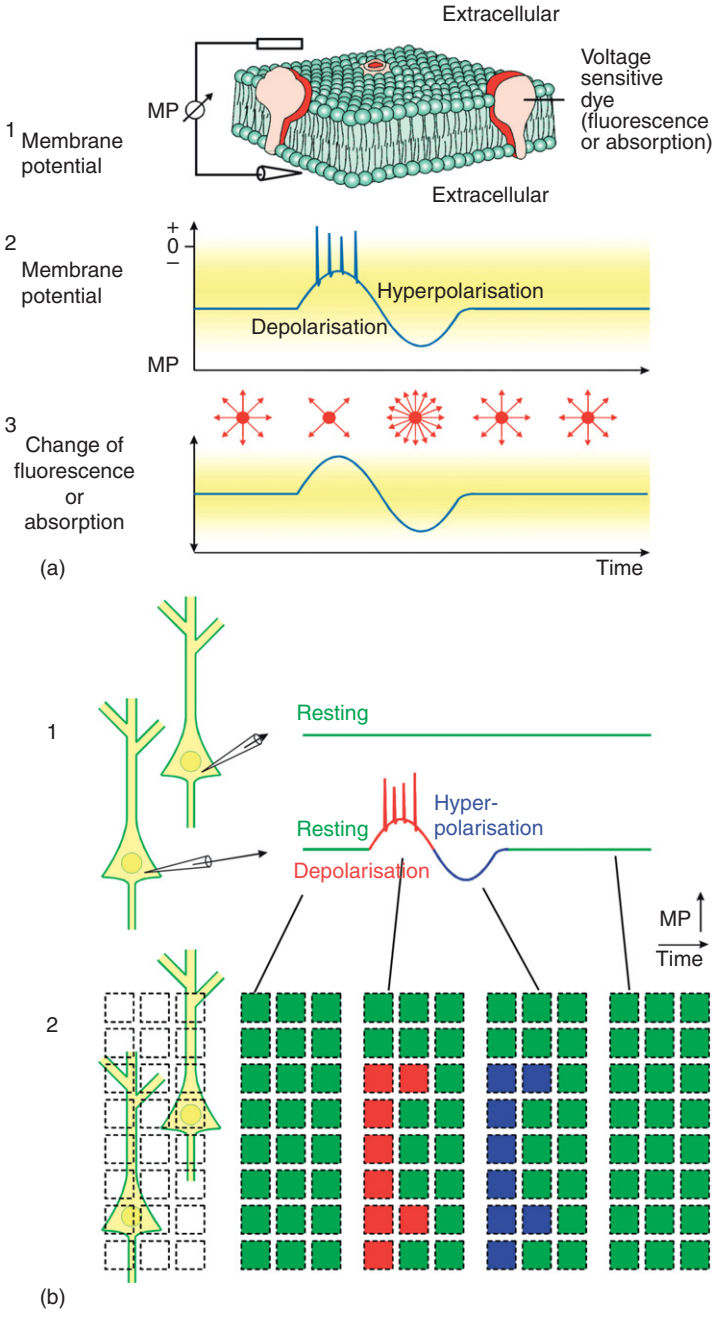


Figure 1 Recordings of neuronal membrane potentials using voltage-sensitive dye imaging. (a) 1 – the dye binds to the lipid bilayer of the membrane and changes in transmembrane potential are reflected in the spectral properties of the dye. Simultaneously, the membrane potential is recorded with an intracellular microelectrode. The voltage sensitive dye produced fluorescence changes when excited at the proper wavelengths. 2 – Changes of the membrane potentials from resting levels by an initial depolarization followed by hyperpolarization. 3 – Corresponding to the changes of the membrane potentials the fluorescence or absorption of the dye varied. With fluorescence dyes depolarization leads to a decrease and hyperpolarization to an increase of radiation (marked by symbols). (b) 1 – Schematic diagram of two neurons; one of which is in the resting state, the other one shows changes in the membrane potentials in depolarizing and hyperpolarizing direction. 2 – With the aid of microscope coupled with matrix photodiodes the different bioelectrical activities of the two neurons can be recorded via different optical behavior. Adapted from Speckmann E-J (2008) Das Gehirn meiner Kunst. Kreativität und das selbstbewusste Gehirn. Münster: Daedalus Verlag.

I evoked activity in all supragranular layers during control condition. However, in some human slices, activity evoked during superfusion with Mg^{2+} free solution was limited to the region in the direct neighborhood of the stimulation electrode in layers I/II. In these slices, the evoked activity pattern typically seen during Mg^{2+}-free superfusion was already apparent under control conditions. Furthermore, in other slices, layer I stimulation evoked 'islands' of

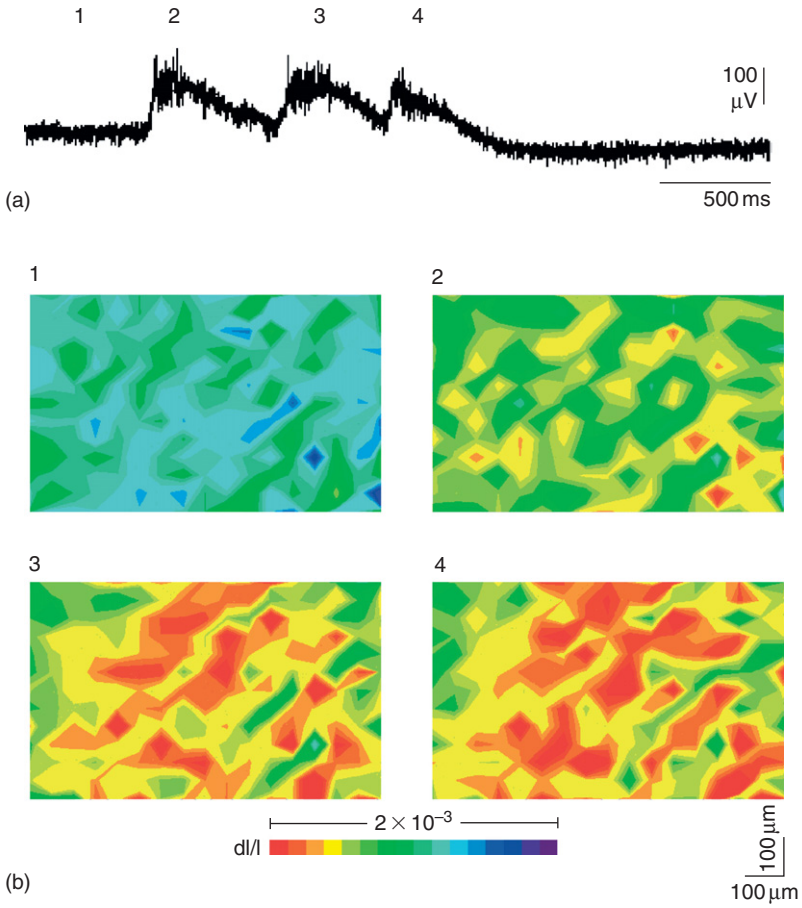


Figure 2 Optical imaging of spontaneous epileptiform burst activity (*area plot*) during application of Mg^{2+}-free solution in human temporal neocortical slices of epileptic patients. (a) Fluorescence signal of a single diode positioned in supragranular layers. The *numbers* indicate the time points at which area plots were constructed. (b) Pseudocolored area plots of fluorescence changes. Area plots (1–4) constructed at different time points of a spike wave complex as shown *above* (a). Calibration of relative fluorescence changes is given at the *bottom* of (b). Adapted from Speckmann E-J (2008) Das Gehirn meiner Kunst. Kreativität und das selbstbewusste Gehirn. Münster: Daedalus Verlag.

excitation, which were inhomogeneously scattered over the whole slice. Clear boundaries usually found around layer IV were missing. The described activity pattern correlates in principle with the neuropathological diagnosis of laminar disorders, as in fact was found for the demonstrated case. These data show that dye imaging can be quite effective in demonstrating abnormal organization and intrinsic epileptiform properties of some resected tissues from epilepsy surgery (Figure 2).

Directions for Future Research

Important future discoveries in this field will require further development of imaging techniques. These techniques should be capable of comparing data obtained from epileptic brain before the surgical intervention (preoperative studies), on the intervention itself (intraoperative studies), and on the period when the part of the brain that has to be removed (postoperative studies). Such technical advances may lead to a better diagnosis and treatment of medically refractory epilepsy.

See also: **Imaging**: Optical Imaging in Epilepsy Research: Overview of Methods and Applications; Optical Monitoring of Circuitry Activity In vitro; Using Intrinsic Optical Signal Changes to Analyse Epileptic Activity and Seizures; **Metabolic Changes**: Glucose Metabolism and $GABA_A$ Receptor Dysfunction in Human Partial Epilepsy; **Non-Synaptic Mechanisms**: Ionic Microenvironment in Living Human Brain and Epileptic Seizures.

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Functional MRI in Basic Epilepsy Research

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Introduction

Functional neuroimaging encompasses several important techniques used to approximate neuronal activity throughout the brain during normal and pathophysiological states. These techniques have proven useful particularly in the study of epileptic seizures, which often involve significant alterations in activity affecting widespread networks throughout the brain. Although functional imaging studies of seizures in animal models have lagged considerably behind investigations of human patients with epilepsy, the recent burgeoning of functional magnetic resonance imaging (fMRI), as well as technological advances in other neuroimaging methods, has resulted in an increase in basic imaging studies of epileptic states. These developments provide a novel and exciting opportunity for epilepsy researchers, as unlike human imaging studies, animal models allow experimenters to control seizures onset and type as well as employ various therapeutic interventions. Furthermore, functional neuroimaging can serve to complement invasive electrophysiological techniques (which possess excellent temporal resolution but limited spatial sampling), as well as histological studies (which permit detailed spatial sampling and resolution but are limited to a single point in time), to allow dynamic mapping of activity in the epileptic brain.

Epilepsy represents a heterogeneous group of chronic disorders associated with intermittent pathophysiological neuronal activity. Thus, one major goal of functional imaging studies of epilepsy is to obtain accurate characterization of neuronal activity within focal brain regions and related networks, as they are affected by each different type of seizure. Knowledge of which brain areas are involved in specific kinds of seizures may facilitate the discovery of targeted pharmacological, surgical, or genetic therapies aimed at interfering with the development or progression of epilepsy. Finally, in addition to mapping seizure origin and propagation, functional neuroimaging can provide a useful tool for understanding the effects of epilepsy on brain networks outside the seizure focus – information that may be relevant to functional brain impairment and cognitive dysfunction during ictal and interictal periods.

The major functional imaging techniques currently employed in animal studies to address these issues include fMRI, positron emission tomography (PET), and magnetic resonance spectroscopy (MRS). Given that each major neuroimaging technique employed in basic epilepsy research measures a different aspect of neurophysiology, the best method to utilize in a particular study is heavily dependent on the nature of the research question. For instance, while most PET and MRS measurements have relatively low temporal resolution, fMRI images can be acquired sequentially in the same experiment, up to a frequency of several per second. Thus, fMRI is a useful tool for tracking changes in neuronal activity during individual epileptic events with relatively high temporal and spatial resolution. In contrast, although PET experiments typically require serial acquisitions over longer time, the increasing availability of novel tracers allows investigation into specific biomolecular changes across multiple

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imaging sessions. This capability makes small animal PET (saPET) a particularly useful tool for studying the slower biophysiological changes inherent to epileptogenesis. Furthermore, MRS can be used to detect subtle metabolic and neurochemical differences in epileptic tissue with high sensitivity and specificity.

The remainder of this article will focus on the use of fMRI in epilepsy research; other articles will discuss other imaging modalities in more detail.

Background

Neuroimaging Signals are Indirectly Related to Neuronal Activity

Although functional neuroimaging studies are used to assess neuronal activity during various states, all neuroimaging signals are only indirectly related to neuronal activity. Neuronal activity encompasses several energy-requiring processes, including presynaptic action potentials, Ca^{2+} currents, neurotransmitter release and repackaging, and postsynaptic potentials. However, functional imaging techniques such as fMRI, PET, and MRS measure changes in cerebral oxygenation, cerebral metabolism and blood flow, and brain metabolite and neurotransmitter concentrations, respectively. Thus, interpretation of functional neuroimaging studies depends on the assumption that alterations in neuronal activity are highly correlated with changes in these neurophysiological parameters, and the inherent limitations of this assumption must be considered carefully.

The Biophysical Basis of the BOLD Signal

In 1936, the Nobel laureate chemist Linus Pauling and his student Charles Coryell discovered that the hemoglobin molecule has magnetic properties that differ depending on whether it is bound to oxygen. While oxygenated hemoglobin is diamagnetic, having no unpaired electrons and zero magnetic moment, deoxygenated hemoglobin has a significant magnetic moment. In the late 1980s, Seiji Ogawa correctly hypothesized that the magnetic susceptibility of deoxyhemoglobin would cause nuclear spin dephasing upon its introduction into the magnetic field of an MRI machine, resulting in a decay of transverse magnetization that depends on the time constant T_2^*. Thus, by inducing a decrease in T_2^*, deoxyhemoglobin acts as an endogenous paramagnetic contrast agent. As a result, the blood oxygen level dependent (BOLD) fMRI signal intensity reflects net oxygen delivery to the brain, which in turn depends on a balance between cerebral blood flow (CBF), cerebral blood volume (CBV), and the cerebral metabolic rate of oxygen consumption ($CMRO_2$).

Interpreting fMRI Results

A well supported theory in the study of neurovascular responses is that an increase in neuronal activity results in enhanced cerebral metabolism, and a compensatory increase in CBF. The increase in CBF usually exceeds metabolic need, resulting in a net increase in oxygenation. Therefore, an increase in neuronal activity typically results in an overall increase in oxygenation and a BOLD activation. The implications of this finding have allowed fMRI to emerge as a powerful tool in the study of both physiological and pathophysiological brain states, such as epilepsy. However, a more controversial question in fMRI has been whether diminished neuronal activity will produce an overall decrease in oxygenation and a BOLD deactivation, and whether fMRI deactivations always represent decreased neuronal activity. Although commonly reported, negative BOLD signals have been less thoroughly studied and controversy over their origin has led to some uncertainty in their interpretation.

Methods

To date, human fMRI studies of epilepsy have far outnumbered basic animal studies of the disease. However, because of issues related to movement artifact, investigations in humans have predominantly been restricted to recordings of nonconvulsive absence (spike-wave) seizures and interictal events. For this reason, anesthetized and paralyzed animal models provide a unique opportunity to study various seizure types in a controlled environment with high-field fMRI. An additional advantage of using animal seizure models in fMRI investigations is the ability to corroborate changes observed in the BOLD signal using invasive electrophysiological techniques in the same or similar preparation. This benefit is particularly useful given controversy in directly relating BOLD signal changes to alterations in neuronal activity.

Using anesthetized and paralyzed rat models, our laboratory has successfully collected high-field fMRI and simultaneous electroencephalogram (EEG) recordings of both convulsive and nonconvulsive seizures, including models of both generalized and partial epilepsy. Previous human neuroimaging studies have examined absence (petit mal) epilepsy, a generalized seizure disorder characterized by 5–10 s episodes of unresponsiveness that are associated with spike-wave discharges (SWD) on EEG. However, these studies have produced inconsistent results regarding whole-brain energetics, and have not clearly identified which specific brain areas are involved. To begin to clarify these discrepancies, we have performed both 7 T and 9.4 T fMRI with simultaneous EEG recordings of SWD in the Wistar Albino Glaxo rat of Rijswijk

(WAG/Rij) rat, an established model of absence epilepsy in which animals display frequent, spontaneous spike-wave seizures.

All fMRI studies of SWD to date have been performed in WAG/Rij rats, genetic absence epilepsy rats from Strasbourg (GAERS), or through chemically-induced spike-wave seizures induced using gamma-butyrolactone (GBL) in normal rats. For simultaneous fMRI/EEG studies of SWD, animals are anesthetized with fentanyl and haloperidol injections every 45 min, paralyzed with tubucurarine to prevent movement, and artificially ventilated. The fentanyl/haloperidol regimen is a drug combination that provides reliable anesthesia without suppressing SWD. A 30 min run of simultaneous EEG (using carbon filament electrodes under the scalp) and fMRI typically allows sufficient time to record 20–50 SWD. Imaging 10–12 coronal slices of 1-mm thickness over 1 s with a 5 s break in between each acquisition permits ample artifact-free EEG recordings to conveniently distinguish images corresponding to 'seizure' versus nonseizure 'baseline.' Generating t-maps comparing images between these two states then allows the identification and quantification of fMRI signal differences in brain regions involved in the SWD.

In contrast to nonconvulsive absence epilepsy, no human fMRI recordings have been collected during generalized tonic–clonic (grand mal) seizures. This gap provides a particularly important opportunity for animal studies of this form of epilepsy. While investigations of spontaneous generalized tonic–clonic seizures have not yet been performed, fMRI studies of seizures induced by chemoconvulsant agents have been completed by several laboratories. For instance, our laboratory has collected recordings of tonic–clonic seizures in anesthetized–paralyzed rats induced acutely by bicuculline administration. After fMRI and EEG recordings of 5 min baseline period, an intravenous injection of bicuculline produces generalized seizures within several minutes. Comparing functional images acquired during baseline with those collected continuously throughout the ictal and postical periods allows the tracking fMRI signal changes throughout the brain during and after these tonic–clonic seizures.

Recent Results

WAG/Rij rats display frequent spontaneous spike-wave seizures that are not blocked by fentanyl/haloperidol anesthesia, thus providing a convenient model to study SWD using simultaneous fMRI and EEG recordings. During these SWD in anesthetized/paralyzed animals, our laboratory observed BOLD signal increases (compared with baseline) in several cortical and subcortical regions, most prominent in the somatosensory cortex, motor cortex, thalamus, and brainstem (**Figure 1(a)**). Recent studies from our laboratory, and work by others, have suggested that fMRI decreases occur in the basal ganglia during SWD in rats. Notably, fMRI signal changes did not involve the whole brain uniformly, as occipital regions were largely unaffected (**Figure 1(a)**). Thus, although SWD are considered generalized epileptic events, these experiments suggest focal involvement of specific brain regions with relatively sparing of other areas. Bench-top experiments have also been performed on animals under the same conditions to measure both electrophysiological signals and CBF (using laser Doppler flowmetry probes). We have observed that recordings in regions showing increased fMRI signals during SWD, such as the barrel somatosensory cortex, display increased CBF, and neuronal firing (**Figure 1(b)**). In contrast, little (if any) change in neuronal firing and CBF was seen in areas lacking BOLD signal changes, such as the primary visual cortex (V1M) (**Figure 1(b)**).

Using anesthetized, paralyzed rats, our laboratory recently completed a multimodal fMRI and electrophysiology study of generalized tonic–clonic seizures induced by intravenous administration of the proconvulsive agent bicuculline. During seizures, large BOLD signal increases were seen in thalamic and widespread cortical regions (**Figure 2(a)**). These changes resembled those recently reported by others during generalized tonic–clonic seizures induced by the drug pentylenetetrazole. Interestingly, however, we also observed ictal fMRI decreases in the hippocampus during several of the recorded events (**Figure 2(a)**). These hippocampal decreases seemed perplexing, as limbic structures, such as the hippocampus are often intensely involved in generalized tonic–clonic seizures. To clarify this unexpected finding, we directly measured neuronal activity and CBF during seizures in this model, in both the hippocampus and cerebral cortex, using microelectrodes and laser Doppler flowmetry probes. These recordings showed that despite opposite BOLD signal changes in the hippocampus and the cortex, both regions showed large increases in CBF, local field potentials (LFP), and multiunit activity (MUA) during seizures (**Figure 2(b)**). Furthermore, whereas the ictal elevations in CBF in both regions were of similar magnitude, MUA showed significantly greater increases in the hippocampus than in the cortex. These results suggest an uncoupling of the BOLD signal from CBF and neuronal activity in the hippocampus of this seizure model, likely resulting from an abnormally elevated metabolic rate ictally that exceeds the delivery of oxygen. This observation serves as an important cautionary reminder that BOLD signal changes may not directly represent similar alterations in neuronal activity. Thus, while fMRI studies can provide significant useful information regarding network involvement of various seizure types, results should be corroborated using alternate means.

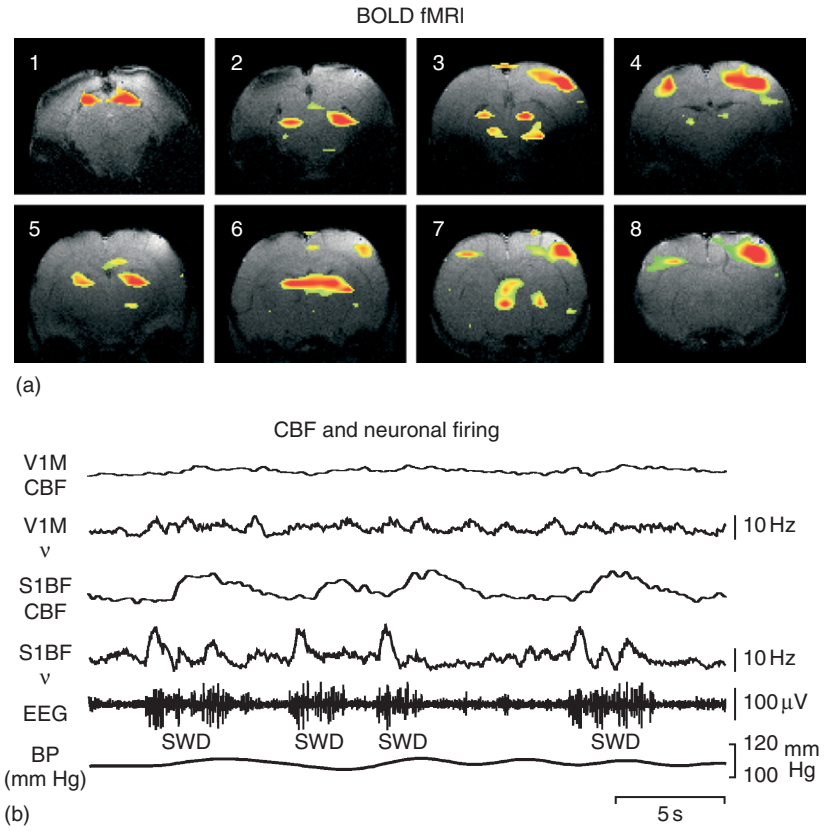


Figure 1 fMRI and electrophysiology recordings during spike-wave discharges (SWD). (a) Example of BOLD fMRI activations during spike-wave discharges. fMRI signal increases during SWD in a WAG/Rij rat under fentanyl-haloperidol anesthesia. t-maps were calculated from 23 SWD pairs recorded during one uninterrupted experimental run and were overlaid onto corresponding high-resolution anatomic images. Eight coronal slices were acquired from back to front (numbered 1–8) at 1-mm intervals, with the first slice at approximately −7.04 and the last slice at +0.40 mm relative to bregma. Bilateral and relatively symmetrical increases in BOLD signal were present mostly in frontoparietal cortex, thalamus, and brainstem nuclei, whereas occipital regions did not show significant changes. (b) Dynamic recordings of CBF and neuronal activity during SWD. Changes in CBF and neuronal activity were recorded by combined Doppler flowmetry and extracellular multiunit recordings obtained simultaneously during multiple episodes of SWD, along with EEG and arterial blood pressure (BP) monitoring in a WAG/Rij rat under fentanyl/haloperidol anesthesia. Spontaneous SWD induce parallel increases in CBF and neuronal firing rate (ν) in barrel cortex (S1BF), and no or very small increases in primary visual cortex (V1M). CBF variations are independent of arterial BP fluctuations. **Figure 1(a)**: reproduced with permission from Nersesyan *et al*. (2004a); **Figure 1(b)**: reproduced with permission from Nersesyan *et al*. (2004b).

To date, most fMRI reports of basic animal epilepsy models have focused on generalized epilepsy, although researchers in our laboratory and others elsewhere have begun to investigate partial seizures. Such studies of partial epilepsy are particularly important, as temporal lobe epilepsy, one of the most common seizure disorders in humans, most frequently presents as a partial seizure disorder. Furthermore, fMRI studies of partial seizures will not only allow insight into the focal regions involved in the seizure, but also provides an opportunity to study the effects of partial seizures on distant uninvolved brain networks. Recent work in our laboratory in a limbic seizure model has demonstrated that during focal seizures, there are fMRI signal increases in the hippocampus, but decreases in the frontal association cortex. Electrophysiology recordings in the frontal association cortex during seizures revealed cortical slow oscillations, resembling those seen in states of deep sleep or anesthesia. These remote network effects of focal seizures may explain behavioral impairment such as loss of consciousness during limbic seizures.

Future Goals

Rapid advances in magnetic resonance technology, including the production of faster and higher field strength hardware, and the development of improved analysis software, have led to increased opportunity for basic fMRI investigations of seizure disorders. In addition, various techniques have emerged that allow experimenters to utilize fMRI not only to evaluate the BOLD signal, but also to measure other hemodynamic responses in the brain. Notably, CBV can be measured throughout the brain using

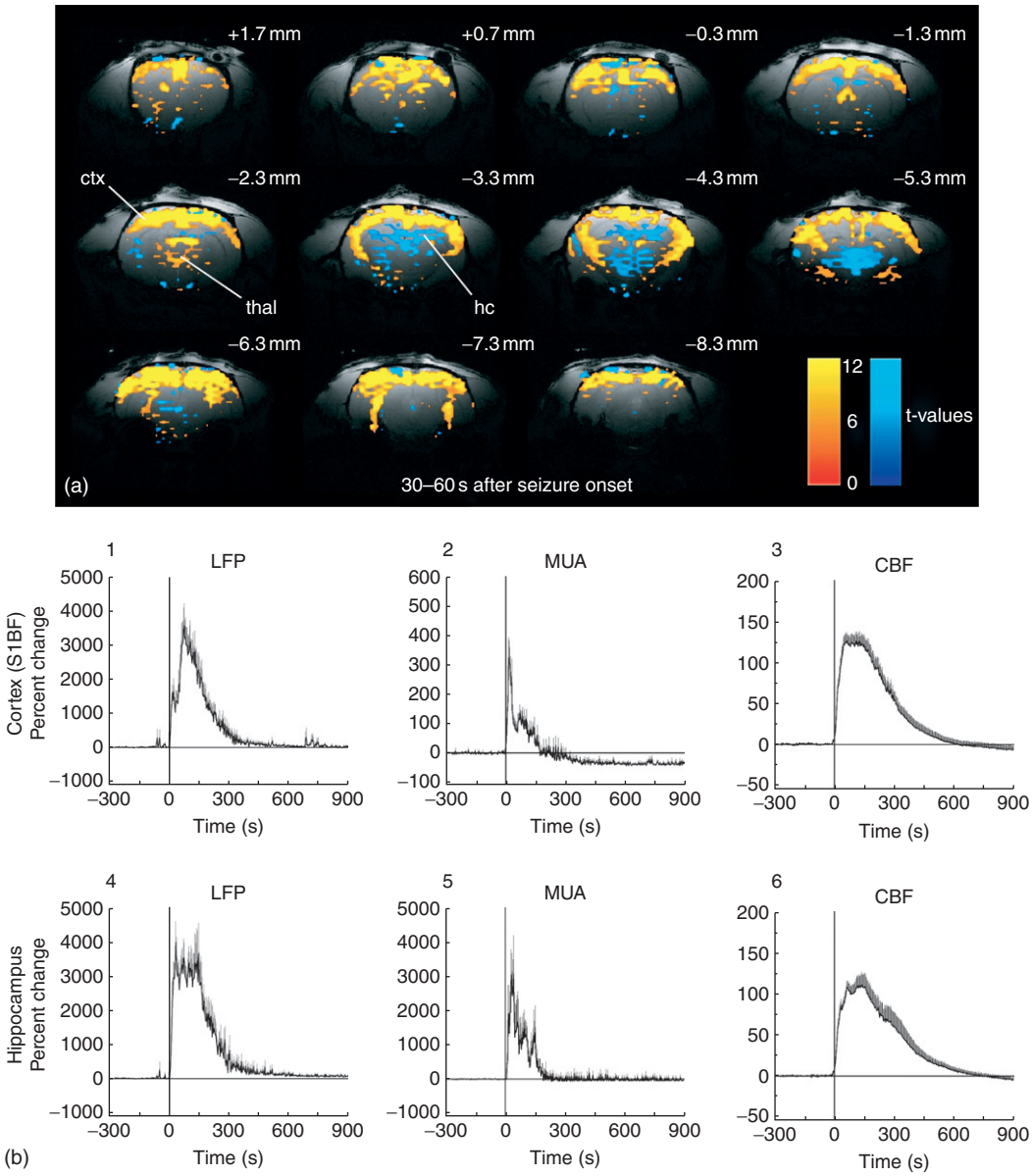


Figure 2 Changes in BOLD signal, CBF, and neuronal activity during bicuculline-induced generalized seizures. (a) Example of BOLD increases and decreases during bicuculline-induced generalized seizures. During the seizure, the BOLD signal showed not only a mixed pattern of widespread increases across the whole brain, including cortex (ctx) and thalamus (thal) but also prominent focal decreases, especially in hippocampus (hc). t-maps are shown for 30 s of data (10 consecutive fMRI images acquired every 3 s) during seizure compared with 30 s of baseline. Maps are superimposed on high-resolution anatomical images. Slices are shown from anterior to posterior, with approximate coordinates relative to bregma. Color bars indicate *t*-values for increases (warm colors) and decreases (cold colors). (b) Neuronal activity and CBF in both cortex (S1BF) and hippocampus show only increases during bicuculline-induced generalized seizures. Average time courses of percent signal change compared with 30 s of baseline data. Time courses are depicted as mean ± SEM (unidirectional only) and straight vertical line at time 0 marks seizure onset. Time courses on the top represent recordings for LFP power (1), MUA power (2), and CBF (3) in the barrel field cortex (S1BF), and on the bottom for LFP power (4), MUA power (5), and CBF (6) from hippocampus. Recording sites were matched to ROIs chosen in fMRI experiments. During seizures, LFP (1) and MUA (2) showed a marked increase in power in S1BF, accompanied by strong increases in CBF (3). Interestingly, hippocampal recordings also showed intense signal increases for LFP (4), MUA (5), and CBF (6) during seizures that were in marked contrast to the only slight changes or even decreases observed in the BOLD signal in the hippocampus. The signal changes in MUA power in the hippocampus during the first 150 s of seizures significantly exceeded those in the cortex ($P = 0.004$, 2-tailed unpaired *t*-test, $n = 43$). Note that *y*-axes in (2) and (5) are not drawn to the same scale. In both regions, the MUA also revealed a decrease in power after termination of the seizures, representing postictal depression commonly observed with this seizure type (postictal depression not visible in (5) due to scale). In (1–6) mean signals are based on the following number of experiments/animals: for cortex (S1BF) LFP (42/24), hippocampus LFP (38/22), cortex (S1BF) MUA (27/16), hippocampus MUA (16/10), cortex (S1BF) CBF (39/23), and hippocampus CBF (35/21). Adapted with permission from Schridde *et al*. (2008).

intravenous paramagnetic contrast during fMRI recordings, and CBF changes can be tracked by employing arterial spin labeling (ASL) and other techniques. Owing to this flexibility, investigators are now able to employ a multimodal approach to fMRI. Furthermore, by recording changes in the BOLD signal, CBV, and CBF during seizures, one can calculate high spatiotemporal resolution maps of the cerebral metabolic rate of oxygen consumption ($CMRO_2$) throughout the brain during these events using what is termed the 'calibrated BOLD' method. $CMRO_2$ is the most direct indication of neuronal activity short of simultaneous invasive electrophysiological recordings. Given that the BOLD signal is an indirect assessment of neuronal activity, the magnitude (or even sign) of BOLD signal changes might not accurately reflect true changes in neuronal activity throughout the brain. This problem was illustrated in the example described earlier of uncoupling between the BOLD signal and CBF in the hippocampus during generalized tonic–clonic seizures. Thus, the increased use of multimodal approaches to fMRI investigations will be an important advance in epilepsy neuroimaging, providing investigators with an opportunity to address the ambiguity inherent to BOLD signal interpretation.

In addition to the availability of multimodal neuroimaging techniques, the increased use of MRI-compatible recording electrodes has further improved interpretability of fMRI by allowing concurrent imaging and electrophysiology recordings within the same experiment. Performing simultaneous electrophysiology recordings with deep microelectrodes during fMRI studies was recently pioneered by Nikos Logothetis and colleagues in animal studies of nonepileptic states, although use of the technique in epilepsy studies has been limited. Our laboratory is now completing a study of partial seizures in rats during simultaneous fMRI, EEG, and hippocampal local field potential recordings. Although technically more challenging than unimodal fMRI studies, electrophysiological recordings provide the most direct assessment of neuronal activity and corroboration of BOLD signal changes.

Given the expanded availability and utility of current neuroimaging tools, there is currently an exciting potential for further fMRI studies of seizures. Although an understanding of its limitation and the use of corroboratory methods are essential, with proper utilization, fMRI will continue to offer a useful and versatile tool for studying epilepsy.

Acknowledgements

This work was supported by NIH R01 NS049307, P30 NS052519, and by the Betsy and Jonathan Blattmachr Family.

See also: **Epileptogenesis**: Surrogate Markers for Epileptogenesis; **Imaging**: Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In vitro; Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Optical Monitoring of Circuitry Activity In vitro; SPECT in Epilepsy Research; Structural Magnetic Resonance Imaging of Epilepsy; **Metabolic Changes**: Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain.

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Optical Imaging in Epilepsy Research: Overview of Methods and Applications

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Introduction

From DNA transcription within a single nucleus to cortical oscillations involving large networks of interacting neurons, different neurobiological activities have their natural temporal and spatial scales over which they are best characterized and studied. A variety of optical imaging methods are now available that are able to capture data from living brain tissue over a remarkable range of scales in time and space. For example, the location and timing of gene expression within individual cells can be measured using transgenic expression of fluorescent proteins, and patterns of neuronal firing activity over centimetres of intact cortex can be mapped using voltage-sensitive dyes. The applicability of an imaging method to a particular problem depends on whether the anatomical structures, molecules, or ions of interest can be selectively labeled with an optical reporter or dye, and whether the labeled target can be detected with an optical system.

All optical imaging techniques share some common features: a preparation is illuminated with light at one or several specific wavelengths, and photons that are reflected, absorbed, or fluoresced from the preparation are recorded by a detector (such as a camera or photodiode). Typically, the photons reflected or fluoresced from the specimen are first passed through a microscope or other optical lens system for focusing or magnifying the field of interest. When an optical dye is used to stain tissue or a cell, investigators use an illumination wavelength (the 'excitation wavelength') that excites the dye molecules to fluoresce and emit photons at a distinctly different wavelength (the 'emission wavelength'). In this way, the signal of interest at the emission wavelength is not contaminated by the photons used to excite the probe at the excitation wavelength. Some optical imaging methods rely on exciting endogenous light absorbing or fluorescing molecules, such as haemoglobin or NADH. Since these techniques depend upon chromophores or fluorescing molecules that are intrinsic to the cells or tissue, they are sometimes called 'intrinsic optical imaging' techniques.

Background and Methodological Issues

Common Imaging Detectors

Charge-coupled device (CCD) cameras

A CCD camera relies on a silicon wafer which has been manufactured to contain an array of individual light-sensitive cells called photosites. Each photosite contributes one element of the whole image that is formed, and is more commonly referred to as a picture element, or 'pixel.' Photosites generate and hold an electric charge that is proportional to the number of photons detected at that given site. The charge stored at each site is then digitized at some resolution (e.g., 16-bit cameras are capable of distinguishing between 2^{16} distinct levels of photon intensity), and software is used to convert the digitized representation of the CCD chip into an image. More expensive CCD cameras mount their detector chips on cooling-devices to reduce electrical noise. CCD cameras offer the special advantage of being able to provide high spatial resolution, but are relatively slow compared to other optical detection technologies. One strategy to improve image-acquisition speed is to include the hardware for digitization inside the camera electronics to directly digitize the data. Since these types of cameras provide a digital output rather than an analog signal, they are referred to as 'digital CCD cameras.' A typical spatial resolution of a CCD camera is 512×512, capable of measuring light at over 260 000 points on a specimen focused on the CCD chip.

Photodiode array

Photodiodes are optical detectors that are capable of converting light into voltage or current. Photodiode arrays used in optical imaging studies typically consist of a few hundred photodiodes, and hence offer much less spatial resolution than a CCD camera. However, this technology offers several significant advantages over the CCD camera, including extremely high data acquisition rates (sufficient to resolve individual action potentials), high dynamic range, and excellent linearity of output (current or voltage) as a function of incident light. This last attribute of linearity is critical for enabling quantitative analysis of optical measurements.

Confocal microscopy

When using classical wide field microscopy for optical imaging, the entire specimen is illuminated with light, and photons reflected or fluoresced by the specimen are passed through a lens system to some optical detector, such as a CCD camera or photodiode array. One disadvantage of this method is that light from areas of the specimen outside the focal plane contribute to the image. This results in a blurring of the image – a feature

that places a fundamental limit on what can be spatially resolved. Confocal microscopy overcomes this limitation by using point-illumination, in combination with a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus information. A laser is used to scan the specimen to illuminate one point at a time, and the fluoresced light from each individual point is detected by a photomultiplier detector, the signal from which is digitized. Software is used to convert the digital data derived from the point-by-point scanned specimen into an image. In this way, it is possible to acquire higher resolution images of cells and their processes, in thicker tissue specimens, than would otherwise be possible. Since the image must be constructed by scanning the specimen one point at a time, confocal microscopy has limited temporal resolution.

Two-photon confocal microscopy takes a different approach to reducing out-of-focus information. This technique involves illuminating a sample with a wavelength that is twice the wavelength of the absorption peak of the fluorophore being used to label the tissue or the cell of interest. Little or no excitation of the fluorophore will occur at this doubled wavelength. However, if a laser generates sufficient photon density at the point of focus, special 'two-photon events' occur; two photons are simultaneously absorbed by the fluorophore, which is equivalent to the absorption of a single photon at the energy equal to the sum of the two that are absorbed. In this way, fluorophore excitation will only occur at the point of focus, eliminating excitation of out-of-focus fluorophores.

General Limitations of Optical Techniques

Associated with each type of optical imaging device and probe is a host of trade-offs that need to be balanced within the context of the goals of a particular experiment. For example, a specific voltage-sensitive dye might successfully be used to stain the tissue of a given preparation to yield a bright fluorescence – but it could also be prone to nonspecific staining which would diminish its signal-to-noise, or it may be unstable over the desired time course due to photobleaching. Photobleaching is the destruction of a fluorescing molecule through its excitation, and is a common property of most optical dyes and all currently available fluorescent proteins. Photobleaching places a limit on the total number of photons that a fluorescent molecule can emit.

As an example of trade-offs among optical detectors, a cooled CCD-camera has low intrinsic noise and can provide high spatial resolution, but it has poor dynamic range relative to a photodiode array and is much slower and a less desirable detector for imaging fast phenomena that undergo optical changes that are small in magnitude relative to their background. In contrast, although a photodiode has significantly poorer spatial resolution than a CCD camera, it is much faster and has a significantly greater dynamic range that makes it ideal for certain types of voltage-sensitive dye applications. Importantly, financial cost is another practical consideration: a two-photon confocal microscope with 'all the bells and whistles' might be out of range for most individual investigators, regardless of the extraordinary advantages of such a system over standard wide-field microscopy. Numerous books and review articles have been dedicated to elucidating the advantages and disadvantages of various optical dyes and technologies, and several useful references are provided in the Further Reading section.

Voltage-Sensitive and Calcium-Sensitive Imaging

Arguably, the use of calcium-sensitive dyes to measure changes in intracellular Ca^{2+} concentration has had the greatest impact of any dye imaging technique applied in neurobiological research; voltage-sensitive dyes are perhaps a distant second. Dyes are also available that are sensitive to a host of different ions, including Mg^{2+}, Zn^{2+}, Na^{+}, K^{+}, Cl^{-}, and pH.

Voltage-sensitive dyes and probes

Two categories of voltage-sensitive dyes are available for staining cells and tissue: (i) absorption dyes, which include RH479 and RH155 and (ii) fluorescence dyes, such as Di-8-ANEPPS, RH461, and JPW1114. Voltage-sensitive dyes are often labeled to denote the person by whom they were first synthesized; e.g., 'RH' dyes were first synthesized by Rina Hildesheim in collaboration with Amiram Grinvald at the Weizmann Institute. Absorption dyes require illuminating the stained tissue with light at the wavelength which is maximally absorbed by the dye; changes in light absorption correspond to alterations in membrane potential. Fluorescence dyes require loading a cell or staining tissue with a dye that is excited at one optical wavelength to fluoresce at a distinctly different wavelength. The changes in emission fluorescence correspond to membrane-potential.

Fluorescent voltage-sensitive dyes are further classified into 'fast-response' and 'slow-response' dyes. Fast-response dyes such as di-8-ANEPPS undergo electrical field-triggered changes of intramolecular charge distribution, and consequently change their fluorescent properties. Their rapid optical response enables measurements of transient potential changes on a millisecond timescale. However, their small fluorescence changes are difficult to detect. Slow-response dyes, which include the rhodamine, merocyanine, and oxonol probes, undergo voltage-dependent changes in their intramembrane distribution. For example, some slow-response dyes work by migrating across the cell membrane, from the outside to inside surface, in response to depolarization; this change is

accompanied by an increase in their fluorescence signal. This transmembrane movement of entire molecules is a significantly slower process than the intramolecular redistribution of electrons in immobile molecules of fast-response dyes.

Whether a specific voltage-sensitive dye is useful for a given preparation is a matter determined by empirical investigation. A website with a current list of voltage-sensitive dyes that have been successfully employed in various specific preparations is maintained by Larry Cohen at Yale, at http://www.med.yale.edu/cmphysiol/cohen/whichdye.html

There has been some recent progress in the development of genetically encoded fluorescent protein voltage sensitive probes (FP-voltage sensors), although further improvements are required for this technology to be widely useful. Three FP-voltage sensors, Flare, FlaSh, and SPARC, have recently been expressed and characterized in mammalian cells, but have yielded poor signal-to-noise because of low plasma membrane expression. Newer constructs (e.g., VSFP2.1) appear to yield improved signal-to-nose, and suggest great potential for future developments.

Calcium-sensitive probes

Calcium is by far the most widely studied ion because of its central role as a second messenger, linking membrane depolarization to important intracellular biochemical changes. Optical imaging of calcium is done for the purpose of studying its role both in signal transduction and as a marker of neuronal activity (since transient increases in intracellular calcium are elicited by action potential firing).

Most commonly used calcium-sensitive dyes fall into two categories: (i) ratiometric dyes such as Fura-2 and Indo-1, which undergo a spectral shift in the wavelength of light they emit in the presence of calcium and (ii) single-wavelength dyes such as Fluo-3 and Fura-red, which do not undergo a spectral shift, but rather increase or decrease their fluorescence at a single wavelength as a function of calcium concentration. The advantage of ratiometric imaging is that the calculation of the ratio of fluorescence at two different wavelengths compensates for differences in dye concentrations, allowing for the quantitative comparison of $[Ca]_i$ between different intracellular domains. This ratiometric approach is important, for example, when comparing changes in a brightly fluorescing cell soma (containing a large number of fluorescing molecules) to changes in a much smaller cell process, such as a dendrite, holding significantly fewer fluorescing molecules.

There have been a number of exciting recent developments in using fluorescent protein markers of calcium in transgenic mice to target specific cell types, with two approaches being most widely investigated to date. The first approach uses a green fluorescent protein (GFP) fluorophore as a sequence insert. The second approach uses 'chameleons' that involve connecting two spectrally different types of GFP by a calcium-sensitive linker. Changes in the degree of fluorescence resonance energy transfer (FRET) occur between the two GFP fluorophores. FRET is a fluorescence labeling technique whereby two different fluorophores are genetically fused to proteins. The emission and excitation spectra of the two fluorophores are matched in such a way that an excited donor fluorophore can transfer energy to another closely proximal acceptor fluorophore, with the net effect that the donor emits less light than it normally would, while the acceptor emits more light at its excitation frequency.

Recent Results

A More Detailed Example: Imaging of Intrinsic Optical Signals (IIOS)

IIOS is based on the principle that action potential firing changes the way neuronal tissue absorbs and scatters light at wavelengths throughout the visible and near-infrared spectrum. These activity-related optical changes are called 'intrinsic optical signals' because they do not require the use of optical dyes. The distinct advantage of IIOS is its simplicity: one need not deal with the issues involved in staining tissue with dyes, and the activity-evoked optical changes can be easily detected with standard CCD cameras using commercially available imaging acquisition and analysis software. Some major disadvantages of IIOS are that it is limited to detecting changes on the surface of tissues and it is not always obvious how the intrinsic optical signals are related – temporally and spatially – to action potential firing. IIOS imaging techniques involve illuminating the tissue with a continuous stable light source at the desired wavelength, and acquiring images with a camera mounted to a standard wide-field microscope (for tissue slices) or an operating or dissecting microscope (for in vivo imaging of the cortex). Our laboratory has been interested in IIOS imaging because of the ease with which it facilitates translational studies. We have used IIOS to study epileptic and normal activity in consenting human subjects in the operating room who are undergoing surgical treatment for epilepsy. These studies are complemented by subsequent animal studies that combine IIOS with more invasive techniques, such as microelectrode recordings and pharmacological treatments, to test hypotheses generated by our observations of the human disease.

The experiments involved in the specific example discussed here were aimed at better understanding the physiological mechanisms underlying the generation of the intrinsic optical signals. It has long been known that the in vivo optical signals are generated by the combination of

at least three components: (i) changes in blood oxygenation, (ii) changes in blood volume, and (iii) blood-independent light scattering changes, possibly due to glial cell-swelling and ion fluxes associated with increased action potential firing. However, the respective contributions of the specific vascular and tissue compartments at various optical wavelengths have not been clearly elucidated.

Rationale

Increases in neuronal activity are known to elicit localized changes in blood volume, and this activity-evoked redistribution of blood in the neocortex is controlled by the pial arterioles lying on the surface of the brain. The firing of action potentials by neocortical neurons results in the dilation of microscopic pial arterioles nearest the active neurons. Molecules such as adenosine and nitric oxide are generated by neuronal activity, and diffuse through the extracellular space to elicit dilation of nearby vessels. In this manner, small changes in neuronal activity are transduced into large changes in blood flow (since flow is related to the fourth power of vessel diameter). As a result of the large increases in blood flow that are elicited by increased neuronal activity, the transit-time of the blood within cortical tissue is greatly reduced, resulting in increases in the oxygen content of blood in the draining venous network. Other imaging modalities, such as BOLD–fMRI, rely upon detecting this increase in blood oxygenation in the veins receiving blood from active cortical tissue.

The light absorption spectra of oxy-hemoglobin (OxyHb) and deoxy-hemoglobin (Hb), show that at certain wavelengths (535 and 810 nm) known as isosbestic points, OxyHb and Hb are indistinguishable. In contrast, OxyHb and Hb are maximally distinguishable at other wavelengths such as 660 nm (**Figure 1**). Several predications follow from the previous discussion. First, intrinsic optical signals observed when illuminating the brain with 535-nm light should be generated by the dilating pial arterioles nearest to populations of neurons firing action potentials. The increase in light-absorbing haemoglobin molecules associated with the vessel dilation suggests that the tissue will absorb more light at this wavelength. Thus, the 535-nm optical signal should be a measurement of activity-evoked blood volume changes, and the tissue should appear to darken with increased activity. Second, optical signals measured at 660 nm should be localized to the veins near active neurons. Since OxyHb absorbs less

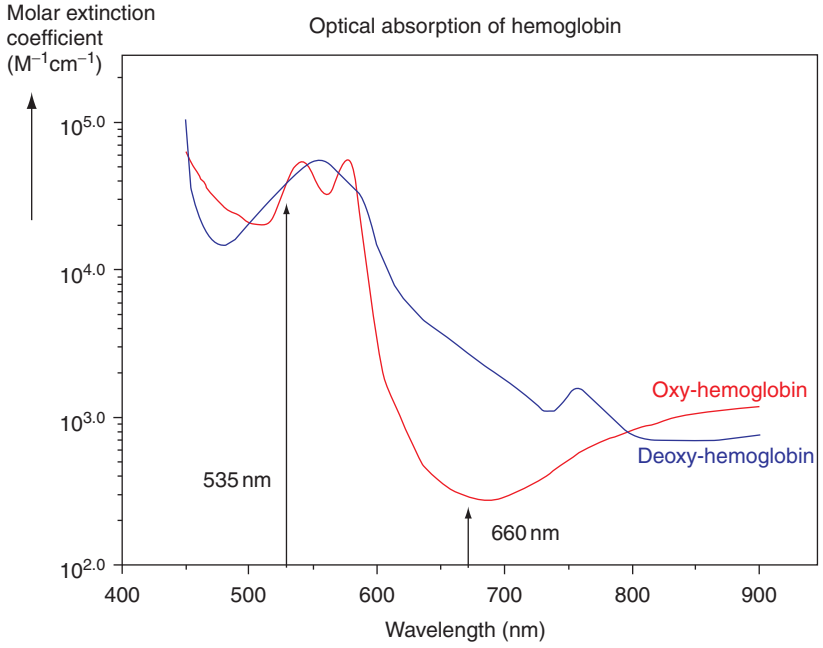


Figure 1 Optical absorption properties of hemoglobin. This plot compares the amount of light that is absorbed by oxy- and deoxy-hemoglobin at wavelengths from 400 through 900 nm. The graphs cross at some wavelengths, such as 535 nm, where oxy- and deoxy-hemoglobin absorb exactly the same amount of light, and hence imaging at these wavelengths is insensitive to differences in blood oxygenation. Optical imaging at such points where the absorption properties are equal (called isosbestic points) would be expected to be independent of changes in blood oxygenation, and hence primarily represent changes in blood volume. At other wavelengths, such as 660 nm, the optical absorption properties of oxy- and deoxy-hemoglobin are clearly distinguishable; changes in the optical signal at such points should represent changes in blood oxygenation. These graphs were generated from publicly available data on the web compiled by Dr. Scott Prahl, Oregon Medical Laser Center at http://omlc.ogi.edu/spectra/hemoglobin/summary.html, tabulated from data by Drs. W. B. Gratzer, Med. Res. Council Labs, Holly Hill, London and N. Kollias, Wellman Laboratories, Harvard Medical School, Boston.

light than Hb at 660 nm, the tissue should appear to become brighter with increasing neuronal activity. The following experiment was designed to test and validate these notions.

General methods

Macaque monkeys (*Macaca nemestrina*) are typically used in our laboratory's optical imaging studies. A 25-mm 'optical window' for acquiring optical and electrophysiological recordings is constructed over the hand motor cortex. This procedure involves a craniotomy and mounting a specially designed stainless steal chamber over the cortex. We typically choose hand motor cortex in optical imaging studies of epileptiform activity; in this way, electrophysiological activity from the cortex can be acquired simultaneously with observations of hand motor movements and EMG recordings from hand muscles. The cortex is stimulated with a bipolar stimulating electrode (5 mm inter-electrode distance) powered by an Ojemann Cortical Stimulator (Integra Life Science Corporation). In the studies shown here, the cortex was illuminated with either 535- or 660-nm light. Images are acquired with at 16-bit cooled digital CCD camera (Roper Scientific) mounted on an operating microscope.

A typical data acquisition trial involves acquiring a series of images at a rate of at least 5 Hz continuously for several minutes. Each trial consists of a 20-s control period prior to stimulation, followed by 4 s of electrical stimulation of the cortex, and ending with a 2–3 min recovery period. Percent-difference-images are generated by subtracting and then dividing the first control-image from all of the other images acquired during the same data-acquisition trial. The percent-difference-images are pseudocolored to facilitate the visualization of small optical changes.

Results

Figure 2 illustrates the results from an experiment aimed at determining the microvascular contribution to the intrinsic optical signal when the cortex is illuminated with 535 and 660 nm light. The top panel shows a low-magnification view of the optical changes evoked by 4 s of electrical stimulation (at 7 mA, which was found to be a stimulation current that was below the threshold for evoking epileptiform afterdischarge activity). As is typical, the largest optical changes at 535 nm occur around one of the poles of the stimulating-electrode, indicating an unequal distribution of current during the stimulation; no optical changes occur within the larger visible blood

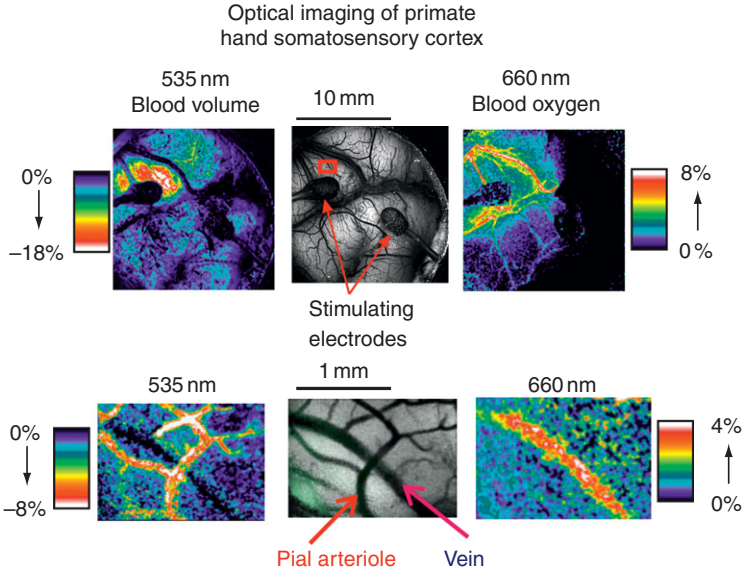


Figure 2 Localization of optical changes at 535 and 660 nm. The top panel shows low-magnification maps of optical changes during 7 mA stimulation, where no afterdischarge activity was elicited by the stimulation. These data are from an individual animal. The middle, top grey-scale figure shows the configuration of the stimulating electrodes, and the red square overlay show region the region whose optical map is plotted at a higher magnification in the bottom pane. Images on the left and right are pseudocolored to indicate their percent changes in the optical signal from baseline conditions. When the cortex is illuminated with 535-nm light (top, left), the largest optical changes are nearest to one of the stimulating electrodes, and distributed diffusely throughout the surrounding tissue. Under 660-nm light (top, right), the optical changes are greatest in the largest veins lying within the cortical sulci. The bottom panel shows optical changes acquired at high magnification, within the area indicated by the red square in the top middle figure. During stimulation, the pial arteriole was observed to undergo a 35% increase in its resting diameter. The 535-nm optical images (bottom, left) show that optical changes are restricted to the pial arteriole, and absent from the venule. The 660-nm images (bottom, right) show that changes are restricted to the nondilating venule. The pial arteriole shown here is approximately 20 μm in diameter at rest, and the venule is 35 μm in diameter. Modified from Haglund MM and Hochman DW (2007) Imaging of intrinsic optical signals in primate cortex during epileptiform activity. *Epilepsia* 48(supplement 4): 65–74, with permission from Wiley-Blackwell.

vessels (**Figure 2**, top left). At this wavelength, all optical changes occur in a negative-going direction (i.e., the tissue appears to become darker because it is absorbing more light). When imaging with 660-nm light, the largest optical changes are observed to occur within the larger veins lying within the sulci, near the site of electrical stimulation – and all optical changes occur in a positive-going direction (**Figure 2**, top right). High magnification optical imaging can be used to resolve the optical changes occurring within the microscopic blood vessels (**Figure 2**; bottom panel). With such magnification, the optical changes appear to be restricted to the dilating pial arterioles at 535 nm and to the venules at 660 nm.

The main conclusions from these data are: (i) optical imaging at 535 nm is selective for blood volume changes; the optical changes are generated by the increased blood volume associated with dilation of the microscopic pial arterioles; (ii) optical imaging at 660 nm is selective for changes in blood oxygenation since the activity-evoked signal is predominantly restricted to the veins that are draining blood from the stimulated cortex; and (iii) the blood volume wavelength at 535 nm appears to localize neuronal activity more accurately than the blood oxygenation wavelength at 660 nm.

In a second type of study, optical imaging at 535 nm (blood volume) was used to study spontaneously occurring epileptiform discharges in a primate seizure model. For these studies, a small epileptic focus was created by soaking a 0.5 mm Gelfoam pledget in bicuculline (100 μM) and placing it on the cortex for several minutes. Spontaneous ictal discharges typically occur for approximately 60 min after the bicuculline-soaked pledget is removed from the cortex. Blood volume imaging suggests that circular waves of propagating seizure activity spread from the site of the acute focus through surrounding nonepileptic cortex (**Figure 3**; top panel). By subtracting images in the reverse manner, opposite-going optical signals can also be observed (**Figure 3**; bottom panel). These results show that during the spread of ictal activity, blood is redistributed from regions in which intense epileptiform discharges have occurred into areas in which the ictal activity is beginning to spread.

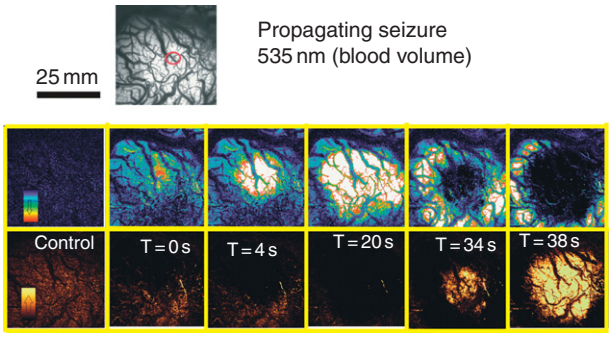


Figure 3 Positive and negative-going optical signals during ictal discharge at the blood volume (535 nm) wavelength. The images shown here were acquired during an ictal discharge spreading over the cortex. An acute seizure focus had been created by applying a 0.5-mm pledget soaked in bicuculline to the cortex for several minutes, in the center of the region shown in the image, within the colored-circular overlay shown in the top gray-scale image. A series of images were acquired during a spontaneous ictal discharge. The top line of rainbow pseudocolored images show positive going signals that are normally associated with increases in neuronal activity. The bottom series of gold pseudo-colored images show the negative going signals that may represent 'blood stealing,' or a redistribution of blood from areas of cortex undergoing a decrease in activity to surrounding areas undergoing an increase in activity. Modified from Haglund MM and Hochman DW (2007) Imaging of intrinsic optical signals in primate cortex during epileptiform activity. *Epilepsia* 48(4): 65–74, with permission from Wiley-Blackwell.

Future Directions

The increasing applicability of optical imaging techniques is the result of ongoing development of imaging hardware and optical reporters and dyes. Advances in imaging hardware are likely to include the development of photodiode arrays and low-noise charge-coupled device (CCD) cameras that are faster, more user-friendly, and have greater spatial resolution than current devices – and will continue to be more affordable as prices for solid state detector technologies fall. Developments in the area of optical dyes include ever-improving optical probes that allow for the measurement of membrane potential, pH, calcium, chloride, and sodium. Perhaps the most exciting recent developments in this area are methods for the transgenic expression of fluorescent proteins. By coupling nucleotide sequences encoding fluorescent proteins to cell-specific promoters, transgenes have been constructed that make specific cell types in a living organism fluoresce. Specific types of neurons, for example, can be made to express fluorescent proteins that allow for unprecedented resolution of morphology, or that have sensitivity to membrane potential, Ca^{2+}, Cl^{-}, and pH. These recent developments in the use of fluorescent proteins may represent the beginnings of a revolutionary leap in the power and applicability of optical imaging methodology.

See also: **Imaging**: Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In vitro; Optical Monitoring of Circuitry Activity In vitro; Using Intrinsic Optical Signal Changes to Analyse Epileptic Activity and Seizures; **Metabolic Changes**: Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain; **Networks**: Determinants of Neuronal Network Activity in Cultured Hippocampus; **Non-Synaptic Mechanisms**: Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity.

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Optical Monitoring of Circuitry Activity In vitro

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Introduction

Nature is inherently colorful, but alas for those of us who want to watch neurons at work, the brain is not. This statement, however, may only have historical significance, for we are in the midst of a revolution that is touching all aspects of biological study, driven by our new found ability to add color. There are three facets to this technological leap: the development of a huge array of fluorescent dyes to explore both structure and function; the ability to introduce the dyes into preparations using molecular biological techniques, microinjection, or chemical tricks (e.g., esterifying the dyes to make them more permeable); and the development of microscopy techniques and equipment to optimize our ability to visualize these dyes. At its extreme, the results are stunning. For instance, it is possible to detect single molecules when bound to a marker fluorescent dye. And without doubt, this revolution will impact hugely on our efforts to understand epilepsy.

The desire to image network activity stems from a frustration with the limitations of electrophysiological recordings. Intracellularly recordings give a high temporal resolution view of that cell's firing pattern or the synaptic barrages that it experiences, but one then needs to put these recordings in the context of the other network activity. Recording electrodes are huge relative to the size of the cells, and so recordings tend to be done in relative isolation; at most, just a handful of such recordings can be made simultaneously.

Electrodes for extracellular recording are similarly bulky, but do provide a sensitive assay of changes in network activity. The signal though, is small and very complicated. It derives from the apparent electric field between two points (the electrodes), reflecting all the ionic fluxes within this field. Unfortunately, it cannot easily be 'reverse engineered' to derive these ionic fluxes. Thus, the entire network activity is integrated into a single signal with almost all spatial information lost. Multicellular recordings do better, but their spatial resolution remains poor; and again, the physical size of the electrodes can be an issue.

The power of functional imaging is that it allows us to collect detailed information from many separate locations simultaneously, often in places beyond the reach of other techniques (e.g., voltage and Ca^{2+} fluxes in dendritic spines), and with greatly increased spatial resolution. The relevance of these techniques to epilepsy research is obvious: seizures are among the most intense activity patterns within the brain, and our understanding will only

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come from an awareness of how the event plays out across the entire network. In short, we need to be able to record from many locations at once, and imaging gives us this capability. The usefulness of imaging, however, comes from an understanding of how activity has been made visible, and an appreciation of the limitations of the techniques, particularly with respect to the trade-offs between temporal and spatial resolution. In this article, we will discuss these issues with a particular focus on the main technique used in our own laboratories: Ca^{2+} imaging of network activity.

Background

There are two main techniques for imaging network activity, utilizing either changes in reflectance of the tissue, or changes in fluorescence. Imaging reflectance is generally referred to as intrinsic optical imaging (IOS), and utilizes subtle changes in the scatter of light caused by neural activitation. In vivo, these include vascular changes (vasodilation/constriction and changes in levels of blood oxygenation), but in vitro, the changes are thought to arise primarily from cell swelling. The signals are small and so generally require averaging or long image capturing over relatively large areas, both significant problems if events are variable and rapid. Furthermore, the underlying biological processes are only manifest over a time course of seconds, so this imaging is slow and of fairly low resolution (100s of microns). These various issues mean that IOS has not been used much in epilepsy research, although it has proved a popular tool to study spreading depression. In this respect, it is worth stating that it can be done very cheaply and almost completely noninvasively (e.g., through a thinned skull).

The remainder of this article will concentrate on imaging fluorescence changes. All fluorescent molecules work the same way. A photon within a particular range of wavelengths (the absorption spectra) is absorbed, raising the molecule to an 'activated state.' The molecule then releases a photon of lower energy (i.e., there is some energy lost in the process) to return to the 'ground state.' Because the released photon has lower energy, the emission spectrum is shifted to a higher wavelength. For chromofluors to be useful as functional markers, their spectral properties must be changed in some way by neural activity. This can happen if either the absorption spectrum is shifted or the absorption efficiency reduced or increased, or if photon emission is modified (again either by increasing or reducing the emission or shifting the spectrum). The fluorescence changes reflect structural changes in the fluorescent molecule, induced by binding or releasing other species such as Ca, or by changes in the electric field. This latter mechanism explains how voltage sensitive dyes (VSDs), embedded in the cell membrane, can indicate changes in the membrane potential.

The first Ca^{2+} dye to be developed was aequorin, but its usefulness was limited by its poor photon emission and the fact that it is consumed by Ca^{2+}. Thus, each molecule detects just a single event, and the signals need to be derived from populations of cells rather than individual cells. The field was really driven forward when Roger Tsien developed a means of modifying the two commonly used Ca^{2+} buffers – EGTA and BAPTA – such that the molecules fluoresced when they bound Ca^{2+}. A second inventive chemical modification then provided an easy means of loading the dye into cells: by adding an ester group on to the dye, Tsien found that the dyes crossed the cell membrane, whereupon they were cleaved by intracellular esterases, thus converting the nonfluorescent esterified molecule into a fluorescent de-esterified one, and fixing the dye within the cell into the bargain.

The usefulness of Ca^{2+} as an indicator of neural activity comes from its unique position as the principal transducer of electrical into chemical signals. All spiking neurons, when they fire action potentials, experience a sharp rise in intracellular Ca^{2+}. Consequently, by monitoring the somatic Ca^{2+} fluxes, one can derive an accurate picture of the cell's firing pattern. With the appropriate microscope and camera, one can thus record the activity, as indicated by their somatic Ca^{2+} fluxes, of 100s of neurons simultaneously, with single cell precision.

A further benefit of the rise of Ca^{2+} imaging is that it has thrust glial cells back into the center stage. Glia also experience large fluctuations in intracellular Ca^{2+}, albeit with the cytosolic Ca^{2+} coming from intracellular stores rather than from the extracellular space (as happens in neurons). This different source gives glial Ca^{2+} transients (GCTs) a distinctive appearance: they generally show far slower rise times than neuronal signals, and when viewed at high enough magnification and speed, the fluorescence signal appears as a wave travelling down glial processes, before smoothly progressing through the rest of the gap-junction coupled glial syncytium. By permitting such activity to be visualized, Ca^{2+} imaging has provided strong evidence for a prominent role for glia in all manner of neural activity, and a particularly important role in epileptogenesis.

Methods

Loading Issues

Different experimenters have used many subtly different loading protocols, the details of which can be found in their various publications. Generally speaking, sections are allowed to settle for 30–60 min following slicing, before

being transferred into a chamber (volume ~5 ml) warmed to 33–36°C. The dye is then added (to 3 ml of ACSF, add 10–30 µl of the following solution: 50 µg dye + 8–45 µl DMSO + 2 µl of 20% pluronic acid (mild detergent) in DMSO – the different amounts reflects experimenter and dye variability (some experimenters also add traces of Cremophor XL, another detergent available from Sigma)). We recommend drawing off some ACSF to mix with the dye solution prior to adding back to the incubation chamber, so that slices are not exposed to high concentrations of DMSO or detergents. The slices are incubated (aerating all the while with 95%/5% carbogen) for 20–40 min, and then washed with ACSF prior to transferring the slices to the microscope perfusion chamber.

All the published work to date on network Ca^{2+} imaging of in vitro preparations have used either neuronal cultures or slices from young animals, generally mice. The reason is simple: brain slices from young mice (<postnatal day 20 = P20) can be loaded with the dye far better than slices from older ones. There are also curious variations in how well different brain regions can be loaded. Thus, loading of neocortex is usually good up to P20, but hippocampal loading is generally poor beyond the first postnatal week, whilst with thalamic labelling, we have had very little success. While we do not fully understand the reasons for these limitations, the answer may relate to another feature of these dyes: the various Ca^{2+} dyes (Molecular Probes currently make more than 30 different dyes) show marked differences with respect to the relative loading of neurons and glia.

There seems to be a spectrum of relative neuronal-glial labelling patterns, with fluo-3 at one end (labelling primarily glia), and then progressing through Calcium Green, Fluo-4, Indo-1, Oregon Green Bapta 1, to Fura-2 (80–95% of Fura labelled cells appear to be neurons). The labelling can be assessed either by co-labelling with the astrocyte-specific marker, sulforhodamine 101, or by careful assessment of the nature of the Ca^{2+} signal (see later text). The difference between the dyes is likely to reflect two properties: the specificity of esterases in a given cell class for the esterified form of the dye, and the dynamic range of Ca^{2+} fluxes seen in that cell class relative to the K_D of the dye. To explain this last point a little further, the effective functional range of a dye, in terms of the Ca^{2+} concentration level, which shows a change in fluorescence, extends from about 0.1^*K_D to about 10^*K_D. If the Ca^{2+} fluxes that a given cell experiences is outside this range, then the dye is not going to show any measurable changes in fluorescence. Generally, dyes that give better glial signals tend to have higher K_Ds, reflecting either a higher baseline cytosolic Ca^{2+} concentration or larger Ca^{2+} fluxes in these cells. Given the clear differences between glia and neurons in these measures, it is perhaps also to be expected that different neuronal populations also show variability.

A related issue is that the act of slicing the brain appears to activate glial populations, and while we have not explicitly tested the hypothesis, it is our impression that the better the quality of the slice, the more the labelling is neuronal. Consistent with this view, organotypic cultures that are labelled days or weeks after preparation – when the glial activation has settled down – can give very good neuronal labelling. Of course, as we have indicated already, glia are increasingly seen as playing a key role in epileptogenesis, and the ease with which they can be loaded with certain Ca^{2+} dyes has been a fantastic stroke of luck for this field.

With respect to age differences, patch clampers have long known that slices from older animals scatter light much more, and consequently the DIC images become worse and worse as the animal gets older. The same will be true for Ca^{2+} imaging in older slices, a problem that working with 2-photon microscopes may conceivably avoid. Another factor, again untested, may be that older brains show increased glial activation following slicing, leading to relatively worse neuronal visualization.

Imaging Issues

In a loaded cell, the onset of neural activity is seen as a sharp deflection in the somatic fluorescence, subsequently relaxing back to baseline fluorescence levels with a time constant of about 0.5 s. Because of the very slow decay kinetics, it is possible to detect events using sampling rates down at 1 Hz. If one is trying to assess levels of participation in interictal events, a slow rate of image capture may be quite acceptable. Obviously, the key is to design the imaging protocol for the job in hand. And for capturing any of the dynamics of an epileptic seizure, it is of course desirable to image much faster than 1 Hz. So how fast can one do Ca^{2+} imaging? Performing a line scan on a part of a dendrite, using a 2-photon microscope with optimal optics (high power, high numerical aperture (NA)), one can easily detect event onsets while sampling at 1 kHz. To do network imaging, however, one needs a wider field of view while still sampling at sufficient spatial resolution to see individual somata. High NA 20× objectives seem optimal, but even then, the light collection is not as good as 2-photon imaging, and the best that has been achieved is imaging at about 60 Hz using a spinning disk confocal and a high speed, cooled CCD camera. On first consideration, this limitation is a real problem: electrophysiological studies of disinhibited slices have reported propagation speeds of 10–100 mm s^{-1}, and so if one is imaging an area of cortex about 400 µm wide (a typical field of view when using a 20× objective), then an event travelling at 40 mm s^{-1} will traverse this field of view in about 10 ms. If one is imaging at 60 Hz, the event will cross the entire field of view within the time course of

a single frame. Fortunately, epileptiform activity can propagate at speeds several orders of magnitude slower than reported for disinhibited slices. Furthermore, what appears to determine whether a seizure propagates rapidly or slowly is the network's own defence mechanism opposing epileptiform spread – in itself a fascinating and hugely important topic.

But before we describe this work, we want to make just a couple more points about methodology. Firstly, it is possible to image at much higher rates (~1 kHz) using VSDs. The signals are rapid, but very small, and thus require spatial averaging. Consequently, one cannot visualize single cells, and the signal is further complicated by the confusion of signals from all the different neural cellular compartments (somata, dendrites, axons). VSD imaging, however, by dint of its speed, does have the potential to address questions that cannot be examined readily using Ca^{2+} imaging, such as propagation in disinhibited slices, or the propagation of afterdischarges.

The second point regards the importance of making electrophysiological recordings synchronous with imaging. All imaging comes at a cost: illumination causes both photobleaching and phototoxicity, whether one uses an epifluorescence system (worst in these regards) or a 2-photon system (best). For these reasons, one must always strive to limit the imaging time. The best way of achieving such limits is to use the electrophysiological recordings to guide when one collects imaging data. Another argument for performing simultaneous imaging and electrophysiology is that all our information regarding epileptiform activity (going back to the first EEG recordings by Berger in 1929) has been electrophysiological in nature; electrophysiology is the frame of reference, and is the only means by which we can interpret our imaging data in terms of prior research. Finally, electrophysiological recordings provide a means of validating our results. As we mentioned earlier, currently we can only do imaging on a limited range of preparations. Our electrophysiological recordings have allowed us to confirm that what we see in a brain slice taken from an adolescent mouse also occurs in adult guinea pig brain slices and in adult cat in vivo – and there is a strong indication that it also reflects the pattern of activity in such phenomena as the Jacksonian march in humans.

Important Results to Date

Targeted Patching

Perhaps the simplest use of Ca^{2+} imaging, and still one of the most powerful, is to identify cells that are doing interesting things within the network. These cells can then be targeted for patch clamping, to allow a detailed electrophysiological and morphological characterization. Such experiments have identified (i) cells which sustain rhythmic (~1 Hz) firing independent of synaptic activity, suggesting themselves as pacemakers for cortical circuits, (ii) other cell classes showing distinctive activity patterns during early network events in hippocampus, and (iii) clusters of neurons participating in so-called 'UP-states.' UP-states are physiological network states that have now been recorded in a number of different preparations (mainly but not exclusively neocortical), both in vivo and in vitro. They are so called because all participating neurons experience a roughly synchronous depolarizing shift of about 10 mV, and the spiking rate switches accordingly from almost quiescent to spiking regularly. Note, however, that the term is generally used to refer to physiological activation when the firing rate is relatively restrained, and is thus different from the well-described epileptic phenomenon of the paroxysmal depolarizing shift (PDS) in which the depolarization drive is so intense that Na-channels fail to deinactivate and successive action potentials are much reduced in amplitude. UP-states may represent the network correlate of working memory.

An In Vitro Model of Surround Inhibition

In our epilepsy experiments, we sought to understand the interplay between inhibition and excitation in ictogenesis. To this end, we chose to use the 0 Mg^{2+} in vitro model, in which epileptiform activity occurs primarily as a result of relieving NMDA receptors of their voltage dependency. Thus, glutamate can open these receptors when the cell is at resting membrane potential, and this sensitivity enhances the excitatory drive. If the slice is kept for a sustained period in Mg^{2+}-free solution, then eventually inhibition also becomes impaired. For these reasons, the 0 Mg^{2+} model is a fascinating preparation in which to study epileptiform activity, because the earliest activity occurs with the inhibitory circuitry intact, but then the activity patterns evolve as the inhibition deteriorates. The model thus shows a wide range of epileptiform activity, and has allowed us to examine the role of inhibition in epileptic circuits.

In particular, we have been able to address a key hypothesis proposed over 40 years ago by Prince and Wilder – that there exists a surround inhibitory mechanism within the network which opposes the spread of ictal events. This proposal arose from in vivo studies, but the difficult nature of such studies has meant that our understanding of this mechanism has stood still for most of the intervening years. We discovered that the earliest epileptiform activity in the 0 Mg^{2+} in vitro model appears to recapitulate the in vivo state very well. With all the benefits that in vitro preparations ensue, including better control of the experimental milieu, access for electrophysiological recording and direct visualization of the

activity of most of the neurons in the circuit using calcium imaging, we have started to unveil the underlying network that provides this critical network protection.

When early 0 Mg^{2+} ictal events are imaged at low magnification (4× objective), the fluorescent signal from individual neurons cannot be discerned, but the progression of the event across the tissue can still be visualized (**Figure 1**). Simultaneous with all our imaging, we also collected patch clamp recordings from layer 5 pyramidal cells, since this cell class is thought to play a key role in ictogenesis. These synchronous recordings showed that there is a powerful inhibitory barrage ahead of the ictal wavefront that matched the timing of network bursts in the cortical territories that had already been recruited to the event. Other patch clamp experiments showed that this feedforward inhibition coincided with excitatory volleys emanating forward from the ictal wave. As a result, pyramidal cells experience rhythmic depolarizations at about 1 Hz ('slow waves' or δ-rhythms). Yet despite the significant depolarizations and the level of the excitatory drive, the cells fired very infrequently. The implication was clear-that the feedforward inhibition was vetoing the excitatory drive. And by doing so, the inhibition restrained the spread of the ictal event.

The crest of each slow depolarization represents a crisis for the network. This was apparent when we next imaged the epileptiform event at higher magnification (**Figure 2**). These experiments showed that the recruitment of new cells occurs only at specific times, coincident with the excitatory barrages. Thus, the ictal event appears to take episodic steps, about once a second, across the network. What determines whether a cell is recruited or not at these crisis points? The answer seems to be that it is the failure of the inhibitory restraint rather than the start of the excitatory barrages that governs the recruitment of pyramidal cells to the ictal event. This important conclusion comes because we know that the initial excitatory volleys, which ordinarily (in the absence of inhibition) would surpass the firing threshold many times over, start 10 s of seconds before the eventual recruitment. This is consistent with prior modelling studies which suggested that the arrangement of inhibitory and excitatory synapses across the pyramidal soma and dendrites – in particular the large inhibitory and absence of excitatory drive on to the soma – allow for a vetoing inhibition, however powerful the excitatory drive might be.

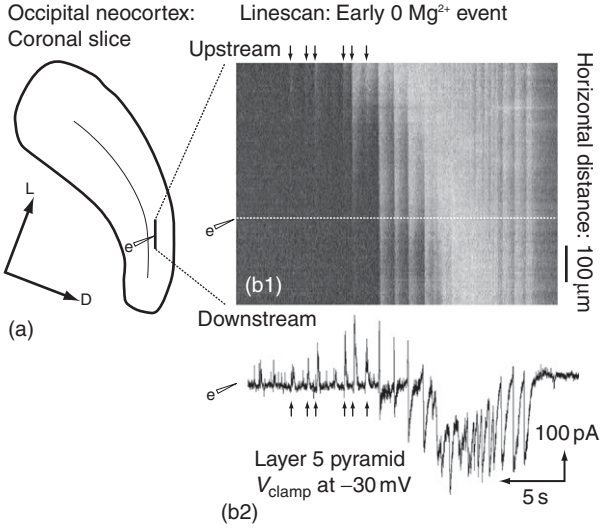


Figure 1 The slow stepwise propagation of zero Mg^{2+} epileptiform events reflects prominent feedforward inhibition. (a) Schematic of a coronal slice showing the recording electrode ('e') and the location of a 'line scan' through layer 5. Imaging was done at 30 Hz using a 10× objective. (b1) Line scan showing the neuropil Ca^{2+} fluorescence as an epileptiform event progresses from top to bottom – distance is plotted on the ordinate, and time on the abscissa. Each row of pixels represents the neuropil Ca^{2+} fluorescence from a bin 40 µm high by 4 µm wide (165 bins = 660 µm total length). The location of the recording electrode ('e') is shown. (b2) The V_{clamp} recording from the layer 5 pyramidal cell plotted below on the same time scale. The cell was held at −30 mV, approximately half way between the reversal potentials for GABA and glutamate, to distinguish inhibitory drives (upward deflections) and excitatory drives (downward deflections). Note the prominent inhibitory volleys (arrowed) corresponding with upstream activity in the line scan. Figure used with permission from Trevelyan *et al*. (2006).

The Role of Glia in Ictogenesis

Pathological clusters of glia, such as glial scars and gliomas, can induce seizures. Yet the etiology of these seizures remains obscure. Glia are known to regulate the extracellular space, for instance by taking up K^+ ions and neurotransmitters, and seizures had previously been presumed to arise from a failure in this function. The recent demonstrations that glia themselves can release neurotransmitters have provided a radically different hypothesis about epileptogenesis in this context – that glia may actively provoke seizures. Glutamate, and other neuroactive substances including nitric oxide, ATP, prostaglandin, have all been shown to be released from glial cells following a transient rises in intracellular Ca^{2+} (glial Ca^{2+} transient, or GCT), although the mechanism of glutamate release remains controversial. The released glutamate can activate local neuronal populations, even triggering epileptiform activity. Perhaps most remarkably, there is evidence that antiepileptic drugs can influence glial activity, seemingly independent of the neuronal effects of these drugs, raising the possibility that their antiepileptic action is mediated through glia and not neurons.

GCTs have also been linked to many other cellular processes, such as regulation of mitochondrial

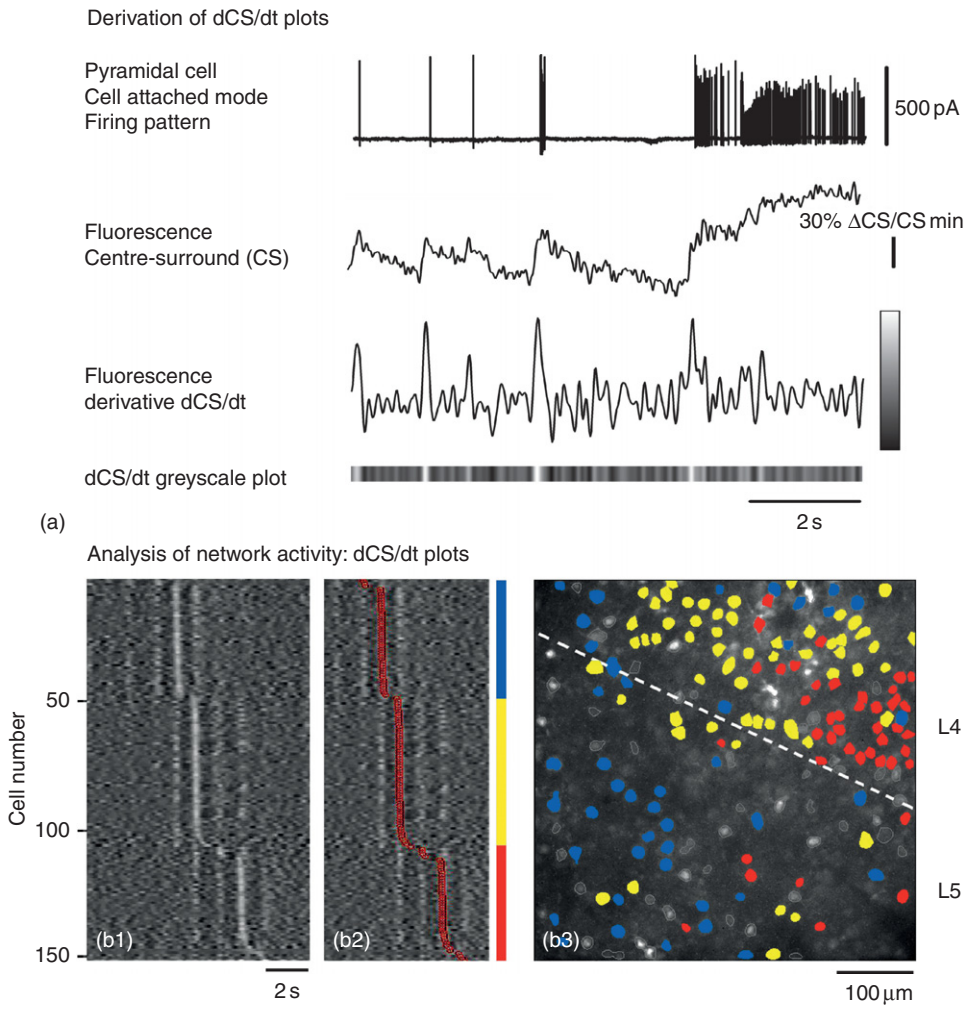


Figure 2 Somatic Ca^{2+} fluxes reveals episodic localized recruitment. (a) The derivation of the cellular Ca^{2+} signal. We recorded the firing pattern of a pyramidal cell (top trace) that had been bulk loaded with the Ca^{2+}-dye, Oregon Green Bapta 1. The second trace shows the somatic Ca^{2+} fluxes – note the sharp rises concomitant with each action potential. The derivative of the Ca^{2+} signal highlights the sudden increases in fluorescent signal, which reflect the sudden recruitment of neurons. The bottom panel shows how this derivative trace is transformed into the greyscale plots shown in **Figure (b)** which shows grayscale representations from 152 identified neurons. (b1) 'Grayscale plot' of the temporal derivatives (dCS/d*t*) of the centre-surround signals from 152 neurons ordered by the time of the maximum for each trace. Each row of pixels represents a dCS/d*t* trace: high rate of change of fluorescence is depicted as lighter grey. Each trace has several peaks. (b2) The same plot, with the single maximum rate of change for each cell marked (red). This ordering clearly shows three main clusters of cells. They are color-coded as shown, and their positions are indicated in the photomicrograph in (b3). Note the tight and largely non-overlapping spatial distributions of the yellow and red groups in layer 4. The lamina borders were determined from the location of the filled cells following post hoc staining for biocytin and matching this up to the electrode location in the field of view (the faint triangular shadow of one of the electrodes can just be seen at the bottom right corner of the field of view). Movie taken using a 20× objective. A movie illustrating this pattern of recruitment can be found in the supplementary information in Trevelyan *et al.* (2006) from which this figure was derived (with permission).

metabolism; morphological remodelling; cell division; control of gene expression; vasoconstriction and vasodilation; apoptosis. Extracellular Ca^{2+} influences gap junction and hemichannel opening. Clearly, GCTs are pivotal to glial behavior, yet there remain many unresolved issues. We know of some triggers (glutamate, ATP, prostaglandins, α-adrenoceptor and muscarinic stimulation), but there are likely to be others. As such, we have only a vague understanding of what determines the amplitude, kinetics and frequency of GCTs in the intact brain. What is clear, however, is that glia can no longer be viewed as passive players in the brain. Rather, they are active participants in neurophysiology and pathology. Our greatest ignorance perhaps concerns how GCTs influence network behavior, such as controlling brain states, rhythms, and pathologies such as migraine and seizures.

Challenges for the Future

Our specific goal is now to identify the interneurons that provide the protective restraint to seizure spread, since these cells represent an important potential target for pharmacotherapy. Our initial data suggest that interictal events may also reflect activation of the protective restraint, leading to an early suppression of a potential ictal event. This observation has suggested a means of identifying the critical interneurons providing the restraint, by targeted patching of those cells which show Ca^{2+} transients only during interictal events. Unfortunately this simple-sounding experiment is confounded by the sheer volume of data required. To capture enough interictal events and the start of a full ictal event involves imaging for 10–100s of seconds, and at 60 frames per second, that represents a large amount of processing that cannot be done within the lifetime of the slice. We are exploring other means of identifying these neurons.

We are also exploring the possibility that the intense feedforward inhibition is related to high frequency oscillations that various researchers have noted to be associated with the transition into a full ictal event. Previous workers have suggested that these oscillations may be culprits in the evolution of the seizure, but our alternative view is that these ripples of activity are actually representative of the tissue trying to stave off the impending ictal disaster.

More generally, there will inevitably be further technological improvements. Genetically engineered Ca^{2+} sensors continue to improve, and these new sensors permit life-long imaging. Also, the recent success in loading Ca^{2+} dyes into neocortex in adult cats in vivo is extremely promising, not only for adding these techniques to the in vivo armory, but also indicating how we might improve our in vitro labelling. Perhaps it will be possible to load the dye prior to sacrificing the animal, in which case the subsequent activation of glia may not be significant because the dye has already been removed from the extracellular space. Finally, a small plea to the community looking to express green fluorescent protein in every cell of interest in the body: The fluorescence spectrum for GFP overlies almost exactly that of the most commonly used Ca^{2+} dyes. For this reason, these dyes cannot be usefully used in tandem with GFP labelling; such is the intensity of the GFP fluorescence that the small fluctuations due to the Ca^{2+} dye are lost in the shot noise (which is proportional to the square root of the number of photons). We hope, therefore, that investigators will start to express a different member of the fluorescent protein family, with a longer wavelength excitation spectrum, to make these animals compatible with fluo-3, fluo-4, calcium green, Oregon green, etc. (Note: fura-2 and indo-1 are compatible already, but not always usable for other reasons).

To conclude, imaging techniques provide a brand new vista for exploring the brain. We have provided here only a small detail of what is being pursued, and what is (or soon will be) possible. Suffice it to say that the future for research in these fields looks colorful, even rosy!

See also: **Glia**/**Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Role of Astrocyte Dysfunction in Epilepsy; **Imaging**: Optical Imaging in Epilepsy Research: Overview of Methods and Applications; **Networks**: Determinants of Neuronal Network Activity in Cultured Hippocampus; **Plasticity**: The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis.

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SPECT in Epilepsy Research

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Introduction

Although about 75% of patients with epilepsy can control their disorder with medication, the remaining population must undergo surgery or live with their condition. A widely cited meta-analysis in the 1990s noted that over 50 000 patients a year in the United States are candidates for surgical intervention. Of the patients that do receive surgical intervention, many (over 70% for temporal lobe epilepsy patients, perhaps the most successful of all groups) will become seizure free. Surgical success is highly contingent upon localization of the epileptogenic zone during the presurgical evaluation.

Over the past 15 years, SPECT (single photon emission computed tomography) has become an important modality for noninvasively localizing or corroborating local epileptogenic foci for intracranial electrode placement or resective neurosurgery. SPECT has also demonstrated great utility for basic neurological research in anatomy, consciousness, neural network connectivity, and neuronal activation in epilepsy. On the basis of three-dimensional mapping of regional cerebral blood flow (rCBF), SPECT provides accurate localization of functional changes in the brain. While SPECT has relatively low spatial and temporal resolution compared to functional MRI (fMRI), it is the only feasible imaging modality available for capturing rCBF images during most seizure types. The SPECT scanner itself is a multiheaded camera that collects gamma particles emitted from nuclear decay of injected radiopharmaceuticals. For ictal SPECT studies, where patients move during seizures, the injection is given shortly after seizure onset, but images can be taken up to 1–2 h later. While many different areas of the brain will absorb the tracer because of natural blood circulation, areas in the seizure focus strongly correlate with areas of increased perfusion; hence, localization of seizure onset is ascertained by areas of ictal hyperperfusion. Ictal SPECT is usually compared to baseline interictal SPECT for each patient, obtained when seizures are not occurring.

Background

SPECT for Clinical Seizure Localization

For seizure focus determination, intracranial electroencephalography (EEG) is often necessary, especially when MRI lesions prove equivocal (i.e., discordant). Unfortunately, intracranial EEG poses some risk of complications (reported as high as 4% in several studies), as well as being quite costly. Moreover, sensitivity of intracranial EEG results is highly dependent upon electrode placement, and inadequate coverage of the brain could result in absent or misleading localization. From a practical standpoint, ictal SPECT is often capable of directly supplementing or replacing intracranial EEG. Indeed, some clinicians recommend omission of an intracranial EEG study if SPECT localizations exactly match the findings from MRI and other noninvasive study findings.

Ictal SPECT has a much greater sensitivity than interictal or postictal SPECT. In the late 1980s, investigators noted that ictal blood flow increases of 3–4 times baseline occur during seizure activity; these findings also indicated that areas of ictal hyperperfusion tend to be large compared with abnormalities noted during the baseline or postictal periods. A metaanalysis subsequently demonstrated that the sensitivity of ictal SPECT ranged from 97% to 100%, exceeding that of postictal SPECT (75–77%) and interictal (43–44%) SPECT.

SPECT vs. Other Imaging Modalities

Beyond the technical level, comparison of SPECT to MRI, fMRI, and PET is greatly complicated by the dissimilar measures (perfusion, structure, blood oxygen level dependent (BOLD) signal, CBF, and metabolism, respectively) provided by each. Naturally, these differences translate to varying application of each technique, as dictated by the specific needs of various epileptic patient populations.

MRI

Having the highest spatial resolution, magnetic resonance imaging (MRI) provides anatomical images that, when coregistered with images from other modalities, can allow for finer structural localization than provided by functional imaging alone. MRI has been found to often correlate with localizations found by other methods; for example, MRI has high sensitivity for detecting tumors and mesial temporal sclerosis, but the structural and functional abnormalities may not always localize in precisely the same area.

fMRI

Measuring BOLD changes, (fMRI can provide high spatial and temporal resolution in comparison with SPECT. fMRI is technically capable of providing information in ictal studies, but this application is rarely used for

practical reasons. EEG-triggered or EEG-monitored fMRI allows seizures to be captured soon after onset, but requires that patients have their seizures inside a scanner (that is either scanning or capable of scanning immediately upon seizure onset). Since most seizures do not occur frequently enough to be captured within reasonable time in the MRI scanner, and since most seizures are associated with significant movement (even minor movements markedly degrade fMRI signals), this approach is rarely used. SPECT alleviates many of these problems, since scans can be performed up to 1–2 h after a seizure. fMRI is useful for imaging interictal spikes, which do occur with reasonable frequency in many patients, and are not associated with movement artifact. The relationship between fMRI localization of interictal spikes and the region of seizure onset as determined by ictal SPECT (and other methods) is under active investigation.

PET

Positron emission tomography (PET) is most commonly used clinically to measure glucose metabolism. In FDG-PET, a glucose analog, 2-fluoro-2-deoxy-D-glucose (FDG), is tagged with the radioactive isotope ^{18}flourine (half-life of 110 min) to create ^{18}F-FDG. In the 40–60 min time-frame after injection, the tracer is taken up by cells with high glucose demands. However, until the fluorine isotope undergoes positron emission decay to form ^{18}O, the tagged sugar will not undergo glycolysis. The decay will eventually produce a pair of gamma particles traveling 180° opposite each other, which can then be detected and used to reconstruct images. Because of the long uptake time for ^{18}F-FDG, PET temporal resolution is much lower compared with SPECT, and PET studies within the ictal time-frame are generally not feasible. Several studies have shown that interictal PET sensitivity appears to be higher than that of interictal SPECT, possibly because of greater decrease in glucose metabolism in the seizure focus as compared with blood perfusion. However, ictal SPECT sensitivity appears to be roughly equal to interictal PET.

SPECT for Investigating Fundamental Epilepsy Anatomy

Temporal lobe epilepsy

Perhaps stemming from its common incidence and the high surgical success rate of resection, temporal lobe epilepsy (TLE) has been widely studied using all image modalities, including SPECT. The difference imaging and SPM-based SPECT analysis methods (see below) were first used and verified with TLE patients. In TLE, SPECT may show propagation from temporal to extra-temporal areas. Our laboratory has used SPECT studies of simple and complex partial seizures that occur with TLE to study network changes associated with loss of consciousness. For complex partial TLE seizures, bilateral hypoperfusion was noted in the bilateral frontal and parietal association cortex, along with hyperperfusion in the upper brainstem and medial diencephalon; for simple partial TLE seizures, no significant changes were found outside the temporal lobe. SPECT studies from other laboratories have also shown a large network to be involved during seizures in TLE.

Occipital lobe epilepsy

In occipital lobe epilepsy (OLE), seizure spread is often rapid, with propagation patterns from the occipital lobe to the temporal, parietal, and frontal lobes. The rapidity of this spread makes localization a challenge, often requiring multimodal studies for presurgical localization. Relatively few comparative SPECT studies have been performed on OLE patients.

A 1999 study of six postsurgical OLE patients found that ictal SPECT localized the epileptogenic region to the occipital lobe in five cases. The unilateral occipital hyperperfusion was often followed by hyperperfusion in the temporal lobe. In two of these patients, ictal SPECT was sufficiently useful to guide intracranial EEG studies, which in turn confirmed the SPECT diagnosis. MRI abnormalities were found in four cases, and three of these cases showed concordance with ictal SPECT. Interestingly, no interictal SPECT studies showed any localizing findings. In contrast, interictal PET findings were concordant, in three patients, with MRI findings, but provided no localization information in the remaining two patients in whom the epileptic foci were found by ictal SPECT. Overall, ictal SPECT demonstrated the greatest diagnostic value in this study, but the patient population was rather limited in size. Further, the ictal/interictal comparison was performed visually (in a blinded manner); more recent SPECT analysis techniques (such as SPM) would be useful in providing more quantitative and comparative SPECT information.

A 2005 follow-up study of 26 postsurgical OLE patients found ictal SPECT to be much less concordant with postsurgical findings than suggested by 1999 study. Difference analysis was utilized, but pixel thresholding was performed by finding areas where percent change in perfusion was greater than 20%. Of the 19 patients imaged with ictal SPECT, only seven had correct SPECT localizations. Intracranial EEG, MRI, PET, and scalp EEG all had greater success sensitivity, with best presurgical evaluation stemming from combined use of several modalities. While ictal SPECT injections were given as soon as possible after seizure onset, the rapidity of OLE seizure propagation may have also hindered clear interpretation of the results. Clearly, the localizing value of ictal SPECT in patients with secondary generalization requires further investigation.

Methods

Radiopharmaceuticals

Several different radiopharmecuticals have been used for SPECT perfusion imaging. Currently, clinical SPECT is performed with either of two ^{99m}Technetium-labeled ligands: ^{99m}Tc-d,l-hexamethyl-propylene amine oxime (^{99m}Tc-HMPAO or ^{99m}Tc-exametazime), or ^{99m}Tc-ethyl cysteinate dimer (^{99m}Tc-ECD or ^{99m}Tc-bicisate). The metastable nuclear isotope ^{99m}Tc has a half-life of 6 h and a gamma photopeak of 140 eV. It decays into the less radioactive isotope ^{99}Tc, and leaves the body within a few days. Whole body radiation exposure for a typical adult trace injection of 20–30 mCi is less than that of a standard head CT. Exposure is carefully limited in children, with reported pediatric dosages starting from 3 mCi. ^{99m}Tc-ECD is chemically stable in excess of the radiotracer half-life. Historically, ^{99m}Tc-HMPAO has been found to remain chemically stable for only 30 min in vitro. However, several methods have been found to better stabilize the compound (including use of methylene blue, ethanol, or $CoCl_2$), and stabilized ^{99m}Tc-HMPAO can be injected for at least 4 h after preparation.

Radiotracers reach the brain within 30 s of venous injection, and brain uptake of both tracers is rapid. Indeed, with both ^{99m}Tc-HMPAO and ^{99m}Tc-ECD, uptake is complete within 1–2 min. There may be differences in brain-to-background ratios between these tracers, but this has little impact on imaging results in clinical studies. Both tracers will cross the blood-brain barrier and become trapped: ^{99m}Tc-ECD is metabolized by enzymatic conversion to form a polar complex, and ^{99m}Tc-HMPAO is converted into a hydrophilic compound. While redistribution of tracer after initial fixation can occur, reverse transport or movement of the tracer is insignificant in practice. Interictal studies tend to be performed at least 24 h (four half-lives) after the ictal study; care must be taken to ensure that about 1 day of nonseizure (both clinical and subclinical) state has passed before initiating an interictal study, to avoid effects of recent seizures on the interictal study.

Timing of Injections

By far the most time-critical aspect of SPECT is the timing of injection after seizure onset. Studies have repeatedly shown that it is desirable to inject within 45–60 s of seizure onset to improve 'capture' of seizure localization. While injection times after this window are not without use (indeed, some information such as lateralization can be gleamed even from postictal injections), localization sensitivity begins to fade as the seizure spreads. Effectively, late injection may cause the seizure focus to appear as a hypoperfusion site, or make the contralateral propagation appear as hyperperfusion. Rapidly spreading seizures that propagate across hemispheres or become bilateral within 15–20 s of onset pose a practical limit for SPECT studies, since injections to assess this time-frame typically cannot be accomplished.

SPECT Study Procedure

Presurgical evaluation using SPECT is typically performed after scalp EEG recordings and fMRI imaging have been completed, but before intracranial implantation of electrodes. The study is usually an inpatient procedure that often takes several days because of the unpredictability of seizure events. Since imaging must be performed within 1–2 h after injection, radiotracer administration must also occur in coordination with scanner time availability.

After preparing the injection site by inserting an IV, nuclear medicine personnel watch for epileptic events, ideally in the same room as the patient. A syringe with correct radiotracer dosage must always be on hand for immediate use; due its short half-life, the dosage may have to be adjusted or replenished several times during a monitoring window.

An epileptic event is determined using both EEG monitoring and in-person evaluation of the behavioral state of the patient. Once seizure start has been determined, the radiotracer is injected immediately.

The SPECT scan itself takes 20–40 min. Motion artifact from patient movement is difficult to avoid because of this extended time period. Image reconstruction on the collected data is performed to obtain tomographic sections. Visual evaluation of the resulting images can often rapidly determine the usefulness of the images for further analysis. Motion, incomplete radiotracer perfusion (because of extravasation at the injection site), delayed injections, and other technical problems can render the study ineffective.

Recent Results

SPECT for Clinical Seizure Localization: Reading and Analysis

Visual assessment

Generally, the 'rating' of SPECT images for localization is first performed visually: an ictal-only study is visually examined (on a perregion basis) for any bilateral asymmetries. Obviously, the nature of this inspection precludes discovery of any diffuse or symmetric focal perfusion changes that occur bilaterally; thus, bilateral seizures may fail to be localized. Additionally, if a region shows hypoperfusion interictally, 'pseudonormalization' may occur during the seizure, so that a focal area of *relative* hyperperfusion could be missed without careful comparison to the interictal

scan. Visual side by side comparison of an ictal and interictal study from the same patient alleviates some of these issues. Complications arise, however, with this type of comparison: patient positioning will most likely be different, thus preventing one to one comparison of slices; likewise, tracer levels will differ because of different injection times, scan times, and blood flow and uptake characteristics that vary day-to-day.

Difference imaging

Researchers in the mid-1990s started to work around these limitations by digitally coregistering the ictal and interictal images, normalizing tracer levels across the brain, and subtracting the interictal from ictal images. Anatomical MRI images were also coregistered. Differences could then be quantified, and anatomy easily visualized. Techiques for performing this type of analysis have been called SPECT difference imaging analysis (DIA), or subtraction ictal SPECT coregistered to MRI (SISCOM). Several parameters must be carefully selected during digital subtraction analysis, including intensity thresholds and coregistration settings. To avoid artifacts and spurious results, it is crucial to visually inspect images, and not to use available algorithms as a 'black box' approach. When used correctly, difference imaging has been found to improve SPECT concordance (sometimes by a factor of two) with intracranial EEG or surgical findings as compared to traditional visual SPECT reading. Nevertheless, difference imaging has its pitfalls: many focal areas of activation are produced by the process, and results are thus still subject to substantial reader interpretation. While reader guidelines and some subjective evaluation will always be required, more sophisticated statistical analysis tools are helpful for more objective, repeatable interpretations.

SPM

Statistical parametric mapping (SPM) has been widely used to analyze many types of neuroimaging data. In this approach, statistical parametric maps of each study are made by applying univariate statistical tests to all voxels in the scans, and then combing the resulting voxel-by-voxel statistical parameters. The maps are, using the null hypothesis, distributed according to t or F probability density functions. The general linear model is used to define the experimental model (covariate, indicator, and confound variables). Problems associated with multiple comparisons are corrected by using Gaussian random fields to adjust voxel statistical significance. Ultimately, areas of clinical interest appear as differences between maps.

Commonly used with fMRI and PET, SPM is now increasingly being used for single-subject ictal–interictal SPECT analysis, using a method referred to as ictal–interictal SPECT analysis by SPM (ISAS, see http://spect.yale.edu/). Several studies have demonstrated the concordance of ISAS results to those using other subtraction techniques, and to surgical outcome. An example of ISAS analysis in a patient with TLE is shown in **Figure 1**. Our laboratory has defined some objective 'reading rules' for ISAS results, validated with a clinical epilepsy population. For a simple SPM ictal–interictal analysis against a predefined healthy control group, nonexperts can be quickly trained to run the software, and the subjective aspects of the program are limited to initial image reorientation and decisions of when to mask extracerebral artifacts. As with other computed analysis methods, however, visual examination of raw and difference images prior to preprocessing is desirable to appreciate potential complicating factors (scanner artifacts, reconstruction issues, etc.).

SPM also permits statistical comparisons of ictal-only studies to be made against healthy normal control groups. Although there are some financial savings if interictal scans are omitted, without within patient comparison of ictal and interictal scans, baseline areas of hypoperfusion may not be appreciated, and thus prevent detection of relative hyperperfusion areas in the ictal-only study. As we have already discussed, using a patient's interictal scan as an intra-subject control prevents this 'pseudo-normalization.'

SPECT for Investigating Fundamental Epilepsy Physiology

Ictal dystonia

Characterized by unilateral sustained muscle contractions, ictal dystonic posturing is a dependable indicator of lateralization of TLE, since dystonic posturing is usually contralateral to seizure onset. Ictal dystonia may be a result of seizure propagation to the striatum and globus pallidus. Understanding the specific networks involved with ictal dystonia in TLE has been the objective of several SPECT studies. One study of 22 patients (15 with ictal dystonia) demonstrated involvement of the caudate nucleus, putamen, and thalamus. These localizations were congruent with other studies on secondary dystonia resulting from infections, trauma, etc. Another SPECT study with a similar population size (13) found statistically significant hyperperfusion in the putamen and mesial temporal lobe associated with ictal dystonia.

Loss of consciousness

SPECT studies investigating loss of consciousness have been conducted in several different patient groups. SPECT studies of TLE by our group and several others suggest that impaired consciousness may be associated with abnormal activity in the upper brainstem/medial thalamus, along with depressed function (bilaterally) of the frontal and parietal cortex. Instead of focusing on one form of epilepsy, other studies have gauged consciousness across various seizure types; findings in one study indicate

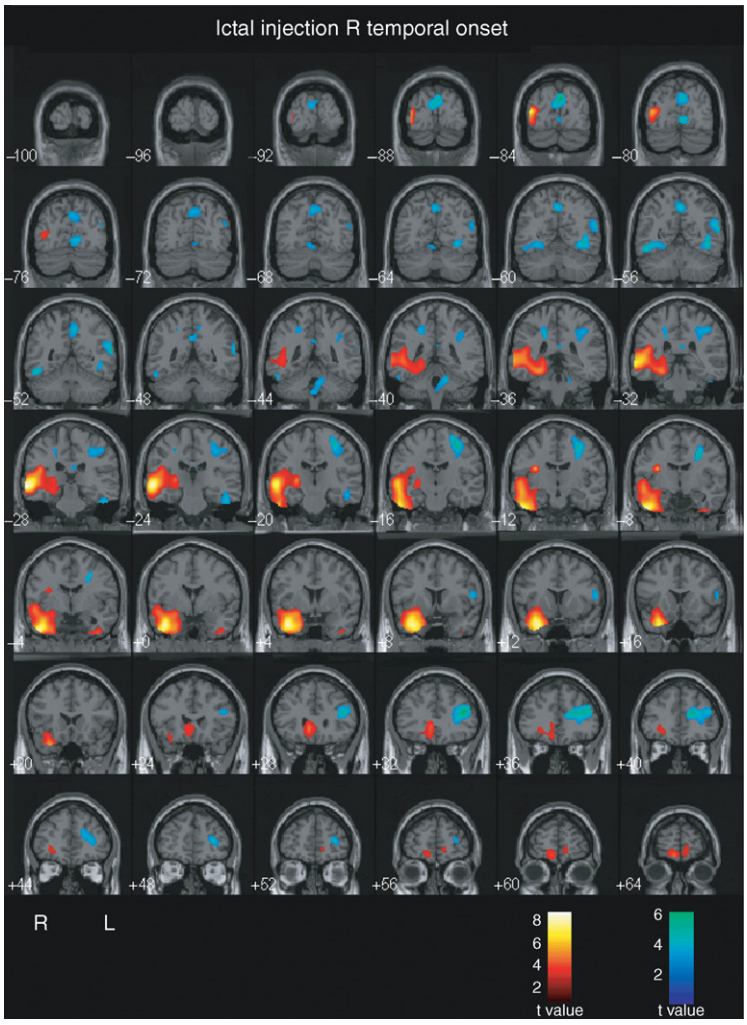


Figure 1 Ictal SPECT in mesial temporal lobe epilepsy (TLE) analyzed by SPM (ISAS). Ictal–interictal SPECT analysis is shown from a patient with right mesial temporal lobe onset, confirmed by surgical localization. Seizure duration was 153s and Tc99m-HMPAO was injected 69s before seizure end. The most significant hyperperfusion cluster was localized to the right temporal lobe (cluster-level significance $P < 0.0001$ corrected for multiple comparisons, Z-score of most significant voxel = 5.06, cluster size, $k = 10429$ voxels). The most significant region of hypoperfusion was localized to the L occipital lobe ($P = 0.035$, $Z = 4.04$, $k = 1625$). Hypoperfusion areas were not found to be helpful for localizing seizure onset. The ictal SPECT scan was background subtracted using the patient's corresponding interictal SPECT, and the difference was then compared to the database of normal SPECT pairs using ISAS (see http://spect.yale.edu). CBF increases are shown as red–white, and decreases are shown as blue–green; color bars indicate t values. Coronal views are shown superimposed on the SPM MRI template. Extent threshold, $k = 125$ voxels, voxel-level height threshold, $P = 0.01$. Reproduced with permission from McNally *et al.* (2005) *Epilepsia* 46(9): 1450–1464.

that impaired conscious is because of secondary hyperperfusion in the thalamus and upper brain stem. Our laboratory has also conducted studies using secondarily generalized seizures induced by electroconvulsive therapy (ECT) for depression. Although occurring under sedation, the induced seizure is similar to generalized epileptic seizures in both clinical and EEG aspects. SPM analysis demonstrated the largest hyperperfusion in the frontal and parietal association cortex, along with midbrain and thalamic involvement. Thus, the seizure's effect on the higher-order association cortex and upper brainstem/diencephalic activating systems may explain the root of impaired consciousness during generalized seizures. Ictal SPECT in epilepsy patients with secondarily generalized tonic–clonic seizures has also demonstrated SPECT changes in these same regions during seizures associated with ictal and postictal unconsciousness. We are currently investigating impaired consciousness in absence seizures, but using fMRI instead of SPECT; high temporal resolution is mandatory in these studies, since absence seizures usually last only 5–10 s, well below effective SPECT injection time.

Secondary generalization

We have recently collected SPECT data on 55 patients with secondarilygeneralized tonic–clonic seizures with pregeneralization, generalization, and postgeneralization radiotracer injections. Preliminary analysis of the results indicates focal, bilateral changes in rCBF during generalization. The data also suggest that epileptogenic lateralization, and in some cases localization to the correct lobe of seizure onset can be reliably determined based on SPECT in secondarily generalized seizures.

Hypothalamic hamartoma

Hypothalamic hamartoma is a rare, abnormal cellular growth (1–3 cm in size) on or near the hypothalamus. The mass does not appear on CT scans, and is isodense and nonenhancing on MRI. A typical symptom of hypothalamic hamartoma is gelastic epilepsy or ictal laughing, presenting with other seizure types (including complex partial). Gelastic seizures typically appear in infancy (3 months–1 year). The hypothalamic hamartoma mass may generate constant seizures, thus entraining surrounding networks. Clinical grading systems exist to denote the degree of attachment of hypothalamic hamartomas to other structures, and hypothalamic hamartomas with broad connectivity appear more often to induce seizures. The seizures (both gelastic and secondarily-associated) do not respond well to anticonvulsant treatment, and surgical resection outcomes are also mixed. EEG studies have demonstrated hypothalamic ictal discharges, and SPECT studies have confirmed that the hypothalamic hamartoma is the epileptogenic center during gelastic seizures, with ictal hyperperfusion. Use of ictal SPECT may allow for earlier detection and removal of hypothalamic hamartomas in some cases, although the diagnosis is usually made based on MRI and clinical features.

Infantile spasm

Infantile spasm (a component of West syndrome) is a rare form of infant epilepsy characterized by clustered spasms, hypsarrhythmia (interictal sharp and slow waves with high amplitude), and profound cerebral developmental abnormalities. Several SPECT studies have been performed to examine the origin and pathways associated with the spasms and hypsarrhythmia. SPECT studies and analyses must be read with regard to both the differences due to patient age (metabolism and perfusion are different for infants and young children, etc.), and to the severe structural abnormalities associated with infantile spasm. As perhaps expected, rCBF abnormalities are quite common: a 1996 study of 20 children for a 4 year period found over 85% had abnormal rCBF, with multifocal decreases in 40% of the children. Several studies have demonstrated that rCBF irregularities may appear in nonsymptomatic areas. Additionally, abnormal rCBF readings may present with normal MRI findings; a 1994 study of seven children all showed unilateral or bilateral hypoperfusion in the temporal lobe without accompanying abnormal MRI.

SPECT and PET studies have shown hyperperfusion or hypermetabolism of the basal ganglia and brainstem during spasms, with associated subcortical activations. Together with studies involving other modalities, the results suggest that a subset of infantile spasm patients have seizures that originate from focal cortical lesions, with additional involvement the basal ganglia and brainstem. Cortical resective surgery or drug therapy has been shown to lead to seizure freedom in some cases.

Conclusions and Directions for Future Research

SPECT is a useful technique; it has the unique capacity to image brain networks involved in human seizures while being unaffected by seizure movement. The ability to obtain ictal images, combined with digital analysis in comparison with interictal blood flow, has greatly enhanced the ability of SPECT to localize seizures for epilepsy surgery, and has shed new light on fundamental seizure networks and mechanisms.

There are many clinical avenues that have not been explored in depth, if at all, using SPECT. Future directions for SPECT research depend, in part, on increased use of the technique, which has been limited in part due to practical logistical issues involved in performing ictal injections. Automation of SPECT injections may help eliminate the time-consuming waiting process, and make studies more economical. While decreases in scanner time may not be technically feasible, advances in computational motion-correction promise to fix a major limitation of this technique. Modernizations being made in imaging software platforms (such as BioImage Suite, http://www.bioimagesuite.org/), in image coregistration, and in statistical analysis will further improve the results that can be obtained from SPECT studies.

Acknowledgements

This work was supported by NIH R01 NS049307, P30 NS052519, the Donaghue Foundation, and by the Betsy and Jonathan Blattmachr Family.

See also: **Imaging**: Functional MRI in Basic Epilepsy Research; Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Structural Magnetic Resonance Imaging of Epilepsy; **Metabolic Changes**: Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain.

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Structural Magnetic Resonance Imaging of Epilepsy

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Introduction

Epilepsy is a relatively diverse group of central nervous system disorders that may be acquired (infection, trauma, febrile seizures, etc.) or have genetic origins. Epilepsy is typically characterized by spontaneous and recurrent seizures. In electroencephalographic (EEG) recordings of brain activity, seizures appear as high frequency electrical discharges. These discharges may originate in, and be confined to a specific brain region with pathological features; for example, temporal lobe seizures (TLS) are often associated with mesial temporal-lobe sclerosis (MTS) – cell loss and gliosis in the hippocampus and related brain regions. However, in other cases, such as Rasmussen's disease, a focal abnormality may not be detected. The diverse etiology of these neurological disorders has prompted their development of numerous animal models to mimic the various manifestations of these disorders.

Electrophysiological and histological methods, applied in both the experimental laboratory and the clinic, have provided direction for treatments. However, these techniques have important limitations, including poor spatial resolution (EEG) and invasiveness (histology). In the last decade, non-invasive methods have gained momentum; they permit serial monitoring (days, weeks, months) and are now a standard tool for augmenting the armamentarium of the epileptologist and neurologist.

Of the various imaging modalities used for diagnosis and therapy monitoring, magnetic resonance imaging (MRI) is the most popular. It has several distinct advantages: it involves no ionizing radiation; multiple 'stains' or 'contrasts' are available from a single study; and both structural and chemical alterations can be probed. Most commonly, structural MRI (T1, T2, DWI) is performed to locate lesions that cause, or are a consequence of, the observed abnormal electrical activity. If lesions are not obvious in an initial 'standard' study, more advanced methods are available. For example, external contrast agents can be injected to detect areas where the blood-brain barrier has been compromised. In other cases, where ischemia is suspected, perfusion-weighted imaging (PWI) may be employed to assess the performance of the capillary bed. Similarly, subtle structural changes (for example, mossy fiber sprouting), may be probed using diffusion tensor imaging (DTI). Physiological information is also

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available via volume selective spectroscopy (MRS) and functional MRI (fMRI). fMRI is particularly useful in identifying critical brain areas (speech, movement, etc.) that must be spared during surgical intervention (typically lobectomy). MRS offers metabolic information that is particularly useful in identifying neuronal loss and regions of necrosis. While magnetic resonance techniques are rapidly becoming the primary investigative imaging modalities, they are often augmented with information available from positron emission tomography (PET) or single photon emission computed tomography (SPECT) – techniques that probe cellular function. Finally, X-rays (computed tomography, CT) remain useful in identifying gross neuro-anatomical abnormalities, such as hemorrhage, necrotic tumors, and abscesses that may produce epileptic-like symptoms.

In the clinic, qualitative visual analysis of MRI data is often the primary method for diagnosis. Since this approach is highly dependent on physician expertise, analyst-independent quantitative approaches have gained popularity. Computational methods have been used to identify volumetric changes (i.e., hippocampal swelling), intrinsic tissue matrix alterations (neuronal loss, glial activation), and connectivity within the epileptic brain (e.g., contra-lateral injury). Typically these analysis techniques require additional data collection, in order to compute such tissue characteristics as diffusion coefficients (DWI, DTI), chemical changes (magnetic resonance spectroscopy, MRS), or tissue relaxation times (T1, T2). The added time required for these analyses limits their use in the clinic. However, they form the backbone within basic science research for determining epilepsy onset, progression, and efficacy of various therapies.

Background

Animal Models of Epilepsy

Rat models are widely used by experimental epileptologists. Partial seizures (arising from a restricted brain regions) can be induced by topical or regional application of amino acids antagonists (e.g., penicillin, bicuculline) or excitatory amino acid agonists (e.g., kainic acid); systemic injection of these agents (also including the cholinergic agonist pilocarpine) induces what appear to be partial seizures, arising from the hippocampus and related regions. Many of these excitatory agonists produce a period of *status epilepticus* (SE, sustained seizures, typically for greater than 10 min) followed by a latent period. After the inductive and latent phases, many animals go on to develop spontaneous recurrent seizures – i.e., become epileptic. Generalized seizures can also be modeled, using electrical stimulation paradigms as well as other chemical induction methods.

MRI of the Pilocarpine Model of Epilepsy

The rat pilocarpine model is characterized by a robust period (1–4 h) of *status epilepticus* that normally results in loss of neurons within various brain regions, including the hippocampus, piriform cortex, amygdala, retrosplenial cortex, and thalamus. Cell loss alters the chemical and physical properties of the brain matrix, and these changes permit tracking of injury progression and cell death using various MR contrasts. Diffusion-weighted imaging in the pilocarpine model has been particularly revealing. In DWI, the signal intensity (contrast) is based upon the relative mobility of the reporter molecule, water. Its motion in the tissue is measured as an Apparent Diffusion Coefficient (ADC) – 'apparent' because the three dimensional motion of water is observed in two dimensions only. ADC measurements suggest that water mobility is reduced within hours of seizure and SE onset, but that mobility gradually normalizes within 24 h. This transient reduction is interpreted as an indication of neuronal swelling, which decreases the relatively mobile extracellular water fraction. During the subsequent 48–72 h ADC values increase. This change indicates an enhanced extracellular water fraction that is temporally correlated with cell necrosis. In other words, as more cells die, water molecules encounter fewer reflective barriers (i.e., less restricted diffusion) and/or diffuse in a less viscous matrix. This reduction in tissue tortuosity (or viscosity) has become a key feature in characterizing ischemic-like injury associated with convulsive seizures (those involving muscle stiffening and contraction/extension of the limbs).

To measure a signal in MRI, it is first necessary to excite the reporter (in the case of DWI, the hydrogen nucleus of a water molecule) and then monitor the rate at which energy is released from the excited nucleus. There are two processes by which this occurs: cooling – where the energy is returned as heat to the environment, and dissipation – where the cohort of excited molecules lose coherence and internally cancel the measurable signal. These processes are characterized by distinct time coefficients, T1 and T2. The latter process is particularly useful in probing the nuclear environment because the loss of coherence is affected by various factors, including viscosity and the presence of paramagnetic compounds. As a guideline, compartments that are relatively viscous have shorter T2s than those containing pure water. In the rat pilocarpine model, T2-weighted image intensities increase (corresponding to increased T2 values) after cellular injury, an observation consistent with edema formation.

Changes in the relaxation or diffusion of the reporter molecule are reflected as intensity-contrasts in the image. A key advantage of these contrasts is the excellent anatomical localization of injury. High resolution imaging allows the investigator to compare imaging data directly with histological findings. This process has proven to be highly

complementary. That is, the MR studies provide unperturbed temporal evolution information, while the histology provides periodic cell based observations. Such analyses lead to an understanding of the evolution of injury following seizure activity, and have shown that each model (and each seizure type) has unique features. For example, in the pilocarpine rat model, changes seen on MRI (reflecting injury) are first apparent in the piriform cortex, and then in the retrosplenial cortex; involvement of the thalamus and hippocampus is apparent only after 72 h. In contrast, seizure-associated damage after injection of the neurotoxin soman (a cholinesterase inhibitor) first appears in retrosplenial cortex. These MRI-based temporal events have been confirmed on histological samples. Such experiments have shown that MRI can provide useful diagnostic and regional information about the onset, latent period, and cellular remodeling that occurs in the brain as a consequence of status epilepticus.

Methods

Pilocarpine injections (without lithium pretreatment) of adult rats were used to reliably invoke SE for 1–4 h. Less frequent convulsive activity often was observed in these animals for the next 4–8 h. Animals were scored for seizure severity using standard behavioral measures. To improve the health and recovery of these animals, moistened chow with fructose was provided. In our experience, mortality typically was about 30%. Animals were imaged at 1, 7, 14, 21, and 30 days following pilocarpine injections. While comprehensive behavioral observations were not conducted, many animals at 21 and 30 days regularly exhibited spontaneous seizures.

After SE induction, the animals were allowed to recover for 24 h prior to MRI. All MRI exams were conducted using a Bruker 4.7 Tesla (T) magnet, with the animals lightly anesthetized by isoflurane. To minimize motion during the MR acquisitions, animals were placed in a custom-built stereotactic head holder. A standard suite of MR sequences was used to acquire T2 and DWI data: T2WI acquisition was performed using a multi-echo spin echo acquisition protocol (6 echo, 20–120 ms), and the DWI data were obtained using two weighting factors ($b = 0\,s\,mm^{-2}$) and ($b = 434\,s\,mm^{-2}$). The T2 and DWI MR data sets were processed to compute T2 coefficient (transverse relaxation, ms) and ADC (water mobility, $mm^2\ s^{-1}$) maps using standard computational methods. Regions of interest (hippocampus, piriform cortex, etc.) were then overlaid on the initial T2 and DWI images, and the quantitative data extracted and summarized.

At the end of the experimental period, all animals were processed for standard histology and immunohistochemistry to evaluate tissue and cellular composition. Prior to histological sectioning, the *ex vivo* brains were re-imaged for high resolution MR microscopy in an 11.7T magnet, using sequences similar to those used for the 4.7T image acquisition.

Recent Results

Diffusion Weighted Imaging

Previously published data from our laboratory and from others have shown that during the initial 24 h after the onset of SE, there are temporally and regionally differential effects within the brain that are evident in the MR images (**Figure 1**). The ADC decreases within the retrosplenial cortex 12–18 h after pilocarpine induced SE, and then returns to control levels by 24 h (**Figure 2**). Perhaps more interesting is the concomitant increase in ADC within the hippocampus. Correlative histological observations indicate that the early ADC decrease reflects

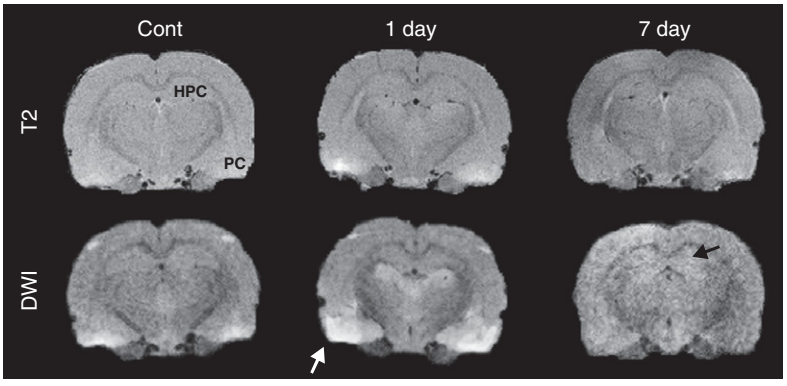


Figure 1 Serial imaging after epilepsy induction. Pilocarpine induced status epilepticus was initiated after control image data (T2, DWI) was obtained. In this example the same animal was repeatedly imaged to provide a temporal snapshot of cellular changes after seizure induction. At 1 day after pilocarpine injection, signal intensity in the weighted DWI within the piriform cortex (PC, white arrow) and midline structures increases. By 7 days there was increased signal within cortical structures, including the hippocampus (HPC, black arrow).

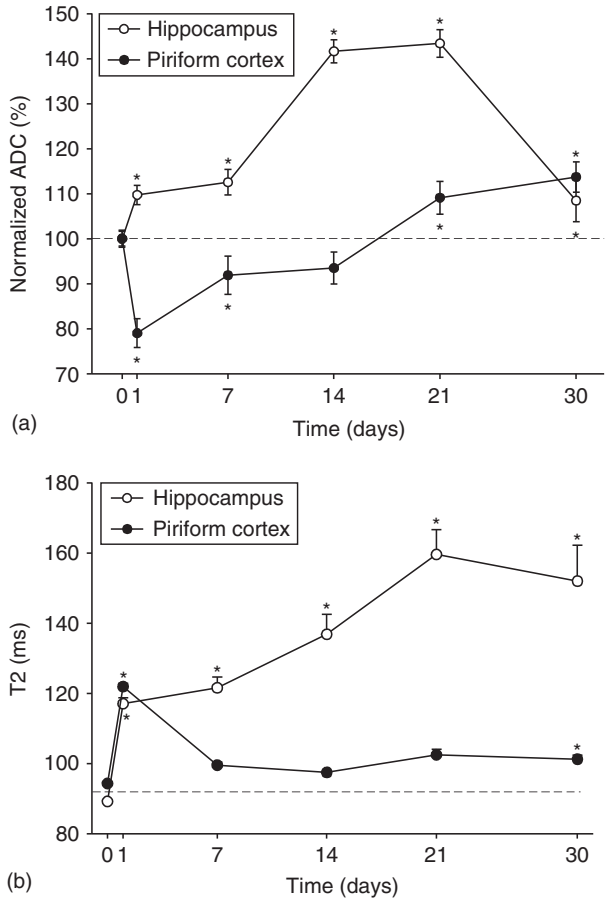


Figure 2 Temporal evolution of quantitative MRI changes. (a) Normalized ADC. The hippocampus and piriform cortex each have different ADC signatures during the first 30 days following seizure induction. Hippocampal ADCs increase during the first 21 days and then slowly return to control values (dotted line). In the piriform cortex there is a rapid decline in ADC, which then slowly increases over the next 30 days. These ADC changes are related to alterations in water diffusion in the tissue within each region. (b) T2 changes in the hippocampus and piriform cortex. T2 elevations in the hippocampus are progressive and become maximal at 21–30 days. This elevation of putative water content (edema) could be indicative of synaptic remodeling and neuronal loss within this region. Conversely, within the piriform cortex, rapid cell death and subsequent pan necrosis leads to an initial increase in T2 values at 1 day, which then quickly decreases back to control levels. Thus, edema formation in the piriform cortex is considerably less than that seen in the hippocampus.

neurodegeneration within the piriform cortex, while within the hippocampus there are few dying neurons.

One of the major advantages of MRI in epilepsy is the ability to monitor the same group of animals for long periods of time. For example, we extended our 7-day study to thirty days. In doing so, we found that ADC values in several regions, including the piriform cortex, gradually returned to control values by 14 days after SE. This normalization time-frame correlates, temporally, with reactive gliosis, reflecting tissue repair mechanisms characteristic of the inflammatory response. Glial cells, although substantially different in size and shape from neuronal cells, apparently produce a biophysically similar tissue matrix with diffusion properties similar to that of the original neuronal tissue. However, the post-SE remediation process is not complete at this 14-day timepoint; ADC values within the piriform cortex continued to increase in the ensuing days, so that at 21 and 30 days following SE, ADC values were significantly greater than controls. These experiments demonstrated that although substantial portions of the principal neuronal layers within the piriform cortex (Layers 2 and 3) are rapidly (24–48 h) lost after the initial seizure induction, the neurodegenerative processes are by no means complete. A growing area of cell loss was apparent in MR images, and was confirmed with periodic histological sampling. Based on the histological data, it appeared that MRI was equally effective in detecting cell loss owing to apoptotic and necrotic processes (**Figure 3**).

In the hippocampus, a different picture emerged. The ADC slowly increased over the first 21 days (to 145% of control) but then returned to near normal by 30 days. Again, these changes were consistent with the corresponding histological evidence; the relatively slow ADC changes in the hippocampus reflected gradual neurodegeneration. Also interesting is the fact that the apparent peak ADC changes within the hippocampus appeared at 14–21 days post-SE, a time period known for synaptic and cellular remodeling (i.e., mossy fiber sprouting (MFS)). In a similar cohort of animals also experiencing pilocarpine-induced SE, extensive MFS occurred within the dentate gyrus at this same time; other investigators have reported that the majority of this remodeling is complete by 30 days – when we see a trend toward normalization of the ADC (**Figure 3**).

More recently, using a variant of DWI – q-space imaging – we were able to further discriminate between what appeared to be neuronal and glial populations within the piriform cortex. In this case, discrimination is based on our ability to isolate different diffusing populations. In other words, water within neuronal cells diffuses, on average, at one characteristic rate, while that in glia diffuses, on average, at a different rate. While our q-space sampling paradigm contained limiting assumptions, it did establish a potential method to evaluate future therapeutic interventions based on cellular targets. More work is needed to validate this technique for its ability to discriminate various cellular populations important in epilepsy.

T2-Weighted Imaging

T2WI provides additional information on brain water content. The T2 coefficient provides an indirect or relative assessment of compartment viscosity. As with the ADC changes, T2 values increased soon after seizure induction (<1–6 h). In the piriform cortex, the average T2 coefficient

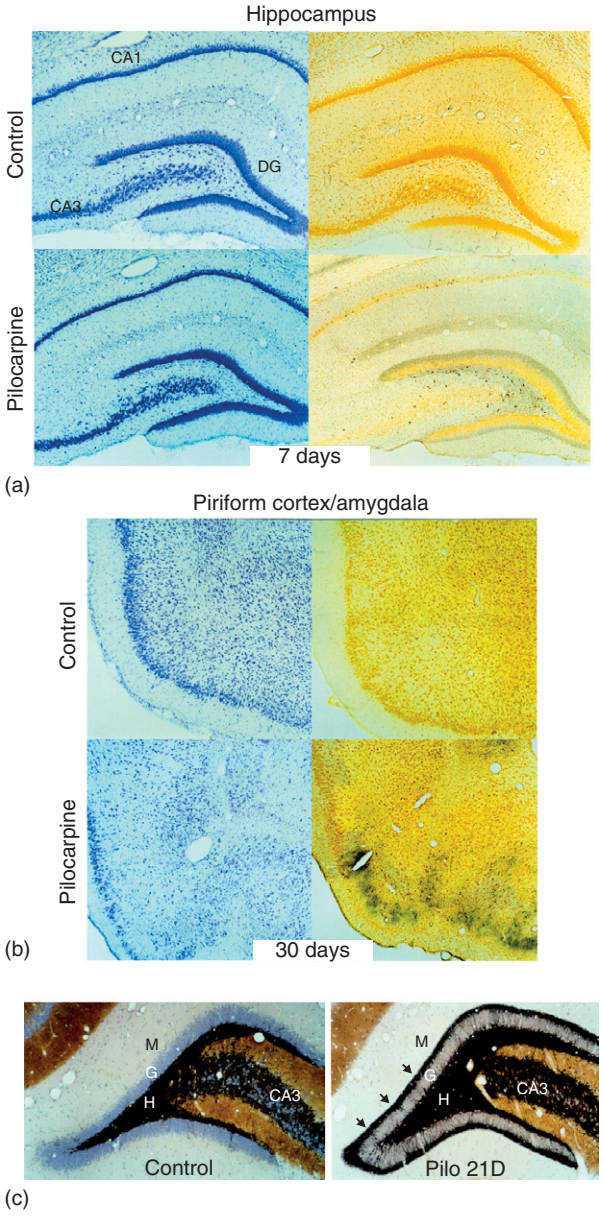


Figure 3 Cellular alterations within the hippocampus and piriform cortex after pilocarpine-induced seizures. (a) The hippocampus exhibits a slow but progressive increase in neuronal death predominantly within the dentate gyrus but also in the CA1 region (left panel, cresyl violet, right panel, silver degeneration stain). Within the hippocampus cell death is maximal at 7 days. (b) In previously published research we have shown that cell death within the piriform cortex is maximal within the first 24 h. The pan-necrotic lesion formed in the first several days is still present at 30 days with significant loss of the principal layers within the piriform cortex. (c) Mossy fiber sprouting (MFS) within the dentate gyrus is maximal within the dentate gyrus at 21 days in the pilocarpine treated animals. The formation of an additional layer of zinc positive (Timm's stain) staining on the molecular layer (M) side of the granule cell layer (G) (arrows) occurs at a time when ADC changes in the hippocampus are maximal. This would suggest that cellular alterations can be visualized with ADC derived MRI data.

value increased by 20% at 1 day post-SE, then returned to control values by 7 days. Of interest was the reappearance of a secondary slow increase in T2 within the piriform cortex at 21–30 days. It is tempting to speculate that this slow increase reflects the new tissue matrix formed during gliosis.

In the hippocampus, the T2 values gradually increased over 21 days post-SE, to 150% of controls, and this level persisted until the end of the observation period (30 days). This observation supports the hypothesis that there is a slow but steady formation of edema that peaks around 21 days. At this time point, the neuronal cell death process is virtually complete within the hippocampus, as is MFS-induced reorganization.

In summary, MR imaging – and in particular, diffusion and relaxation contrast imaging – is sufficiently sensitive to detect tissue changes that follow severe epileptic activity. In addition, the standard clinical sequences can be extended to provide quantitative insights – if increased acquisition and analysis times are not a limiting factor. There is emerging evidence that cell populations in the brain have distinct diffusion signatures, and that these characteristics may be accessible from detailed diffusion measurements. Chronic diseases such as epilepsy require serial measurements to determine the status of injury. MR technology provides an essential and versatile window for this type of measurement.

Future Directions

Conventional MR procedures provide excellent images with sub-millimeter resolution of brain structures. These imaging approaches have been applied in the laboratory setting to provide quantitative measures that characterize regions of interest. By correlating knowledge from complementary technologies (i.e., other imaging methods, histopathology), subtle features can be extracted that offer additional insights into the disease process.

MR spectroscopy has the ability to provide data on key cellular metabolites such as *N*-acetylaspartate acid (NAA – a neuronal marker), creatine/phosphocreatine (Cr – an energy metabolite) and choline (Cho – a membrane component). Lactate peaks often appear transiently on MRS images during injury, and thus provide sensitive information about the level of injury.

Diffusion tensor imaging (DTI) is a variant of diffusion-weighted imaging that can assess tissue microstructure in vivo. The microstructure of the white matter fibers preferentially limits the movement of water molecules diffusing perpendicular to the fiber direction; this privileged water diffusion is termed 'anisotropy.' Numerous quantitative parameters can be extracted from DTI data that

describe altered microstructure (primarily white matter) within the brain. Only recently have investigators begun to use DTI – experimentally and in the clinical setting – to study or diagnose epilepsy. This DWI variant has the potential of providing additional prognostic and diagnostic information about regional tissue status.

Functional MRI (fMRI) uses the hemodynamic response of the brain to various stimuli as a reporter of brain activity. fMRI is used extensively for brain mapping studies, including pre-surgical evaluation of epilepsy patients. With the appropriate cognitive, motor or sensory test, fMRI may define seizure-affected and normal brain regions. However, due to the extensive post-processing required, this technique is not likely to become part of routine clinical practice. fMRI evaluation in animal studies is difficult because some anesthesia is generally required to reduce motion artifacts of animal subjects, and inhalation anesthetics are known to reduce blood flow. Development of alternative methods to reduce movement, and or to compensate for movement during data processing, may increase the use of this technique in the laboratory.

Finally, many of the new advances in MRI are arising from computational methods that extract relevant MR parameters quickly and efficiently. Enhanced statistical techniques, wavelet analysis, histogram-based analysis and other data extraction methodologies will provide additional perspectives on the onset, evolution, and treatment of epilepsy.

Acknowledgements

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Using Intrinsic Optical Signal Changes to Analyze Epileptic Activity and Seizures

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Introduction

Epileptic activity and seizures represent highly dynamic phenomena potentially activating brain tissue in various regions of the brain. Analyzing such epileptiform activity, and the pathways through which it spreads, requires tools that provide both excellent temporal and spatial resolution. Optical imaging is capable of yielding information on this scale and is therefore well-suited to study the onset and spread of seizure activity. There are two main forms of optical imaging. 'Extrinsic' optical imaging relies on the use of indicators that change their optical

describe altered microstructure (primarily white matter) within the brain. Only recently have investigators begun to use DTI – experimentally and in the clinical setting – to study or diagnose epilepsy. This DWI variant has the potential of providing additional prognostic and diagnostic information about regional tissue status.

Functional MRI (fMRI) uses the hemodynamic response of the brain to various stimuli as a reporter of brain activity. fMRI is used extensively for brain mapping studies, including pre-surgical evaluation of epilepsy patients. With the appropriate cognitive, motor or sensory test, fMRI may define seizure-affected and normal brain regions. However, due to the extensive post-processing required, this technique is not likely to become part of routine clinical practice. fMRI evaluation in animal studies is difficult because some anesthesia is generally required to reduce motion artifacts of animal subjects, and inhalation anesthetics are known to reduce blood flow. Development of alternative methods to reduce movement, and or to compensate for movement during data processing, may increase the use of this technique in the laboratory.

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Introduction

Epileptic activity and seizures represent highly dynamic phenomena potentially activating brain tissue in various regions of the brain. Analyzing such epileptiform activity, and the pathways through which it spreads, requires tools that provide both excellent temporal and spatial resolution. Optical imaging is capable of yielding information on this scale and is therefore well-suited to study the onset and spread of seizure activity. There are two main forms of optical imaging. 'Extrinsic' optical imaging relies on the use of indicators that change their optical

properties (e.g., fluorescence or absorbance) with variations in cellular activity (i.e., changes in neuronal membrane potential). With the use of voltage sensitive dyes, temporal resolution is excellent (within milliseconds), but as dyes are toxic, this approach is used in experimental studies only. In contrast, 'intrinsic' optical imaging is based on signal changes associated with tissue itself and is entirely noninvasive. This article deals with intrinsic optical imaging and its relevance to the study of different forms of epileptic activity, including their regions of onset and spread, with special consideration of experimental studies carried out in the brain slice preparation, in vitro.

Background

Basics of Intrinsic Optical Imaging

The processes which occur when light interacts with living cells (or tissues or organs), leads to loss of light intensity (attenuation), resulting from scattering, absorption, and fluorescence. Scattering indicates that photons undergo elastic collisions; that is, no energy is lost and the photon merely changes direction. Light reflectance and transmittance are affected in opposite ways. The more a medium scatters light, the more the light is reflected and the less is transmitted. Higher frequency (shorter wavelength) light at the blue end of the visible spectrum is scattered considerably more in cells and tissues than lower frequency (longer wavelength) light. For this reason, near-infrared radiation is more effective for noninvasive optical detection through bone and tissue, as less energy is lost through scattering. When light is absorbed by tissue, its energy is dissipated as either thermal energy or fluorescence.

Physiological processes

The physiological processes underlying changes in the intrinsic optical signal (IOS) are still elusive. The key data (and opinions) that have been published are summarized in the following paragraphs. In early studies, optical changes in the nerve trunk of the walking leg of the shore crap were found to correlate with nerve activation. The signal change consisted of an increase in light transmission in association with the period of electrical stimulation, followed by a prolonged decrease in transmission afterwards. Using optical and mechanoelectric movement detectors, investigators showed that rapid swelling in nerve fibers, related directly to the mechanisms underlying the action potential, is responsible for this IOS change. In experiments on single squid giant axons, combined electrophysiological and optical methods identified fast and slow scattering changes. The fast scattering changes were dependent on membrane voltage, whereas the slow scattering changes were caused by periaxonal volume changes.

A system of considerably higher complexity is represented by the mammalian brain slice preparation. This preparation permits investigators to study neuronal activation induced by electrical stimulation, and changes because of spontaneous neuronal activity under pathological conditions, across a fabric of cortical regions. In brain slice preparations, both transmitted and reflected light can be analyzed. In hippocampal slices, stimulation-induced IOSs are abolished in the presence of Ca^{2+}-free artificial CSF (ACSF) or by addition of kynurenic acid, suggesting that postsynaptic activation may be important for an IOS. Other studies clearly showed, however, that postsynaptic activation is not a prerequisite for an IOS; a reproducible IOS is associated with epileptiform discharges induced by low Ca^{2+} ACSF in which synaptic transmission is blocked. A widely held hypothesis suggests that cell swelling or shrinkage of the extracellular space (ECS) volume, associated with neuronal activation, may be the most important factor in generating the IOS; changes in plasma viscosity, cellular volume, and membrane potential have all been suggested to underlie the IOS change in slice preparations, and postsynaptic activation and shrinkage of the ECS are assumed to be important. However, the mechanisms and components underlying the signal generation are still elusive.

Methods

Combined Hippocampal–Entorhinal Cortex Slice Preparation

The experiments in our laboratory are performed on combined hippocampal–entorhinal cortex slices taken from Wistar rats. The animals are decapitated under deep ether anesthesia. The brains are rapidly removed and washed with cold ACSF containing (in mM): NaCl 124.0, KCl 3.0, $MgSO_2$ 2.0, $CaCl_2$ 2.0, NaH_2PO_4 1.25, $NaHCO_3$ 26.0, and glucose 10.0 (pH 7.4), oxygenated with 95% O_2, 5% CO_2. Brain slices (400 μm) are cut in a nearly horizontal plane using a vibratome. The slices contain the temporal cortex and the perirhinal cortex as parts of the neocortex, the entorhinal cortex, the subiculum, the dentate gyrus (DG), and the ventral hippocampus. Slices are stored in ACSF and are individually transferred to an interface recording chamber, placed on a transparent membrane illuminated from below, and continuously perfused with prewarmed (35 °C) and carbogenated ACSF. Epileptiform activity is induced either by omitting Mg^{2+} or Ca^{2+} from the ACSF, or by adding 4-aminopyridine (4-AP) to the ACSF.

Electrophysiological Recordings

The recordings are started after more than 1 h of equilibration of the slices in normal ACSF. Bipolar stimulation electrodes are placed into the perforant path and a recording electrode is placed into the granule cell layer of the DG. The viability of slices is tested by the single stimuli to the perforant path and observation of the extracellular field potential in the DG. After viability testing, the stimulation electrode is removed from the chamber. The recording electrode is moved from the DG to the medial entorhinal cortex, as amplitude and duration of seizure-like events are the largest in layers IV and V of this region. A second, ion-sensitive, microelectrode is used in some experiments, placed in the temporal cortex to monitor spontaneous epileptiform activity after omission of Mg^{2+} from the ACSF.

Optical Imaging

The slices are transilluminated from below using a halogen lamp supplied by a stabilized voltage-regulated power supply. Homogeneous illumination is achieved by a curved glass rod (Ø 8 mm) placed directly underneath the slice below the transparent floor of the interface chamber. The slices are viewed from above with an upright binocular microscope Video images using a CCD camera (8 bit, Sanyo, Osaka, Japan) are obtained via a monocular phototube. During the experiments, images are stored on videotape using an S-VHS video-recorder. The complete video signals are converted at a 12.5 MHz ratio into 240 lines by 640 320 pixel per line employing a frame-grabber board and in-house software. The 8 bit information of each converted pixel represents one of 256 grey levels. The first image used for the analysis is captured 5–10 s before the stimulus-induced onset of the seizure-like event, and the last image is obtained at least 45 s after the electrical offset. Our aim is to examine alterations of the IOS with time. Thus, the first image in a series serves as a control which is subtracted from each of the following images. These subtracted images disclosed areas in the slice where light transmission has changed (at the time the image is obtained) relative to control transmission levels. Pseudo-colored images are calculated also by subtracting the control image from subsequently acquired images and assigning a color lookup table to the relative pixel values.

The series of subtraction images typically contains between 200 and 800 images with a variable temporal resolution. During seizure-like events the intervals ranges from 50 ms to 2 s. In order to detect changes in light transmittance induced by the different manipulations, subtracted images are data-reduced by averaging four neighboring pixels, and covered with a grid of 81 regions of interest by employing in-house software. To graph data from specific slice areas, square regions of interest (10×10 pixels) are placed over subiculum, medial entorhinal cortex, lateral entorhinal cortex, perirhinal cortex, and temporal cortex and in the hippocampus proper in CA1 and CA3, and the DG. The average digital intensities are sampled and stored. The figures and plots illustrate relative differences in light transmittance (T). The magnitude of the intensity difference ($\triangle T$) is expressed as a percentage of the digital intensity of the control image in a series. Changes in light intensity ($\triangle T/T$) typically range from 0.4% to 5.12% during the discharges, whereas noise usually is below 0.1%. Therefore, the signal-to-noise ratio ranges from 4 to 51.

To analyze propagation of a single seizure-like event in time and space, subsequently captured images are compared. Corresponding pixels in consecutive images which exceed a previously chosen threshold of light intensity change (clearly above the optical noise) are marked. By this approach, a circumscribed area of IOS associated with epileptiform activity may be displayed. Changes in this area exhibit specific spread patterns from one image to another, providing information about region of onset, direction and extent of propagation, and the velocity of spread (the latter determined by analysis of the temporal interval between images).

Recent Results

Mechanisms

As indicated above, the mechanisms and components underlying the generation of IOSs are still elusive. In particular, whether IOSs are generated by neuronal or glial changes has been controversial, as has the manner in which subcellular compartments contribute to the signal change.

The notion of a predominant glial contribution to the IOS is on the basis of initial data indicating that IOSs are attributable to a furosemide-sensitive uptake of K^+ into glia cells and to a concomitant water influx causing cell swelling. However, subsequent experiments have modified this view, since a reduction in signal amplitude of only 11% was observed in the presence of furosemide. In addition, there is evidence that furosemide, in the concentrations used in these experiments, exerts nonspecific and pleomorphic effects – including reduced neuronal excitability, interference with glial volume regulation mechanisms, and pronounced changes in extracellular pH. Investigators have also argued for an important role for neurons as a source of the IOS change, primarily based on data generated in excitotoxicity experiments. Bath application of the glutamate agonists *N*-methyl-D-aspartate or kainate produced a large signal change reflecting cell swelling. This response was not prevented

or reduced by furosemide, a blocker of the $Na^+/K^+/2Cl^-$ transport system that is prevalent in glial cells. The changes were distinctly laminar compared with more widespread changes seen with glial swelling.

In recent experiments in our laboratory, we have tried to gain some insight into the contribution of glial changes to IOS generation. We have employed antidromic stimulation, and application of fluoroacetate (FAC) as a tool to selectively block glial cells. FAC significantly decreased the slope and the maximum amplitude of the IOS, without affecting most electrophysiological parameters. This result may indicate that under glial blockade with FAC, light scatter increases – perhaps due to changes in glia conformation.

On a subcellular level, changes in size of mitochondria also appear to contribute to the generation of the IOS change during neuronal activation. Using a dual-angle scatter ratio optical imaging system, investigators demonstrated that an increase in light scattering occurred in hippocampal slices following *N*-methyl-D-aspartate treatment and concomitant swelling of mitochondria. In our laboratory, we have chosen a different approach to gain information about the contribution of mitochondria in the generation of the IOS. Combining tissue activation and blockade of mitochondrial activity – via inhibition of complex I with rotenone – we analyzed the effects on signal amplitude. Under rotenone conditions, we saw significantly decreased amplitudes of the IOS.

In conclusion, our data show that the amplitude of the IOS change is on the basis of both glial and mitochondrial changes during tissue activation. This result indicates that the IOS changes not only reflect tissue activation, but also contain information regarding the underlying types of activation and participating cellular components.

Origin and Spread

Intrinsic optical imaging is advantageous for studying the spread of epileptiform activity since it allows for simultaneous monitoring of changes over an entire preparation. We have used this technique in vitro to investigate seizure-like events (SLEs) which are characterized by 'tonic' and 'clonic' features, resembling seizures in vivo. In the low Mg^{2+} model, we demonstrated, for example, that discharges originated in the entorhinal cortex and spread to the temporal neocortex. We have compared origin and spread patterns of discharges in both the low Mg^{2+} model and in the 4-AP model. Although SLEs induced by low Mg^{2+} solutions were initiated predominately in the entorhinal region, there was no consistent region of onset or spread in the 4-AP model. This difference was explained by the differing mechanisms in the two models; in the low Mg^{2+} model, epileptiform discharges result from unblocking the NMDA receptor – which is more richly expressed in entorhinal cortices, whilst 4-AP is an antagonist of various K^+ channels found ubiquitously.

Age Dependency

Tissue from juvenile rats

It is well known that infants and young children carry a higher risk of developing seizures and epilepsy than adults. Experimental studies have suggested that this sensitivity is because of discrete periods of altered seizure susceptibility and expression during development. Whether and/or how this increase in seizure susceptibility during development may be reflected in regions of seizure onset, spread patterns, and spread velocity are poorly understood. We have combined extracellular microelectrode (ionsensitive electrode) recordings with intrinsic optical imaging to study the spatiotemporal patterns of seizure onset and spread during development in low Mg^{2+}. We examined rats from three age groups: 4–6 days (age group I), 10–14 days (group II) and 20–23 days (group III). Seizure susceptibility was the highest in age groups II and III. In age group I, SLEs originated mainly in the hippocampus proper. SLEs in age group II originated mainly in the entorhinal cortex, and this tendency was even more pronounced in age group III. Seizure invasion of the hippocampal formation, via the perforant path–DG and via the subiculum, was seen in age group I and II. By contrast, in age group III, seizure invasion into the hippocampus was exclusively via the subicular pathway. The velocity of spread at which SLEs propagated within different regions of the slice increased with postnatal age. The characteristics of onset, spread patterns, and propagation velocities, as revealed by this study, provide insight into the evolving properties of the developing brain.

Tissue from old rats

The highest incidence of epileptic seizures has been reported to occur in the elderly. There is little doubt that the high incidence of cerebral structural lesions (i.e., vascular, infectious, neoplastic, neurodegenerative, traumatic), metabolic disturbances, and the potentially proconvulsive consequences of comedication play important roles. It is also well known that there are pronounced alterations in the plastic properties of the aging brain in the absence of structural lesions. However, the effects that those changes in brain plasticity may have on seizure susceptibility remain unclear. We, therefore, were also

interested in the effects of old age on seizure onset, spread patterns, and propagation velocities in the absence of structural lesions. To this end, we employed slices from 'senile' animals; in the case of Wistar rats, senescence is said to be at about 2 years of age. The main findings of this study were: seizure-like events in tissue from aged (compared to young adult) animals tend to arise in more than one onset region, they show a less extensive spread to adjacent areas, and are characterized by decreased propagation velocity in distinct anatomical regions. Furthermore, we saw less pronounced activity-induced reductions of the ECS-volume and a smaller increase of activity-induced extracellular K^+ concentration. These results suggest that the changes in plasticity that occur during the process of aging exert pronounced effects on spread patterns and propagation velocities. The restricted seizure spread and reduced propagation velocities may, in part, be explained by our findings of significantly reduced activity-dependent ECS-volume changes and the less pronounced K^+ increases in aged tissue compared to controls.

Future Goals

In future studies it would be of great interest to further elucidate the underlying mechanisms that contribute to the generation of the IOS change. Such experiments should include investigation not only of the role of neuronal elements, but also detailed studies into conformational changes of subcellular elements. A further area of great interest is the analysis of plasticity changes of the brain following insults such as status epilepticus. It is known that status epilepticus may cause pronounced plasticity changes, rendering the neuronal network more vulnerable to the development of seizure activity. However, there is a paucity of information about the exact time course of changes in the wake of status-like events, and information regarding the evolution of changes is lacking. A better insight into the sequence of plastic changes is the prerequisite if preventive therapeutic options are to be considered. In such experiments, it would be plausible to combine in vivo and in vitro experiments. Brain slices could be prepared from animals following experimental status epilepticus in vivo. The in vitro experiments using electrophysiological methods and intrinsic optical imaging could then be carried out at different time points after status epilepticus.

Acknowledgements

This article is dedicated to Dr. Katharina Buchheim (May 17, 1965–December 1, 2005), who made significant contributions to the field and whose career was prematurely curtailed.

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INFLAMMATION

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Cerebrovascular Diseases, Seizures, and Epilepsy

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Introduction

A seizure is a paroxysmal event due to abnormal, excessive, hypersynchronous discharges from an aggregate of central nervous system (CNS) neurons. This abnormal electrical activity can be reflected in various clinical/behavioral manifestations, ranging from dramatic convulsive activity to experiential phenomena not readily discernible by an observer. Although a variety of factors influence the incidence of seizures, approximately 5–10% of the population will have at least one seizure during their lifetime, with the highest incidence occurring in early childhood and late adulthood. The term 'seizure' needs to be carefully distinguished from 'epilepsy.' Epilepsy refers to a condition in which a person has recurrent seizures due to a chronic or genetically predetermined underlying process. A subject with a single seizure does not necessarily have epilepsy, and an epileptic patient may rarely experience seizures. In fact, epileptic patients oscillate unpredictably between the 'ictal' state (seizures present) and the 'interictal' state (often no clinical symptoms). The distinctions between seizures and epilepsy, and between ictal and interictal activities, are clinically very well understood. However, sometimes confusion exists among nonclinicians in the research setting (including basic researchers) where interictal bursts or acutely induced seizures (in vitro or in vivo) may be falsely referred to as 'epilepsy.' This issue of accuracy becomes increasingly important when dealing with intravascular or cerebrovascular mechanisms of seizures or epilepsy, as we will attempt to elucidate in the following paragraphs.

Background

Seizures, Epilepsy, and the Blood–Brain Barrier

Seizures are a result of a shift in the normal balance of excitation and inhibition within the CNS. Given the numerous properties that control neuronal activity, it is not surprising that there are many different ways to perturb this normal balance, and therefore precipitate seizures. One of the main determinants of neuronal firing rate and synchronicity is the extracellular potassium level. Potassium controls glial and neuronal resting potential, repolarization, ion channel conductances, cerebral blood flow, and Na^+/K^+ pump activity. The complexity of CNS potassium homeostasis underscores its importance in the mammalian brain. The process involves different cell types (neurons, glia, and endothelium), several supercellular mechanisms (spatial buffering, cerebral blood flow), and a strictly controlled segregation of potassium concentrations between the blood (4.0–5.0 mM) and the brain parenchyma (2.5–3.0 mM). Other molecular elements that may either participate in seizure onset or decrease seizure threshold are brain levels of albumin, antibodies, or drugs.

The blood–brain barrier (BBB) serves as the main gate preventing the free communication of systemic cells and molecules with the brain parenchyma. The BBB is a specialized system of capillary endothelial cells that protects the brain from harmful substances in the blood stream, while supplying the brain with the nutrients required for proper function. Unlike peripheral capillaries, the BBB strictly limits transport into the brain through both physical (tight junctions) and metabolic (enzymes) barriers. Failure of the BBB may occur as a

consequence of intraparenchymal or systemic pathologies. These parenchymal pathologies include malformations of cortical development (MCD), brain tumors, seizures, and multiple sclerosis. Systemic pathologies causing BBB failure may be due to hypertension, stroke, blood hyperosmolarity, or systemically mediated inflammatory processes (due to the production of TNF-α, IL-1β, IL-6, histamine, arachidonic acid, or reactive oxygen species) (**Figure 1**). Investigators have hypothesized that disruption of the BBB will lead to seizures. This prediction is based on the following experimental findings:

1. Seizures are episodic and may have a cerebrovascular trigger. Patients with epilepsy have seizures intermittently and, depending on the underlying cause, many patients are completely seizure-free for months or even years. This sporadic appearance of seizures implies that there are precipitating factors that induce seizures in patients with epilepsy. Numerous groups, including ourselves, have described a number of vascular/blood-related factors that may be tipping the fragile epileptic brain toward seizures.
2. The normal brain is capable of seizing under appropriate circumstances if the BBB is transiently breached.
3. Opening of the BBB lowers the threshold for seizures. This effect implies that there are various underlying endogenous factors that influence seizure threshold. Whether these factors are the same in a chronically epileptic patient as those factors involved in the generation of acute seizures (e.g., in 'normal' brain) remains unknown.

A variety of conditions have a high likelihood of resulting in a chronic seizure disorder. For example, severe penetrating head trauma is associated with up to a 50% risk of subsequent epilepsy. The high propensity of severe trauma leading to epilepsy suggests that brain injury results in long-lasting, pathologic changes in the CNS that transform a presumably normal neural network into one that is hyperexcitable. This process is known as

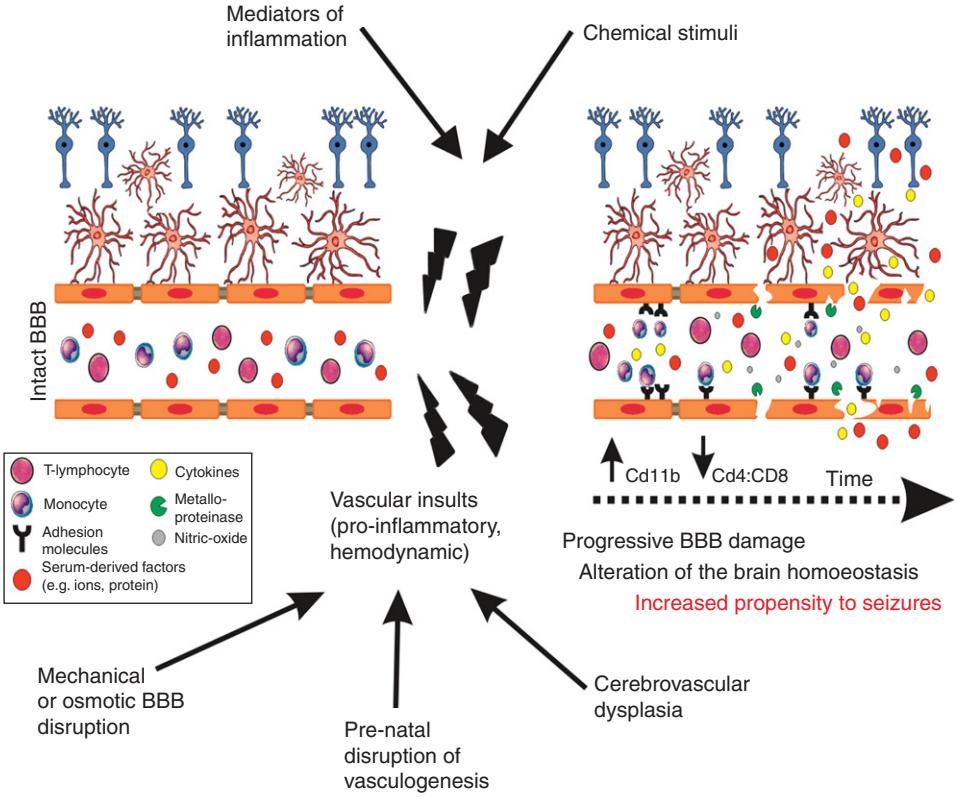


Figure 1 Acute seizures can be induced by a variety of manipulations of the neurovascular unit. Blood–brain barrier (BBB) damage occurs in response to pathological stimuli initiated intravascularly such as proinflammatory and hemodynamic changes. Activation of the circulating white blood cells and subsequent increase in vascular adhesion is responsible for breakdown of the BBB and cellular transmigration into the parenchymal side. Activation of specific enzymes such as metalloproteinases is associated with degradation of the vascular matrix and alteration of the BBB permeability. Constitutive cerebrovascular alterations as observed in malformations of cortical dysplasia are also associated with leaky BBB. We propose systemic intravascular pathological events leading to alteration of the BBB permeability to be a factor contributing to seizures.

epileptogenesis, and the specific changes that result in a lowered seizure threshold can be considered *epileptogenic factors*. Precipitating factors are responsible for causing a single seizure in someone without epilepsy. Precipitating factors may involve intrinsic physiologic processes, such as psychological or physical stress, sleep deprivation, or hormonal changes associated with the menstrual cycle.

Seizures and epilepsy are commonly observed in conjunction with stroke, traumatic brain injury, and central nervous system infections – all conditions known to compromise BBB function. It remains debatable whether the compromised integrity of the BBB is a factor involved in the etiology of epilepsy or secondary to such pathologies. In support of the etiological role the BBB plays in seizures is the fact that BBB disruption (BBBD) after acute head trauma is a well-known pathologic finding in both animal and human studies. BBB disruption may persist for weeks to years after the injury and may colocalize with abnormal EEG activity. The increased interest in osmotic opening of the BBB, as a viable mechanism of increasing drug delivery to the brain, provides an opportunity to explore the connection between BBBD and seizures in a controlled clinical environment. Osmotic opening of the BBB, by intravascular infusion of a hyperosmolar bolus of mannitol, is mediated by vasodilatation and shrinkage of capillary endothelial cells. Cell shrinkage results in widening of the interendothelial tight junctions to an estimated radius of 200 Å. The marked increase in BBB permeability to intravascular substances (10–100-fold for small molecules) following this osmotic disruption procedure is due to both increased diffusion and bulk fluid flow across the tight junctions. The permeability effect is largely reversed within minutes. In rodents, loss of BBB integrity by intraarterial hyperosmotic mannitol has been shown to rapidly lead to EEG changes consistent with epileptic seizures (spike/wave complexes interspersed with decreased EEG voltage that persisted for several hours after the BBB disruption event).

Given these findings, it is not surprising that seizures are the primary complication of osmotic BBBD. Indeed, seizures occur in a relatively large number of patients undergoing such procedure (13–55%). This high incidence was initially attributed to meglumine iothalamate, a known epileptogenic agent used as a contrast agent for CT. However, seizures associated with BBBD continued to occur (albeit with decreased frequency) when BBBD was monitored by radionuclide scanning rather than CT. To date, the correlation between level of BBBD and probability of seizure occurrence has not been carefully examined.

Recently, seizure onset was monitored in patients undergoing osmotic BBBD followed by intraarterial chemotherapy (IAC) to treat primary brain lymphomas. Procedures were performed under barbiturate anesthesia. Focal motor seizures occurred immediately after BBBD in 25% of procedures and originated contralateral to the hemisphere of BBBD. No seizures were observed when the BBB was not breached and only IAC was administered; the only predictors of seizures were positive indices of BBBD – namely, elevation of serum S100β levels and CT scans. The effect of osmotic BBBD was also evaluated in naïve pigs (**Figure 2(a)–2(c)**). Identical procedures generated an identical result; sudden behavioral and electrographic (EEG) seizures correlated with successful BBB disruption. The contribution of tumor or chemotherapy to acute seizures was in this case excluded. This study thus demonstrated a correlation between the extent of acute BBB opening and development of seizures in humans and in a large animal model.

Brain Development, Vasculogenesis, and Epilepsy

The evidence that epilepsy affects about 1% of the adult population and 4% of children supports the view that it is primarily a disorder of childhood. Various types of epilepsies diagnosed at a young age are included into three 'etiological categories': (1) epilepsies due to genetic abnormalities, (2) epilepsies resulting from disruption of normal developmental processes, and (3) epilepsies associated with a perinatal insult. One of the most common causes of neurological disabilities is malformations of cortical development (MCD). MCD result from abnormal neuronal positioning during corticogenesis, and are believed to be the morphological and perhaps physiological basis of several neurological diseases, including mental retardation and autism. MCD are commonly associated with epilepsy, and the clinical spectrum of epilepsies associated with MCD is broad.

In addition to neurogenesis, vasculogenesis also plays an important role in MCD and epilepsy. Some reports have emphasized that methylazoxymetanoic acid (MAM)-treated animals display an array of pathological features that include cerebrovascular changes. MAM-treated animals are obtained by prenatal exposure to the antimitotic agent MAM at gestational day E15. As adults, these MAM-exposed animals exhibit vascularization of the malformed cortex, with severe damage of the layered distribution of vascular trunks. Investigators have therefore hypothesized a decisive role of neuroblast migration and maturation in cortical angioarchitectural development. Thus, in addition to neuroblast toxicity, a parallel process targeting the vasculature may be responsible for MCD in MAM-treated animals.

Prenatal vasculogenesis is required to support normal neuronal migration and maturation. Altering this process leads to failure of normal cerebrovascular development and has a profound implication for CNS maturation. Previous data demonstrated that prenatal exposure of rats to the angiogenesis inhibitor, thalidomide, results in

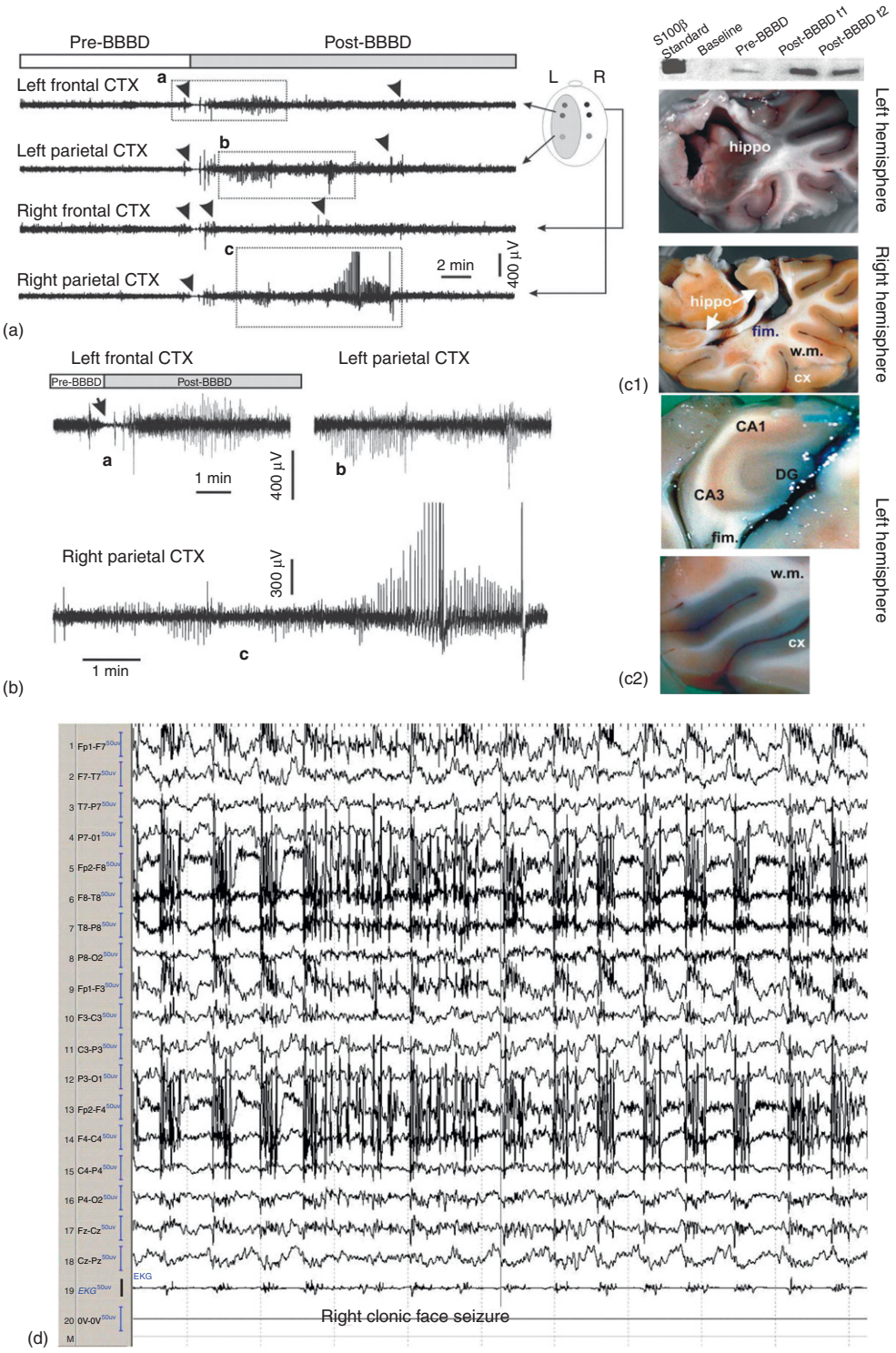


Figure 2 Examples of EEG recording to exemplify ictal patterns of seizures induced by some of the stimuli shown in **Figure 1**. EEG correlates of acute BBB disruption: a widespread increase in BBB permeability leads to seizures. (a) EEG recordings revealed a sharp increase in activity after BBBD in a naïve pig during BBB osmotic disruption. A hyperosmotic bolus of mannitol was injected in the carotid artery. The BBB disruption was performed on the left hemisphere. Subsequently, the EEG activity was predominant in this hemisphere. Motor seizures occurred controlaterally on the right side. The traces in (b) are magnified segments as indicated by the *dashed boxes* in (a). The *arrows* point to the EEG slowing that followed mannitol infusion (see also **Figure 4**). Filters used for viewing during collection were 0.08 and 300 Hz (low-pass and high-pass filters, respectively); sampling rate was 1000 Hz. (c1, c2) Serum S100β increase occurred immediately post-BBBD, as assessed by Western blot. Morphological demonstration of successful hemispheric BBB disruption by Evans blue staining. Note the ubiquitous leakage in the left hemisphere and the absence of extravasation in the right. w.m, white matter; DG, dentate gyrus, fim, fimbria. (d) EEG recording from a patient after BBB disruption.

significant morphological alterations in cortical and hippocampal regions. Abnormal neuronal development was associated with vascular malformation and a leaky BBB. Protein extravasation and uptake of fluorescent albumin by neurons was commonly associated with abnormal cortical development that usually results in neuronal hyperexcitability.

A recognized etiological link between cerebral vasculature and epilepsy is seen in the Sturge–Weber syndrome (SWS) (also called encephalotrigeminal angiomatosis), characterized by neurocutaneous angiomas that involve the leptomeninges and the skin of the face (the so-called port-wine stain). Leptomeningeal angiomas, as demonstrated by structural neuroimaging, may be unilateral or bilateral in the parietal and occipital regions. SWS is clinically characterized by seizures, which may be intractable, and transient focal 'stroke-like episodes' (such as hemiparesis and hemianopsia), headache, developmental disorders, and mental retardation. SWS may be caused by residual embryonic blood vessels and their secondary effects on surrounding brain tissue. A vascular plexus develops around the cephalic portion of the neural tube, under ectoderm destined to become facial skin. Normally, this vascular plexus forms at week 6 and regresses around the ninth week of gestation. Failure of this normal regression results in residual vascular tissue, which forms the angiomata of the leptomeninges, face, and ipsilateral eye. Neurologic dysfunction results from secondary effects on surrounding brain tissue, and blood flow abnormalities in the angiomas may cause increased capillary permeability, stasis, and anoxia. Venous occlusion might actually cause the initial neurologic event – either seizure, transient hemiparesis, or both. One of the most characteristic findings of SWS on MRI is gadolinium enhancement of angiomas that are undetectable on CT scan. BOLD-MR venography may also be sensitive in detecting early venous abnormalities in SWS, earlier than conventional MRI sequences.

BBB Dysfunction and Seizures: Which Comes First?

It was recently demonstrated that exposure of the cerebral cortex in vivo to bile salts resulted in BBB opening with subsequent extravasation of serum albumin into the brain extracellular space, and that this disruption caused the generation of epileptiform discharges within 4–7 days. Moreover, it was reported that interferon-γ induces seizures in patients with focal BBB disruption. Finally, investigators demonstrated that in rats, osmotic opening of the BBB determined encephalographic ictal activity and acute iatrogenic opening of the human and porcine BBB promotes seizures. Serum-derived factors that include normal constituents (e.g., glutamate, potassium, serum proteins, and white blood cells), disease-related products (e.g., IL-1β, TNF-α, IL-6, and antigens), xenobiotics, and heavy metals are normally excluded from the CNS by the BBB. To various degrees, all of these compounds may promote acute brain dysfunction and seizures. In particular, the involvement of cytokine production has been extensively studied in humans and in experimental models of epilepsy after the induction of *status epilepticus*. Recent reports have shown that IL-1β elevation in blood precedes status epilepticus while others have shown that interfering with the IL-1β system prevents onset of seizures. Taken together, these results strongly support a role for cytokines in the development of seizures.

How Do Systemic Inflammatory Events Affect the Brain?

Accumulating evidence supports the existence of an immune response in the brain during systemic inflammatory processes. In particular, components of Gram-negative bacteria such as lipopolysaccharide (LPS), when injected intraperitoneally or systemically, trigger an upregulation of MCP-1, IL-6, IL-6r, gp130, or TLR-4 in the circumventricular organs (CVOs), choroid plexus, leptomeninges, and endothelial cells along the cerebral blood vessel. Systemic injection of LPS, recombinant IL-1β, TNF-α, or IL-6 triggers proinflammatory signal transduction pathways within different populations of cells in the CNS, without being associated with substantial neurodegenerative processes. This interplay between the peripheral immune response and the CNS may play a role in the development of brain pathologies such as seizures and epilepsy. Interestingly, when LPS was administrated chronically in rats, an increased permeability of the BBB was observed as a consequence of the systemic inflammatory stimuli.

Methods

Osmotic Opening of BBB

An hyperosmotic bolus of mannitol is injected directly into the carotid artery, provoking a rapid opening of the BBB (within few seconds from the injection). This technique allows the study of the effect of acute BBB damage on physiological parameters such as brain electrical activity, as recorded by EEG. Two 35-kg Yorkshire pigs were placed under anesthesia using isofluorane (1–1.5%). Anesthesia was induced by intramuscular ketamine with the addition of xylazine (Rompun; 1–2 mg kg^{-1}) and atropine (0.02 mg kg^{-1}). In the supine position, the femoral artery was cannulated and under fluoroscopic guidance, the internal carotid artery was catheterized. Mannitol (1.4 M) was administered intraarterially via the catheter at a predetermined rate of 3–12 $cm^3 s^{-1}$ for 30 s. EEG data (13 channels, including ground, reference, EKG, and impedance electrodes) were collected using a Nihon

Kohden EEG System (Nihon Kohden, Tokyo, Japan). These tracings were collected on a Nihon Kohden JE910-A jack box, using sterile stainless steel subdermal needle electrodes (13×0.40 mm with 0.5×27 gauge) manufactured by Axon Systems, Hauppage, NY, USA. The system was electrically isolated from the pig and the equipment used in performing angiographic procedures.

EEG Recording and Video Monitoring

Stereotactic electrode implantation was performed under pentobarbital anesthesia (45 mg kg^{-1} i.p.), using the Kopf stereotactic frame and a stereotactic atlas of the rat brain. For intrahippocampal recordings, bipolar twisted stainless wire electrodes (0.1-mm diameter, 0.5-mm vertical tip separation, Medwire, New York, NY, USA) were placed bilaterally in the dorsal hippocampus (LH: left hippocampus, RH: right hippocampus, A: -3.9 mm, L: ± 3.0 mm, D: -2.5 mm from Bregma). EEG data were sampled at a rate of 100 Hz.

Animals and Tissue Sample Preparation

Status epilepticus was induced using the pilocarpine and the lithium–pilocarpine models of temporal lobe epilepsy. Rats (male Sprague-Dawley 200–250 g) were injected with LiCl (3 meq kg^{-1}) followed 20-h later by methylscopolamine (1 mg kg^{-1}, i.p., Sigma-Aldrich) and pilocarpine (30 mg kg^{-1}); methylscopolamine (1 mg kg^{-1}, i.p., Sigma-Aldrich) was injected 30 min before pilocarpine (320 mg kg^{-1} i.p.) to prevent peripheral cholinergic side effects. Animals were anesthetized (isoflurane) during recurrent motor seizures and at onset of status epilepticus (SE), as assessed by behavioral observation (stages 4 and 5 of Racine's scale) and EEG recordings. Blood was withdrawn from the right heart ventricle; brain areas (hippocampus and entorhinal cortex) were resected and frozen.

FACS and Cytokine ELISA

Fluorescence-activated cell sorting (FACS) allows the separations of subpopulations of white blood cells (WBCs) and the evaluation of the activation of such immune cells. In particular, we assessed the percentage of activated T lymphocytes (CD3+), monocytes, granulocytes, and macrophages cells (all positive for CD11b+) after pilocarpine or lithium chloride treatments. Blood samples were withdrawn before development of SE. For FACS analysis, 150 µl of whole blood (or brain homogenate) was incubated with a combination of specific antibodies recognizing different populations of WBCs. Specifically, mouse anti-rat CD8a-FITC, CD3-R-PE, and CD4-PE-Cy5 (BD-Pharmingen) were added (2, 5, and 5 µl, respectively) to blood samples. Mouse anti-rat CD11b-FITC and CD3-R-PE were added (2 and 5 µl, respectively) for the determination of neutrophils/monocytes. Blood samples were analyzed by the FACS-Core facility at LRI. IL-1β, TNF-α, and IL-6 ELISA test kits were purchased from Pierce Biotechnology Inc. and tests performed as described by the vendor.

FITC–Albumin Solution Composition

A microangiographic technique based on the injection of fluorescent probes enables confocal visualization of the entire brain micro- and macrovasculature. Simultaneous observation of functional changes in BBB permeability was also possible with utilization of appropriate microangiographic molecules. To this end, fluorescent albumin was used, since albumin does not extravasate abluminally if the BBB is intact. The fluorescent albumin solution was prepared by reconstituting 500 mg of bovine desiccate albumin-fluorescein isothiocyanate (FITC-albumin, MW 69 KDa; 12 mol mol^{-1} albumin; Sigma, St. Louis, MO) in 50 ml of phosphate buffered saline (0.1 M PBS) lacking magnesium and calcium ions. Presence of vascular leakage was evaluated by visual inspection and by confocal microscopy.

Recent Results

Lithium and Pilocarpine: Animal Models of Immunomediated Epilepsies?

Pilocarpine is a nonselective muscarinic agonist with a relatively high affinity for CNS muscarinic receptors. It is commonly used to induce an experimental model of temporal lobe epilepsy. In different animal species, intraperitoneal injection of pilocarpine leads to a convulsive status epilepticus (SE) that persists for several hours. The initial SE is thought to be triggered by a cholinergic activation of excitatory neurons in specific brain regions that include limbic cortices. Although there is a paucity of studies directly assessing pilocarpine penetration across the BBB, a recent report demonstrated that intracerebral pilocarpine does not exceed micromolar concentration ($\sim$200 µM) in rats sacrificed immediately before or at SE. Since this drug shows a relatively poor brain penetration, the CNS effect must be mediated by micromolar concentrations of pilocarpine.

Unfortunately, in spite of the extensive use of systemically injected pilocarpine to induce SE in vivo, very few studies have examined the proconvulasant effects of pilocarpine when applied directly to the brain. Interestingly, when pilocarpine was directly injected in a rat brain, the dose required to induce status epilepticus was three to four orders of magnitude higher than (1) what was predicted by the muscarinic receptor affinity constant or (2) the pilocarpine brain levels measured after intraperitoneal injection (350 mg kg^{-1}) at onset of SE. In in vitro

studies on hippocampal slices, direct application of micromolar (10 μM) concentrations of pilocarpine was reported to induce epileptiform activity. However, ictal epileptiform discharges were observed only when the potassium concentration was enhanced to 7.5 mM or after prolonged perfusion of pilocarpine. Similar conclusions were recently reported using brain slices and in an in vitro whole brain preparation.

In addition to the well-described histological alterations induced by pilocarpine SE, magnetic resonance studies in the rat in vivo show that pilocarpine treatment is associated with an enhancement of BBB permeability. Whether such changes are the consequence of the SE or are due to a peripheral effect of pilocarpine, independent from the epileptiform activity, is not known; however, data support a peripheral mechanism for BBB opening occurring prior to SE (**Figure 3**).

Recent results have demonstrated a remarkable inflammatory component in pilocarpine and lithium–pilocarpine acute seizures. **Figures 3** and **4** show examples in support of the systemic origin of triggers that may lead to pilocarpine– and lithium–pilocarpine-induced seizures. Activation of cholinergic neurons was originally recognized as the only factor triggering pilocarpine SE. However, a peripheral muscarinic effect of systemically injected pilocarpine cannot be excluded. After pilocarpine i.p. injection in vivo,

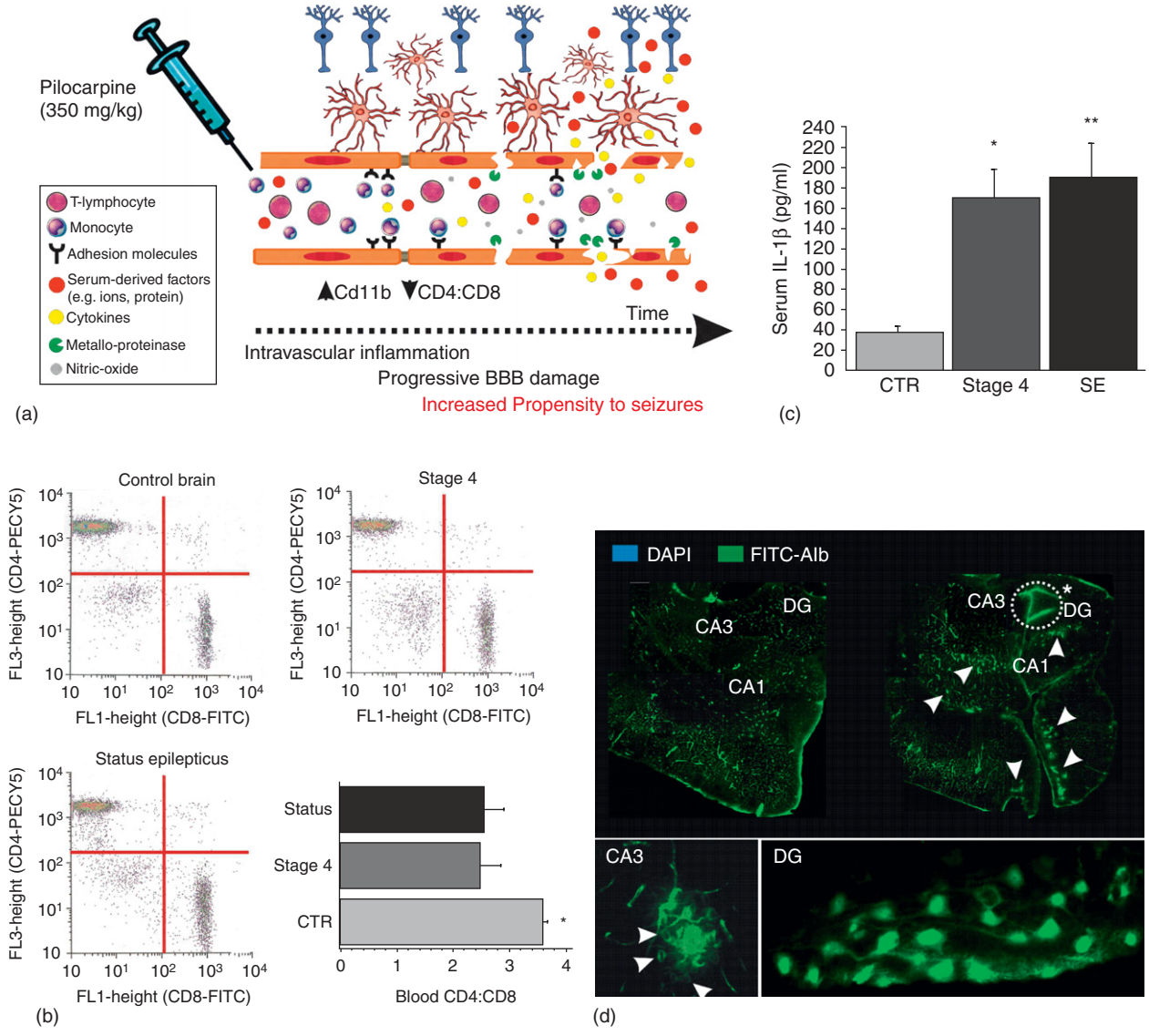


Figure 3 Pilocarpine-induced systemic proinflammatory changes in rats. A common experimental model of status epilepticus (SE) utilizes intraperitoneal administration of the cholinergic agonist pilocarpine preceded by methyl scopolamine treatment. In vivo, after pilocarpine i.p. injection (350 mg kg^{-1}), increased serum IL-1β (c), decreased CD4:CD8 T-lymphocyte ratios (b) and focal BBB leakage, and neurons containing fluorescent albumin (d) were observed. Early systemic events increasing BBB permeability may promote entry of serum-derived cofactors into the brain leading to pilocarpine-induced SE. Disturbance of brain homeostasis represents an etiological factor contributing to pilocarpine seizures (a).

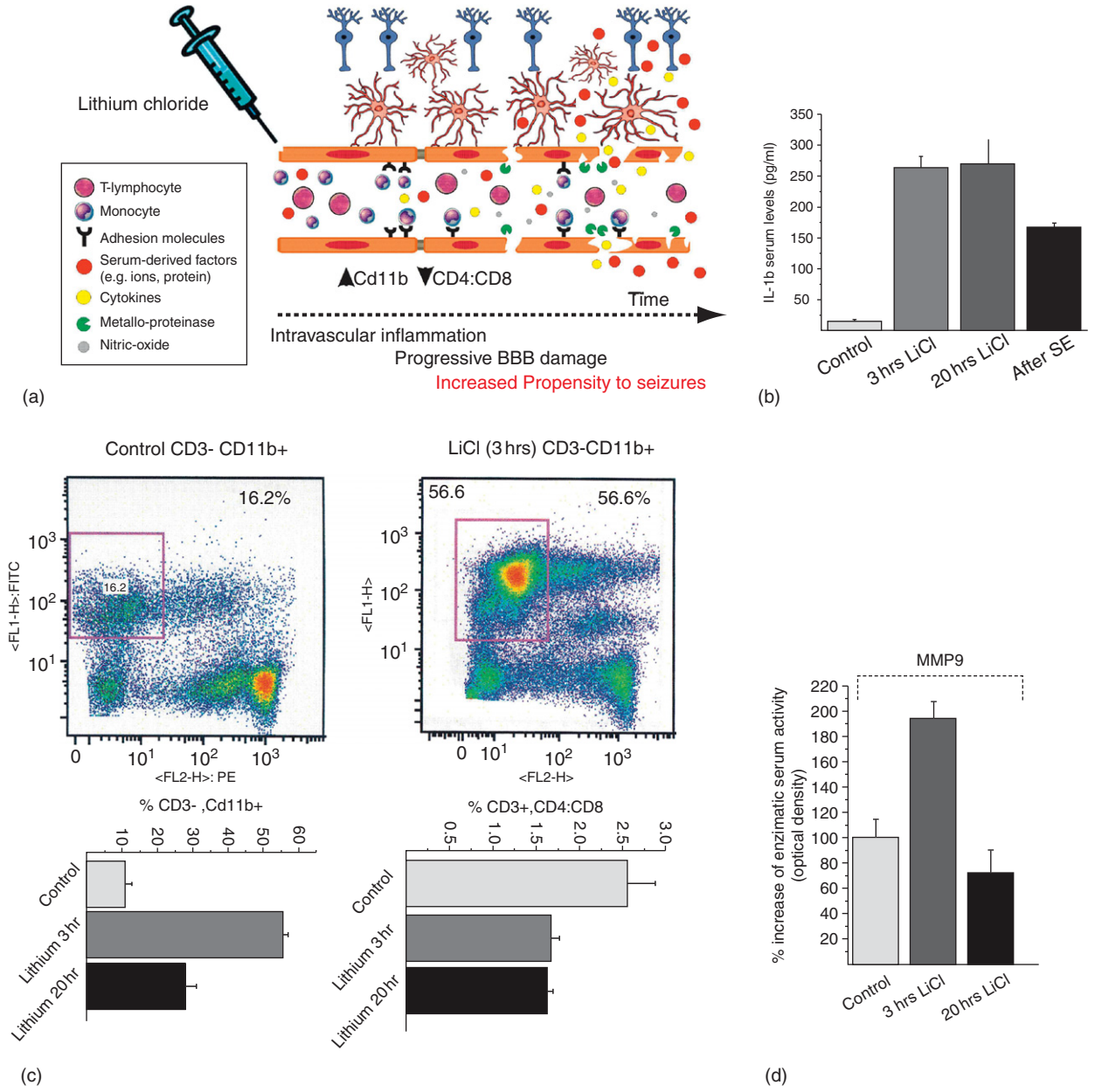


Figure 4 Lithium chloride-induced systemic proinflammatory changes in rats. Comparably to what was observed in the pilocarpine model of temporal lobe epilepsy, LiCl induces systemic proinflammatory changes and BBB damage. In particular, a profound activation of monocytes (CD11b+) was observed starting 3 h after LiCl administration. CD4:CD8 T-lymphocyte ratio was also decreased. The cytokine IL-1β was increased in blood and MMP-9 was activated. Of relevance is the fact that MMP-9 is the effector of vascular damage remodeling. These findings demonstrate the occurrence of proinflammatory changes before the injection of pilocarpine, preceding the onset of seizures, thus proposing a complementary mechanism contributing to seizure onset.

serum IL-1β rapidly increased, CD4:CD8 T-lymphocyte ratios decreased, and focal BBB leakage was observed (Figure 3). In vitro, pilocarpine failed to trigger significant synchronized epileptiform activity when applied at concentrations identical or higher to blood levels measured in vivo. Intense electrographic seizure-like events occurred in brain slices only in the copresence of levels of K^+ (6 mM) that mimic BBB leakage. These results suggested that early systemic events that increase BBB permeability may promote entry of cofactors (e.g., K^+) into the brain, subsequently leading to pilocarpine-induced SE. Focal pilocarpine accumulation into the brain, resulting from BBB leakage, could also contribute to seizure generation, as demonstrated in the isolated in vitro whole brain preparation. Therefore, disturbance of BBB was suggested to be an etiological factor contributing to pilocarpine seizures.

Another model of SE relies on the use of pilocarpine preceded by systemic treatment with lithium chloride.

Status epilepticus can be induced by the intraperitoneal administration of 30–35 mg kg^{-1} pilocarpine only if preceded by lithium chloride (3 meq kg^{-1}). Interestingly, the pharmacokinetic properties of lithium show a peak serum level at 30 min after administration and a rapid decay with complete clearance from the bloodstream after 22 h. Previous data demonstrated that the ideal interval between administration of lithium and pilocarpine, required to maximize seizures, is about 2 h, yet most studies used an interval of 20 h. Interestingly, lithium is a proinflammatory agent, in as much it can mimic white blood cell activation seen under conditions of acute inflammation. Additionally, lithium exposure may facilitate or promote seizures in the absence of cholinergic stimuli. We recently tested the hypothesis that exposure to lithium may replicate some of the inflammatory events observed after exposure to high concentrations of cholinergic agonists (e.g., pilocarpine 350 mg kg^{-1}). The results are shown in Figure 4. Rats responded to lithium (at 3 mEq kg^{-1} dosage), before exposure to pilocarpine, with EEG changes resembling those seen in patients undergoing lithium treatment for bipolar disorder. The hematologic changes in lithium-treated rats (before pilocarpine administration) consisted of systemic proinflammatory changes identical to those seen after exposure to convulsive levels of pilocarpine (Figures 3 and 4). Both pilocarpine (convulsive dose) and lithium alone caused extravasation of FITC-labeled albumin into the brain, consistent with BBB disruption.

Our current hypothesis is that lithium lowers threshold for seizures that occur after muscarinic action on neurons. This interaction would help explain the mechanism underlying the lithium–pilocarpine model, where lithium levels should have dissipated by the time the cholinergic drug is administered. Our data suggests that pilocarpine-induced seizures are not due solely to a direct action of

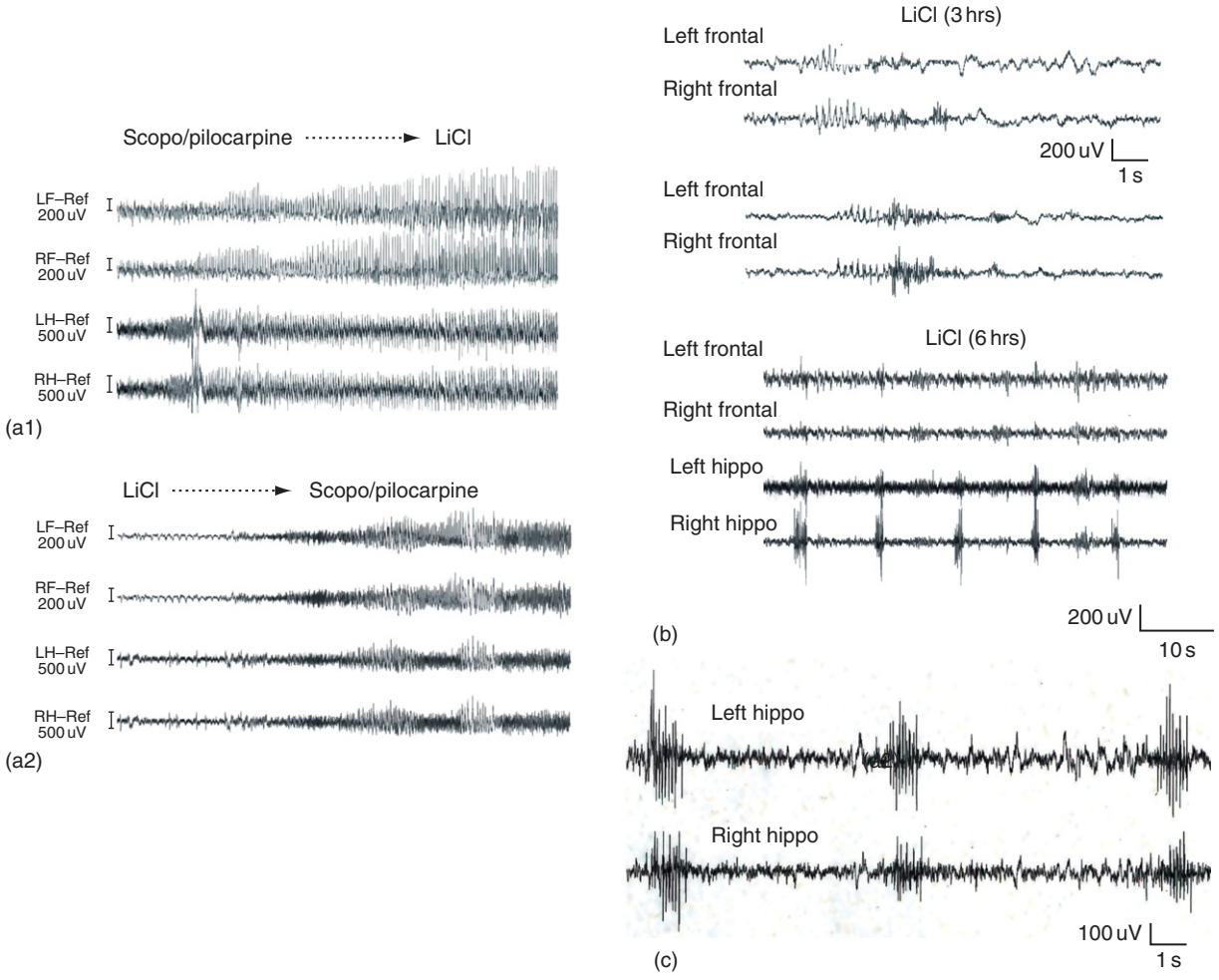


Figure 5 Systemic treatment with pilocarpine and lithium chloride induces EEG changes by a mechanism involving systemic inflammation and BBB damage. A change in the behavior (head nodding, stiffing of the dorsal muscles, and repetitive chewing) was observed in the majority of rats injected with LiCl. EEG analysis revealed an increase in the occurrence of low-frequency beta and theta waves starting 2–3 h after LiCl injection, at the time of ongoing intravascular inflammation and BBB damage (see Figures 3 and 4). Six hours later, a faster activity developed.

the drug on CNS neurons. If lithium and pilocarpine 'cooperate' to reduce seizure threshold – by inducing an inflammatory response – then the temporal sequence of exposure should not be crucial to the seizure-inducing process. This possibility was directly tested by comparing seizure induction by the 'traditional' paradigm of exposure (lithium then pilocarpine) to the opposite sequence (lithium *after* subconvulsive pilocarpine). **Figures 5** and **6** show that either approach produced SE, further unraveling the current belief that the seizure-inducing effect of lithium and pilocarpine is specific to their actions on neuronal targets.

Future Directions

In summary, there is growing evidence supporting the role of alterations in cerebrovascular function in determining seizure propensity. While seizures are a result of neuronal oversynchronization and excitability, the propensity for abnormal firing may be dependent on systemic events. Particularly within this context, the distinction between seizures and epilepsy is critical; specific treatments designed to target these two distinct pathologies may have considerable benefits. In particular, the detection and treatment of the 'first' seizure, as a secondary manifestation of an acute vascular event (e.g., stroke, hemodynamic, systemic inflammation) could prevent the initiation of processes leading to cognitive impairment or to epilepsy. In this scenario, seizures could be controlled by targeting the prodromic (vascular) events rather than the seizure itself. This approach would open the possibility of employing drugs other than classical antiepileptic medication. Of importance will be the discovery of mechanisms linking damage of the BBB to the alteration of neuronal excitability. While imbalance of the ionic brain content could represent one factor involved in such a cascade, other serum-derived factors (e.g., albumin as recently proposed) could play a role. Moreover, experimental evidence demonstrates the seizure-promoting effect of both acute and chronic opening of the BBB, in naïve or epileptic animals, suggesting the possibility of multiple vascular pathways of altering neuronal excitation.

Seizures elicited during iatrogenic vascular interventions (e.g., cardiopulmonary bypass, endarterectomy) have been associated with alteration of cerebral blood flow,

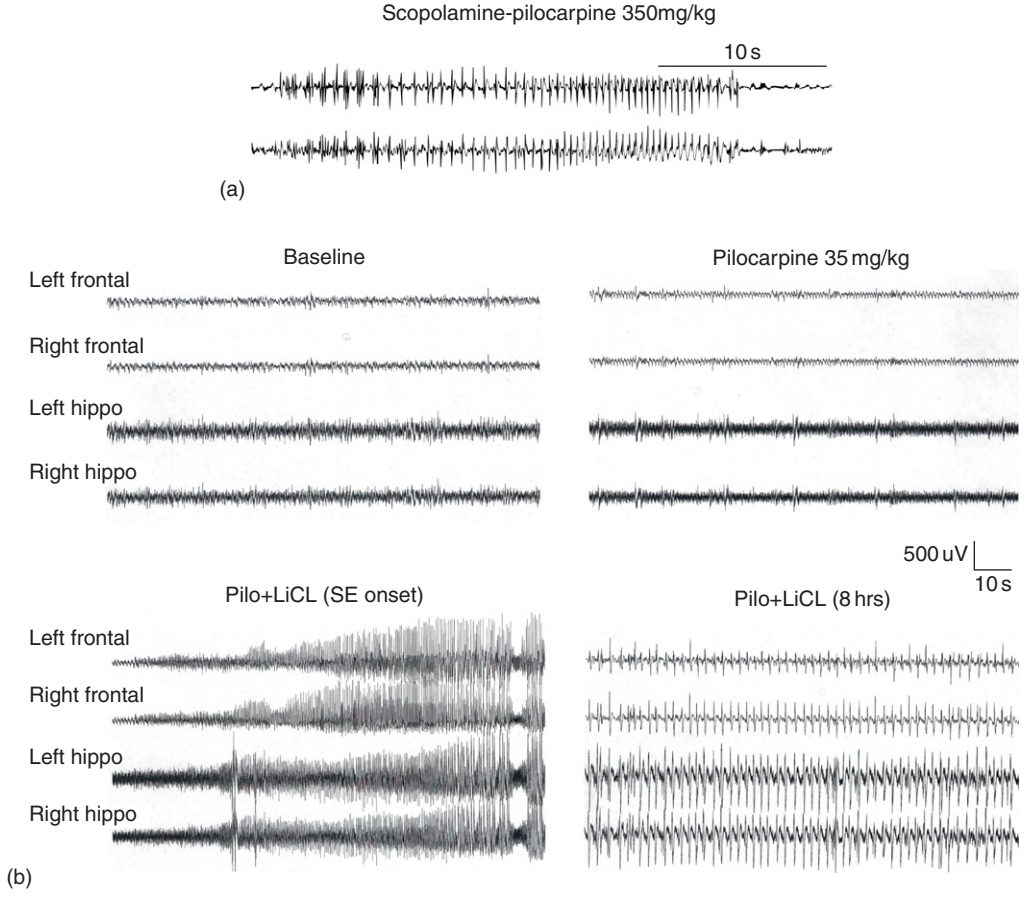


Figure 6 Status epilepticus was elicited by the combination of LiCl and pilocarpine independently from their sequence of administration. No significant EEG changes were induced by the low dosage of pilocarpine (35 mg kg^{-1}).

activation of circulating white blood cells, and increased permeability of the BBB. If systemic intravascular inflammation is involved in the occurrence of seizures, then prophylactic control of systemic vascular inflammatory processes and preservation of BBB integrity (e.g., using antiadhesion molecule or antiinflammatory drugs) may represent alternatives and/or complementary pharmacological approaches to prevent seizures and possible subsequent epileptogenesis.

See also: **Aging**: Poststroke Epilepsy and the Aged Brain; **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; **Cell Damage/Excitotoxicity**: Excitotoxicity and Neurodegenerative Disease; **Inflammation**: Inflammatory Responses and Cytokines; Modulation of Neuronal Excitability: Vascular Changes and Inflammation.

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Connections between Infections and Seizures

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Introduction

About 5% of patients with an infection of the central nervous system will experience a seizure. A seizure may be the presenting symptom, or only one manifestation of the infection. Common infections of the central nervous system that may present with seizures include: herpes simplex, cytomegalovirus, arbovirus, human immunodeficiency virus, neurocysticercosis, malaria, toxoplasmosis, bacterial meningitis, and brain abscess. The seizures may be due to direct invasion of brain tissue by the infecting organism, production of toxins by the organism, or production of inflammatory mediators by the brain. Infectious processes in the brain can lead to breakdown of the blood–brain barrier and brain edema. Severe systemic infections can also be associated with seizures, even if the infection is not present in the central nervous system. In this latter instance, the seizures are most likely due to hypoxia or some other severe metabolic change (such as hyponatremia) that is the result of the overwhelming infection. In all cases, treatment for the seizures associated with infection usually involves treatment of the underlying infection.

Bacterial infections in the central nervous system are less likely to cause seizures than do viral infections. However, generalized convulsions may occur with bacterial meningitis. Compared with other bacteria, infections with *Haemophilus influenzae* are more commonly associated with seizures. The mechanism of the seizure induction is not known. As with other signs of infectious processes, it has been postulated that the seizures are either a result

activation of circulating white blood cells, and increased permeability of the BBB. If systemic intravascular inflammation is involved in the occurrence of seizures, then prophylactic control of systemic vascular inflammatory processes and preservation of BBB integrity (e.g., using antiadhesion molecule or antiinflammatory drugs) may represent alternatives and/or complementary pharmacological approaches to prevent seizures and possible subsequent epileptogenesis.

See also: **Aging**: Poststroke Epilepsy and the Aged Brain; **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; **Cell Damage/Excitotoxicity**: Excitotoxicity and Neurodegenerative Disease; **Inflammation**: Inflammatory Responses and Cytokines; Modulation of Neuronal Excitability: Vascular Changes and Inflammation.

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of the primary infection or due to the inflammatory response to the bacterial infection. To date, there are few data to support either possibility.

Encephalitis due to a viral infection of the brain can be associated with both focal and generalized seizures. Viral encephalitis is characterized by neuronal and glial degeneration, inflammatory infiltration, edema, and tissue necrosis. Herpes simplex virus is the most common pathogen associated with seizures in cases of viral encephalitis. Seizures occur in ~85% of children infected with Japanese encephalitis and up to 10% of adults with West Nile virus. Equine encephalitis, St. Louis encephalitis, cytomegalovirus, and rabies have also been reported to cause seizures. Finally, it has been estimated that 2–5% of HIV-infected patients have seizures because of the primary infection of the brain by the virus.

The issues surrounding the relationship between herpes virus infections of the central nervous system and seizures are quite complex. Infection with herpes virus, particularly herpes virus 6B (HHV-6B), is quite common. A number of researchers have suggested that there is a causal association between infection with HHV-6B and seizures in infants aged less than 1 month. Recently, however, this causal relationship has been questioned; it could be that the infection is causing the seizures, but it is also possible that infants susceptible to the infection are those most likely to have seizures. The evidence distinguishing these two possibilities is not clear. Herpes viruses can also result in a latent or persistent infection. This persistent infection has been implicated in febrile seizures and some forms of epilepsy – in the absence of symptomatic encephalitis. HHV-6B has been found in astrocytes within resected tissue from a substantial proportion of patients with temporal lobe epilepsy. The current hypothesis is that the presence of the virus is a result of an early infection and its continued presence somehow lowers the seizure threshold or increases the likelihood of developing epilepsy. This hypothesis is potentially appealing, given that herpes is easily reactivated, particularly by other viral infections. It is possible that reactivation of the virus results in a series of consequences that result in a lowering of the seizure threshold. For example, reactivation of HHV-6 may alter gene expression in astrocytes, which in turn alters glutamate pathways. Thus, the seizures could be due to a direct action of the reactivated virus, or to a secondary action initiated by the presence of the virus in the latent form. Again, the available data do not provide definitive support one way or the other.

Other infections that involve the central nervous system have been reported to cause seizures. Neurocysticercosis, infection of the central nervous system with the cyst form of tapeworms, is the most common parasitic infection of the brain. In developing countries, up to 50% of adult-onset epilepsy is due to neurocysticercosis. Malaria is estimated to spread into the central nervous system in approximately one-third of the cases, and cerebral malaria can present with seizures. The incidence of acute seizures in neurosyphilis is 14–60%. Infectious diseases in which seizures have been reported, but are not typically the presenting feature, include rubeola, schistosomiasis, trichinosis, paragonimiasis, echinococcosis, trypanosomiasis, typhus, and amebiasis.

In summary, infections of the central nervous system are clearly associated with seizures, either during the acute infection or as a delayed response. The mechanisms underlying these seizures are not understood and are, most likely, dependent on the infectious agent and time course of the infection. In addition, inflammatory processes in the brain have been implicated in a number of cases, and could be the underlying cause of seizures in some, if not all, infections.

Background

Studies of infectious causes of seizures have been limited by a number of significant issues. Seizures are most commonly studied in the laboratory in rodents – rats or mice. However, infectious processes are quite different in rodents than in humans; in particular, rodents are generally resistant to a number of organisms that commonly infect humans. Conversely, agents infectious to rodents may not be infectious in humans. When a common agent infects both species, the infection may follow a different course in each species, making comparisons difficult. Host responses to the infecting organism can also be different. Finally, work on infectious agents in the laboratory is carried out under significant constraints, and some pathogens are difficult to grow and maintain in the laboratory. Two infectious agents that have been studied in animal models are Herpes simplex type 1 (HSV-1) and neurocysticercosis.

HSV-1 is a neurotropic virus that can travel through the central nervous system by retrograde axonal transport and has been shown to have an affinity for the temporal region of the brain. In patients with encephalitis due by HSV-1, 38% will have seizures. The symptoms associated with HSV-1 infection are thought to be due to both viral and host factors. As a herpes virus, HSV-1 also has the ability to become latent. In order to study the mechanism of the seizures in HSV-1 infection, attempts have been made to produce an animal model of HSV-1 infection with adequate recapitulation of both the acute and chronic characteristics of the infection.

Mice, rats, and rabbits have since been used in generating models of HSV-1 infection. The virus must be grown in cultured monkey kidney or baby hamster kidney cells. Mice have been infected by corneal scarification, rats by intranasal inoculation, and rabbits by injection into the olfactory bulb. In most publications, animals are

reported to have symptoms of a systemic viral infection. The severity of the symptoms is relative to the dose and route of administration of the virus. There is a significant mortality rate in all of these approaches, and viral shedding has been measured in ocular and nasal secretions from the infected animals.

Seizures have been documented by observation and electrical recording in these models. Both generalized and partial seizures have been reported. The severity of the seizures seems to correspond to the severity of the viral infection, as determined by the systemic symptoms. Brain slices have also been obtained from infected animals, and recordings have demonstrated abnormal excitability with depolarized membrane potentials and a lower threshold for burst generation. In one study using rats, in animals with seizure activity, inflammatory infiltrates and hemorrhagic lesions were detected in the trigeminal ganglia, olfactory bulb, amygdala, hippocampus, piriform cortex, entorhinal cortex, and the spinal trigeminal ganglia. Astrocytic hypertrophy has also been demonstrated. These rodent models have successfully modeled human infection with herpes, in that they exhibit inflammation and neuronal death, hyperexcitability, and seizures. However, these models have considerable mortality and have not yet yielded answers to the questions about the mechanisms underlying the seizures.

Hippocampal slice cultures have also been infected with HSV-1 by adding the virus directly to the culture medium. Acute infection can induce epileptiform activity and neuron loss, suggesting that this model could be used to examine the mechanisms by which the virus causes cell loss and hyperexcitability. Whether any mechanism determined in this model would relate to the human infection remains to be determined.

Another type of infection that has been studied in animals is neurocysticercosis, an infection of the human central nervous system by the helminth *Taenia solium*. Since pigs serve as the intermediate host, humans become infected with the tapeworm form (taeniasis) by eating undercooked pork that is infected with the egg form. The larvae hatch and cross the intestinal mucosa to enter the bloodstream. They exit the bloodstream and migrate into tissues, including the brain, where they mature into cysts called cysticerci. These cysticerci are viable parasites, which modulate and inhibit the host immune response. The presence of cysticerci in the brain does not normally produce symptoms. Individuals who have died of other causes have been found to have viable cysticerci in the brain. Those patients who do have seizures invariably have a prominent inflammatory infiltrate around the cysts (**Figure 1**), including the presence of proinflammatory cytokines and an altered blood–brain barrier. Since the viable cysticerci can suppress the host immune response, it has been suggested that the seizures are not a result of the parasitic infection per se, but rather result from the host response initiated by dying cysts. It is not known what causes the seizures, but it has been postulated that one or more epileptogenic substances are released by the dying parasite or produced by the surrounding inflammatory cells. There is no animal model for neurocysticercosis, but a model has been developed to examine the seizures produced by the dying parasites.

Methods

Taenia solium, the human tapeworm, is not infectious in rodents, but a closely related helminth – *Taenia crassiceps* – can produce cysticercosis in mice. However, it will not produce infection of the brain. Inoculation of mice with *T. crassiceps* leads to infestation of the peritoneal cavity with a large number of cysts. In the peritoneal cavity, the parasites eventually begin to die and granulomas form around the cysts. These granulomas mature through a series of defined stages (1–4), depending on the amount of intact organism remaining. By stage 4, only host cells and debris remain, without clearly identifiable parasite elements.

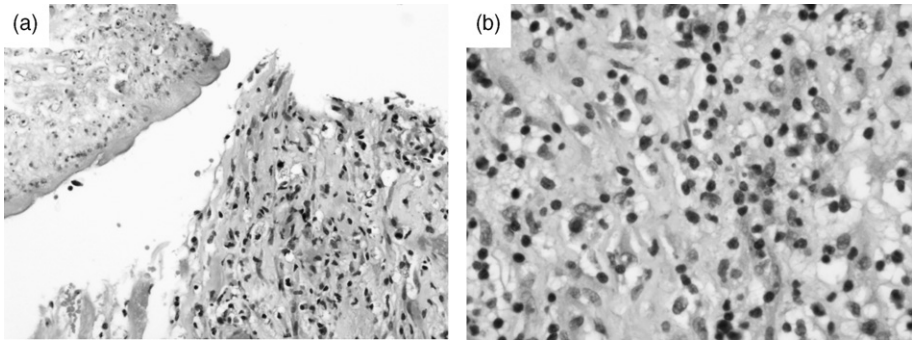


Figure 1 Histopathology of human infection with neurocysticercosis. Two images from the right frontal lobe of a 27-year-old man with new onset seizures are presented. In (a) (40×), both the degenerating parasite (upper left) and the surrounding brain with inflammation (lower right) can be seen. The cuticular surface of the worm is well preserved in this section. The higher power (400×) photomicrograph in (b) shows the brain parenchyma filled with lymphocytes, plasma cells and an occasional eosinophil. Hematoxylin and eosin stain. Photomicrographs courtesy of Dr. J. Clay Goodman, MD.

To test whether the seizures are a result of the host response to the dying cyst, granulomas were harvested from mice infected with *T. crassiceps*. A portion of the granuloma was saved for histological analysis and the remainder was homogenized in phosphate-buffered saline. The extract prepared from the granulomas was then injected into the brain of an adult rat to mimic the presence of these substances in neurocysticercosis. Preliminary data showed that injection of the extract into either the hippocampus or the amygdala could produce electrographic seizures. With the animals under low levels of anesthesia, no behavioral seizures were observed after injection of extract. The hippocampus and amygdala were chosen because of their relatively low seizure threshold. A single injection of 10 μl extract (containing 25 μg total protein), or control substance, was administered and the animals were monitored continuously for at least 1 h. Control experiments with known convulsants determined that the recorded electrical activity had to reach at least twice the amplitude of the baseline activity to be defined as epileptiform, and that the onset of the epileptiform activity should occur within 3 min to be considered a direct effect of the injected material. After termination of each experiment, the brain was examined to confirm accuracy of the injection and recording sites.

Recent Results

Initial experiments determined that injection of 10 μl of phosphate-buffered saline had no effect on the recorded electrical activity. Each extract prepared from an individual granuloma was tested in at least two animals. Extracts from stage 1 and 2 granulomas consistently produced an epileptiform discharge in the hippocampus that started within 1 min of injection and lasted a mean of 44 s (range of 25–76 s). Extracts from granulomas in stage 3 or 4 did not reliably produce seizures. Once the epileptiform activity had stopped, it did not recur. Injections of extracts from nonstimulated host cells prepared from spleen of uninfected mice and from homogenized viable parasites did not produce epileptiform activity. This study provided direct evidence that a substance produced in early stage granulomas, but not a substance released by the degenerating parasite, can initiate seizure activity. This suggests that the inflammatory reaction by the brain may be the sole source of the epileptogenic activity.

Future Goals

To really understand the relationship between seizures and infections of the central nervous system, there is need for better animal models. This will require a thorough understanding of the pathophysiology of the infectious process in both humans and whatever experimental animal is chosen. For the case of neurocysticercosis, we would like to know how the cyst suppresses the immune response. What leads to death of the organism, to the formation of the granuloma, and to stimulation of the host immune response? What is the role of the inflammatory response in the genesis of the seizures? For herpes infections, we would like to explain the direct effect of an acute infection on neuronal and glial function. Are there changes in cellular function when the virus is latent? How is the virus reactivated in the brain, and is the effect of a reactivated virus different from that of a new infection? There is still much that needs to be learned about infections in the brain and how they lead to seizures.

In addition, the infecting organism needs to be clearly defined, and means to grow it reproducibly in the laboratory developed. While some questions can be answered in in vitro systems, such as slice cultures or acute cultures of dissociated cells, it is clear that many issues must be addressed in vivo – e.g., where there are questions about the role of the blood–brain barrier or inflammatory processes.

The seizures in patients with neurocysticercosis are thought to be provoked by active inflammation. The seizures often resolve as the inflammation subsides, suggesting that one of the mediators of the inflammation is the cause of the seizures. This idea that the inflammation is a major contributor to the seizures has been voiced in studies of many different infectious processes. Inflammation is a host response to injury. Local and sustained inflammatory responses have benefits in infectious disease, generally limiting bacterial replication and spread. However, sustained and/or inappropriate inflammation is the cause of a number of human diseases. Until recently, the brain was considered to be resistant to inflammation or immune activation. While we now know that the brain is not 'resistant,' the immune responses in the brain are somewhat different than those seen peripherally. The brain exhibits a number of key features of inflammation, including glial activation, edema, an acute phase response, and synthesis of cytokines, free radicals, adhesion molecules, and other markers of the inflammatory response. Finally, immune cells do invade brain tissue. Most inflammatory mediators are expressed at very low (or undetectable) levels in the normal brain, but can be rapidly induced. For example, infection will stimulate microglia to transform into macrophages, which will move to the site of infection. Activated microglia produce cytokines and trophic factors that can either damage or protect surrounding cells.

A number of cytokines have been investigated for a possible link to seizures. Interleukin 1 (IL-1) is a proinflammatory cytokine that is released following damage; it can, in turn, induce growth factors and nitric oxide synthase, and affect a number of neurotransmitter systems. There is evidence for both a proconvulsive and anticonvulsive action of the β form of IL-1. Tumor necrosis factor-α (TNF-α) is also an inflammatory mediator in

the brain. It can have opposing actions (pro- or anticonvulsant), depending on which receptor it binds to and the duration of the inflammatory process. TNF-α and IL-1 also control expression of other cytokines, which can in turn modulate neuronal excitability by a direct action on ion channels or by inducing gene transcription. The role of these cytokines in those seizures that are associated with infections of the brain is not known. Both IL-1 and TNF-α, as well as a number of other bioactive substances, have been shown to be present in the inflammatory granulomas found in neurocysticercosis. The contribution of these substances to the seizures seen in this disease is an area of active investigation.

Another goal of research into the link between seizures and infection is to develop better treatment strategies for these seizures. Clearly, the primary treatment is aggressive management of the underlying infection, especially for bacterial infections. However, consider the case with neurocysticercosis. The helminth does not appear to cause seizures while alive. So, should an asymptomatic patient be treated with antihelminthic drugs, which will cause death of the parasite? Should this antihelminthic treatment be combined with a potent antiinflammatory drug? In a patient with seizures, should an antiinflammatory agent be used alone or with an anticonvulsant? These issues continue to be debated. In viral infections of the central nervous system, will aggressive antiviral therapy reduce the incidence of later seizures? Should there be an attempt to treat a latent infection? Can reactivation of herpes be detected before seizures begin? Finally, can understanding how infectious processes lead to seizures improve the treatment of idiopathic seizures?

See also: **Inflammation**: Inflammatory Responses and Cytokines; Modulation of Neuronal Excitability: Vascular Changes and Inflammation; Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; **Seizures**: Seizure Initiation and Termination; **Treatments (non-Antiepileptic Drugs)**: Ginseng and Other Herbal Treatments for Epilepsy.

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Inflammatory Responses and Cytokines

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Introduction

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inflammatory reactions in a large variety of maladaptative changes occurring in epileptic tissue, including neuronal hyperexcitability and cell damage, glial cell activation and proliferation, and alteration in the blood-brain barrier (BBB) permeability. These observations suggest that inflammatory reactions in the brain may be implicated in the pathogenesis of seizures, thus opening new perspectives to their pharmacological treatment using non-conventional anti-inflammatory approaches.

Background – Expression Studies

Inflammatory Molecules in Experimental Models of Seizures

Seizure induced in rats or mice by electrical stimulation or intracerebral application of chemoconvulsants increases the production of prototypical inflammatory cytokines, such as interleukin(IL)-1β, IL-6, and TNF-α prominently in glia, and in some instances also in neurons. Using experimental models of status epilepticus in which epileptogenesis eventually gives rise to spontaneous seizures, researchers found that the levels of expression of cytokine mRNAs and their related proteins, levels which are barely detectable in normal healthy brain, are rapidly ($\leq$30 min) increased after seizure induction. This induction is transient and decays within a few days, except for IL-1β, which remains upregulated during epileptogenesis and in chronically epileptic tissue. Evidence for increased production of cytokines and other downstream inflammatory mediators in brain has been also reported in genetic models of audiogenic seizures and in kindling. Cytokine expression is induced in brain areas specifically involved in seizure generation and propagation. Glial cells, including both astrocytes and microglia, represent the main sources of proinflammatory cytokines in the brain; cytokine receptors, however, are expressed both by glia and neurons, suggesting that inflammatory molecules may be soluble mediators that can establish functional interactions between glia and neurons. Recently, it has been shown that IL-1β and its signal-transducing receptor (IL-1R1) are highly expressed during epileptogenesis in the astrocytic endfeet impinging on brain microvasculature and in endothelial cells of the BBB. Interestingly, the brain areas where these phenomena were observed coincided with areas of alterations in BBB permeability, resulting in serum albumin parenchymal extravasation and in neuronal damage.

The induction of proinflammatory cytokines is responsible for the NFkB-dependent transcription of various inflammatory genes and the synthesis of the related proteins such as cyclooxygenase-2, chemokines, major histocompatibily complex class I and II, and Toll-like receptor family members. The cascade of inflammatory mediators can, in turn, activate adaptive immunity mechanisms leading to infiltration into the brain parenchyma of T and B cells, and dendritic or NK cells. However, adaptive immunity does not appear to contribute to brain inflammation in models of temporal lobe epilepsy (TLE) or in human epileptogenic tissue from TLE patients, in contrast to what has been reported for Rasmussen's encephalities.

Inflammatory Molecules in Human Epileptogenic Tissue

An increased level of proinflammatory molecules has been demonstrated in brain tissues surgically removed from patients with drug-resistant epilepsies using immunohistochemistry, western blot, and mRNA analysis. In particular, IL-1β and its signaling receptor IL-1R1, as well as NFkB activation were found in reactive glia and surviving neurons in TLE hippocampal specimens (as compared with postmortem hippocampal tissue from neurologically unaffected subjects or surgically removed tissue from epileptic patients with an extrahippocampal tumor). In TLE patients, increased BBB permeability was detected in the same areas showing activation of the IL-1 system. Finally, scattered T and B lymphocytes and NK cells were found mainly associated with microvessels but not in brain parenchyma. These changes observed in human TLE tissue overlap with those observed in experimental models.

Brain tissue analysis also shows evidence of inflammation in tuberous sclerosis, focal cortical dysplasia (FCD), and glioneuronal tumors (GNT) – all conditions associated with epilepsy. In particular, expression of NFkB and TNF-α has been reported in dysplastic neurons and giant cells in tubers. Strong induction of the IL-1β system has been found in the neuronal dysmorphic neurons, balloon cells, and glial components of FCD and GNT specimens. Interestingly, the density of IL-1β- and IL-1R1-positive cells in FCD and GNT specimens is positively correlated with the frequency of seizures evaluated before tissue resection. The presence of perivascular lymphocytes, scattered microglia nodules and a dramatic increase in the expression of several cytokine genes have been described in brain specimens from patients with Rasmussen's encephalitis and active seizures.

Among the various cytokines measured in plasma and cerebral spinal fluid, IL-6 is the one consistently and significantly upregulated in patients with recent tonic-clonic seizures. Increased serum levels of proinflammatory cytokines have been described in West syndrome and in children with febrile seizures. The observation that brain inflammation occurs also in epileptic disorders that do not features an inflammatory pathophysiology raises the possibility that this phenomenon may be a common substrate contributing to the pathogenesis of seizures in various form of epilepsy with different etiologies.

Methods

Adult male Sprague-Dawley rats, and various strains of adult wild-type and transgenic mice, were used for inducing seizures and to investigate the expression and role of inflammatory mediators. Seizures were induced in freely moving rodents by intraperitoneal or intracerebral injection of chemoconvulsant drugs, such as kainic acid, bicuculline and pentylentetrazole. Hyperthermia-induced seizures were induced in immature rodents to mimic febrile convulsions. Seizures were monitored by electroencephalographic (EEG) and behavioral analysis. Systemic or intracerebral application of pro- or anti-inflammatory compounds to rodent models of seizures was carried out to investigate the role of inflammation in seizure onset and recurrence.

Recent Results

Functional Studies: Modulatory Effects of Cytokines on Seizures

To understand the functional consequences of inflammatory mediators in the epileptic brain, three main experimental approaches have been used:

1. Proinflammatory molecules were injected into the rodent brain before the convulsive stimulus, to mimic a preexisting inflammatory state and to exacerbate the production of these molecules during seizure;
2. A chronic inflammatory state in brain was induced by administering lipopolysaccharide (LPS), a bacterial toxin, to rodents or by using transgenic mice that overexpress specific cytokines, and seizure susceptibility was then assessed;
3. Pharmacological agents interfering with the cytokine systems were injected in rodents, and their effect on seizure susceptibility was assessed.

Role of IL-1β. The application of IL-1β in the rodent hippocampus shortly before the convulsive stimulus prolonged the duration of seizure activity. In contrast, intracerebral injection of IL-1 receptor antagonist (IL-1ra), the naturally occurring competitive antagonist of IL-1R1, mediated powerful anticonvulsant effects *via* blockade of IL-1 signaling. Accordingly, mice overexpressing the human soluble form of IL-1ra in astrocytes showed a reduced susceptibility to seizures. Intracerebral injection of IL-1β or systemic administration of LPS, which induces high levels of IL-1β and other cytokines in brain, decreased the threshold for seizure induction in an experimental model of febrile seizures in immature rodents; in contrast, seizure threshold was raised in mice with a genetic deletion of IL-1ra gene and after intracerebral application of IL-1ra. These findings suggest that an increase in IL-1β levels, as it occurs during seizures, infection or various brain damaging events has proconvulsant effects since an experimentally-induced increase in IL-1β levels prolongs seizure duration and lowers the threshold for seizure induction. These data are reinforced by the evidence that inhibition of IL-1β synthesis in rodents, using selective inhibitors of interleukin-converting-enzyme (ICE or Caspase-1) significantly reduced the frequency and the duration of seizures. Thus, these data support a crucial role of IL-1β in the mechanisms of ictogenesis.

Role of TNF-α. Pharmacological studies have shown that the injection of TNF-α into the mouse hippocampus significantly reduced seizures via interaction with TNF-α type II receptors (p75). Transgenic mice with low to moderate overexpression of TNF-α in astrocytes show decreased susceptibility to seizure, while mice with high overexpression of TNF-α develop signs of neurological dysfunction, including seizures. These findings highlight the importance of the tissue microenvironment (i.e., cytokine concentration, receptor subtype predominantly involved) in determining the final outcome of an increase in this cytokine on brain tissue excitability.

Role of IL-6. Transgenic mice overexpressing IL-6 in astrocytes show an increased susceptibility to seizures induced by glutamatergic agonists, as well as a loss of GABA- and parvalbumin-positive neurons in the hippocampus which may be implicated in their propensity to develop seizures. High overexpression of brain IL-6 is associated with the occurrence of age-dependent neurodegenerative changes and sporadic spontaneous seizures. Recent evidence, however, shows that IL-6 attenuates hyperthermia-induced seizures in developing rats, thus highlighting a possible dual role of this cytokine on neuronal excitability.

Role of other cytokines. Limited information exists about the effects of other inflammatory cytokines on seizures. For example, IL-2 facilitated sound- and chemoconvulsant-induced seizures in mice, and relatively high doses of this cytokine can cause recurrent seizures, although transiently. Intraventricular injection of IL-3 increased spike-and-wave discharges, and epileptic-like activity was observed after in vitro application of interferon to neuronal cultures or hippocampal slices. Finally, it has been shown that administration of LPS to rodents can facilitate spike-and-wave discharge activity in genetically seizure prone rats, and decreases the threshold to pentylentetrazole-induced seizures in mice, suggesting that a proinflammatory state in the brain can increase neuronal excitability and the propensity to develop seizures.

Mechanisms Underlying Cytokine Modulation of Neuronal Excitability (Fig. 1)

Initial studies, mainly focusing on IL-1β and TNF-alpha, attempted to explain the mechanisms by which cytokines

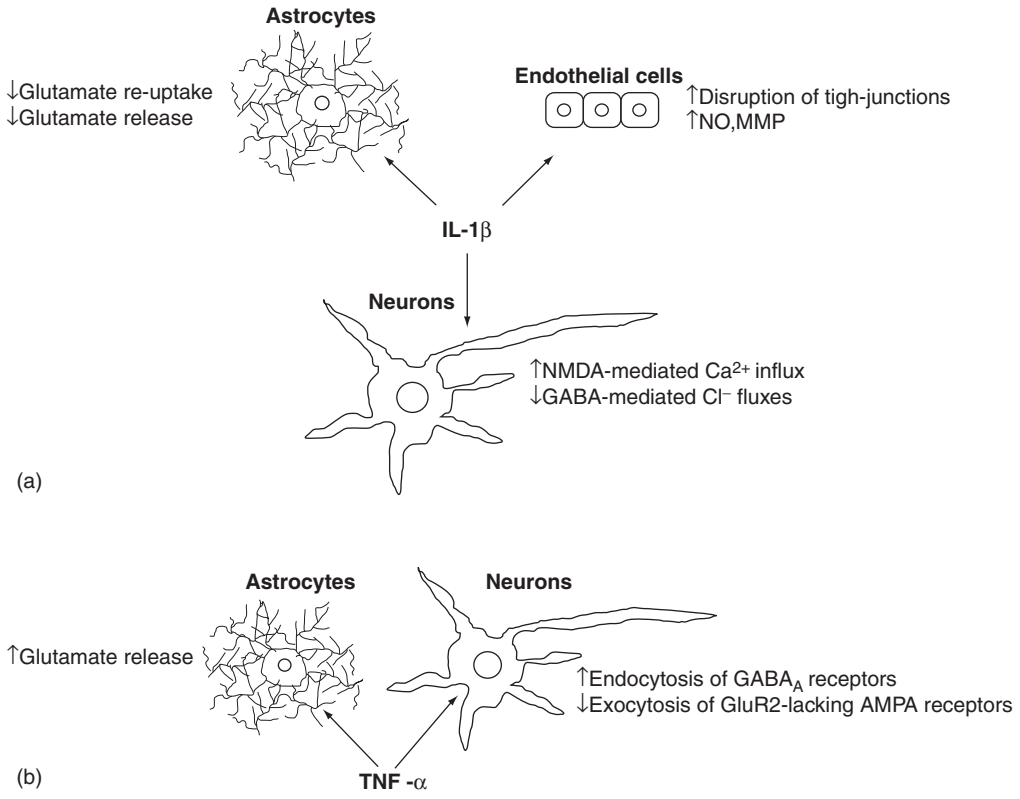


Figure 1 This schema depicts some of the mechanisms so far explored possibly underlying the increase in neuronal excitability mediated by inflammatory cytokines. Experimental data suggest that functional interactions between cytokines, glutamate, and GABA may result in increased excitatory strength. Changes in BBB permeability induced by cytokines may also contribute to enhancement of neuronal excitability (see text for details). NO, nitric oxide; MMP, matrix methalloproteinase.

may deleteriously affect neuronal excitability. The main approach was to investigate whether cytokines alter the function of classical neurotransmitters that are known to play a pivotal role in neuronal network excitability, such as glutamate and GABA. IL-1β has been reported to affect glutametergic neurotransmission by at least three routes: (1) by increasing extracellular glutamate concentration via inhibition of astroglial reuptake; (2) by increasing astrocytic or neuronal release of glutamate; and (3) potentiating *N*-methyl-D-aspartate (NMDA) receptor function via enhanced tyrosine phosphorylation of the NR2B subunit, resulting in increased neuronal Ca^{2+} influx. The dendritic colocalization of IL-R1 with NMDA receptors in hippocampal neurons supports their functional interaction mediated by IL-1β induced post-translational NMDA receptor changes. In addition to its interactions with glutamatergic transmission, IL-1β also reduces GABA-mediated Cl^- fluxes in hippocampal slice preparations.

The effects of IL-1β on endothelial cells of the BBB may also play a role in altering neuronal excitability in surrounding brain tissue. IL-1β can increase BBB permeability to serum albumin *via* disruption of tight junctions possibly mediated by the cytokine-mediated production of nitric oxide in endothelial cells as well the activation of methalloproteinases. The impairment in BBB permeability may lead to chronic neuronal hyperxcitability via parenchymal extravasation of serum proteins; moreover, a recent study reported a positive correlation between the extent of BBB leakage and the frequency of spontaneous seizures in rats.

A functional interaction between TNF-alpha and AMPA receptors was recently shown. This cytokine, by interacting with its type I receptor (p55) which contains an intracellular 'death domain' involved in cell damage, increased the surface expression of Ca^{2+}-permeable AMPA receptors at the neuronal plasma membrane, thus amplifying glutamate responses. TNF-alpha also promoted the endocytosis of $GABA_A$ receptors, thus decreasing inhibitory synaptic strength. This cytokine has also the ability to increase the release of glutamate from astrocytes.

These effects of IL-1β and TNF-alpha can also underlie their detrimental effects on neuronal survival to an excitotoxic insult since these cytokines can exacerbate necrotic and apoptotic neuronal cell loss, depending on their concentration, the time the brain tissue is exposed to the cytokine vis-à-vis with the injurious event and the type of cytokine receptors involved.

Conclusions and Future Goals

The activation of inflammatory pathways in brain areas involved in seizure inititiation and propagation has been well documented in experimental models as well as in chronic epileptogenic tissue from drug-refractory epileptic patients. Moreover, specific inflammatory processes triggered in the brain by seizures persist during epileptogenesis until the occurrence of spontaneous convulsions. The availability of clinically relevant experimental models of epilepsy is instrumental to study the chronological sequelae of inflammatory events following different kinds of brain injuries associated with a high risk of developing spontaneous seizures, and the role of brain inflammation in the epileptogenic process. Several studies are still required to further investigate the functional interactions between inflammation and epilepsy:

- analysis of the profile of inflammatory markers in blood and/or CSF, evaluated at the first seizure and during follow-up, to determine whether some inflammatory molecules can be predictive for the occurrence of epilepsy, particularly in those symptomatic cases with a high risk of developing epilepsy;
- characterization of inflammatory markers in those patients in which anti-inflammatory drugs appear to modify seizures;
- characterization of the detailed molecular signaling processes activated by inflammation to highlight new potential therapeutic targets;
- definition of the genetic backgrounds regulating the expression of the inflammatory response in epileptic patients, to determine whether there is a genetic predisposition to develop brain inflammation following brain damaging events.

If a link between brain inflammation, epileptogenesis and ictogenesis is established, this finding will provide strong support for the development of novel pharmacological strategies for inhibiting seizures using anti-inflammatory treatments directed against specific inflammatory pathways, and possibly for arresting epileptogenesis. The expression of inflammatory molecules is constitutively very low in the brain, suggesting a limited role of these molecules in normal brain physiology, thus targeting specific inflammatory mediators for controlling seizures may also reduce the chances of neurological side-effects.

See also: **Inflammation**: Cerebrovascular Diseases, Seizures and Epilepsy; Connections between Infections and Seizures; Modulation of Neuronal Excitability: Vascular Changes and Inflammation; Neurodegeneration, Neuroprotection and Inflammation in the Brain.

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Modulation of Neuronal Excitability: Vascular Changes and Inflammation

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Introduction

After many decades of animated debate in the scientific community about putative mechanisms, including neurodegenerative pathways, involved in epileptogenesis in the most common animal models of limbic epilepsy (e.g., those based on kainic acid or pilocarpine administration), we are still far from a commonly accepted explanation. In our opinion, one of the major theoretical limitations is represented by the fragmented approach to this problem. Biological mechanisms involved in epileptogenesis and in the brain response to an ictogenic insult have been rarely studied from a holistic point of view. Electrophysiology, neuropathology, neuroimmunology, and other biomedical approaches have focused independently on particular aspects of the complex pattern of brain alterations associated with the epileptic condition. Our goal has been to adopt an integrated and multidisciplinary approach that can take into account different factors – in in vivo studies of alterations of the 'whole brain' – in epilepsy. We attempt, therefore, to combine electrophysiological, neuropathological, and neuroimmunological data, considering the multiple players involved in vivo in this dangerous game.

Background

A wealth of data, obtained both in human and animal studies, indicate that perturbations in neurovascular integrity and breakdown of the blood–brain barrier (BBB) may lead to neuronal hypersynchronization and epileptiform activity. However, the mechanisms underlying these processes are still unclear. Furthermore, inflammatory processes related to the activation of brain vessel endothelia and/or BBB leakage have been reported, but no explanations have yet been provided for the relevant mechanisms of action.

Seizures bring about, by definition, electrical alterations of the electroencephalogram (EEG). For this reason, experimental neurobiological studies of the etiopathogenesis of epilepsy are historically based on electrophysiology and, in particular, on the study of neuronal discharges. This focus has led to a relative neglect of two other potentially key (and probably crucial) factors – vascular and inflammatory processes (and their combined role).

In our investigations, we recently showed that pilocarpine-induced status epilepticus (SE) produces marked vascular effects in a neocortical (somatosensory) and an allocortical (cingulate) brain region of Wistar rats (**Figure 1**). We reported that vascular alterations, characterized by hyperemia and neurons degenerating by apoptotis, are evident in the supragranular layers of the somatosensory cortex and in superficial (II/III) layers of the cingulate cortex 12 h after SE (which lasted 4 h) (**Figure 2**). Deeper layers (V, VI) exhibited early edema and necrotic cell death. These results suggest that cortical injury caused by pilocarpine-induced SE may involve different mechanisms in different cortical layers, probably due to laminar differences in blood vessel organization and regulation.

Blood–Brain Barrier

The BBB is a selective barrier formed by tight junctions between endothelial cells, acting primarily to limiting the penetration of different substances from the blood into the brain. The BBB presents a 'physical barrier,' which forces most molecular traffic to take a transcellular route across the BBB, rather than moving paracellularly through the junctions. The high density of the tight junctions that compose the BBB restricts the passage of substances from the bloodstream much more than endothelial cells in capillaries elsewhere in the body. Astrocyte cell processes, called 'end-feet,' surround the endothelial cells of the BBB (forming the so-called *glia limitans*), providing biochemical support to the endothelial cells. BBB disruption is associated with various neurological disorders. During the past years, there has been increasing interest in BBB alterations in epilepsy, both in humans and in animal studies.

BBB Alterations in Epilepsy

Technical advances in neuroimaging procedures in humans and in animal models have contributed knowledge about cerebrovascular alterations consequent to seizures, demonstrating that the BBB can be reversibly disrupted by pathologic processes, leading to – and caused by – epileptic activity. Vascular alterations have been correlated with neuronal and glial cell degeneration in different animal models of chemically induced acute or

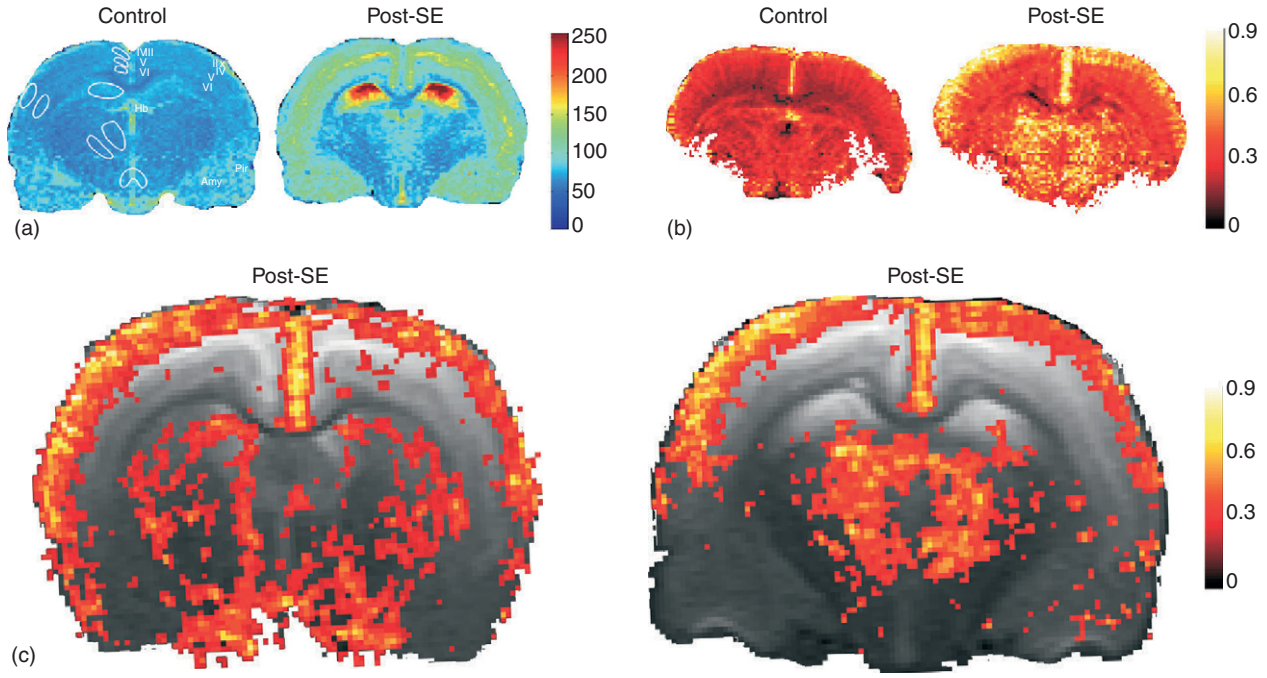


Figure 1 (a) T2 parametric maps of control brain and in postacute phase of status epilepticus (SE). (b) Regional cerebral blood volume (rCBV) maps in control and post-SE animals. Pseudocolor images refer to one rostrocaudal levels (–2.5 mm from bregma). (c) Maps generated to show the highest values of rCBV increase (cut off at 0.40 arbitrary units) at two different levels (a: 0.26 mm from bregma, b: –2.5 mm from bregma) superimposed to T2-weighted images (T2W) in the same post-SE animal. In the cerebral cortex the simultaneous detection of the two signals shows clearly that rCBV peaks in the superficial layers, overlying the T2W hyperintensity. An increase of rCBV is also evident in the dorsolateral striatum and medial basal forebrain, as well as in the medial thalamus. Modified from Fabene PF, Marzola P, Sbarbati A, and Bentivoglio M (2003) Magnetic resonance imaging of changes elicited by status epilepticus in the rat brain: diffusion-weighted and T2-weighted images, regional blood volume maps, and direct correlation with tissue and cell damage. *Neuroimage* 18: 375–389.

chronic seizures. Recently, BBB permeability disruption in limbic regions of the rat brain, shortly after SE as well as in the latent and chronic seizure phases, has been reported. BBB leakage likely contributes to the progression of epilepsy. In epileptic rats, artificial opening of the BBB induces a persistent increase in the number of seizures, and BBB permeability disruption is positively correlated with chronic seizure frequency. Acute vascular failure seems to be sufficient to cause seizures in the absence of other central nervous system (CNS) pathology, not only in animal models but also in humans.

Bridging the Gap Between BBB Alteration and Neuronal Hyperexcitability

We now need to bridge the gap between BBB leakage, documented in both human and rat epileptic brain tissue, and neuronal hyperexcitability. By definition, a seizure is a paroxysmal hypersynchronous discharge generated by CNS neurons. Since seizures are electrical alterations, it is important to address the question of what mechanisms link vascular alterations to the electrical activity of a given neuron. Are such mechanisms passive or active? Are they sufficient to explain the pathology, or do we need a wider theoretical framework?

The role of albumin in epileptogenesis has been recently studied in detail. Focal BBB opening by direct cortical application of albumin-containing solution can lead to the generation of an epileptic focus in rats. In particular, extravasated albumin accumulates in astrocytes and neurons in the hippocampus of temporal lobe epilepsy (TLE) patients, as well as in microglia, astrocytes, and neurons of the rat epileptic brain. Interestingly, blood volume is consistently highest in regions with highest concentration of degenerating neurons and glia. Furthermore, adult rats undergoing focal BBB disruption exhibit over time a reduction in cortical volume compared with control rats, associated with reduced number of neurons and increased number of astrocytes, as well as increased threshold for spreading depression and increased propagation velocity of evoked epileptic potentials.

Similarly, by means of long-lasting focal neocortical BBB disruption or direct exposure to serum albumin in in vivo studies in rats, as well as albumin exposure in brain slices in vitro, it is possible to induce hypersynchronous propagating epileptiform field potentials. In these experiments, direct brain exposure to serum albumin was

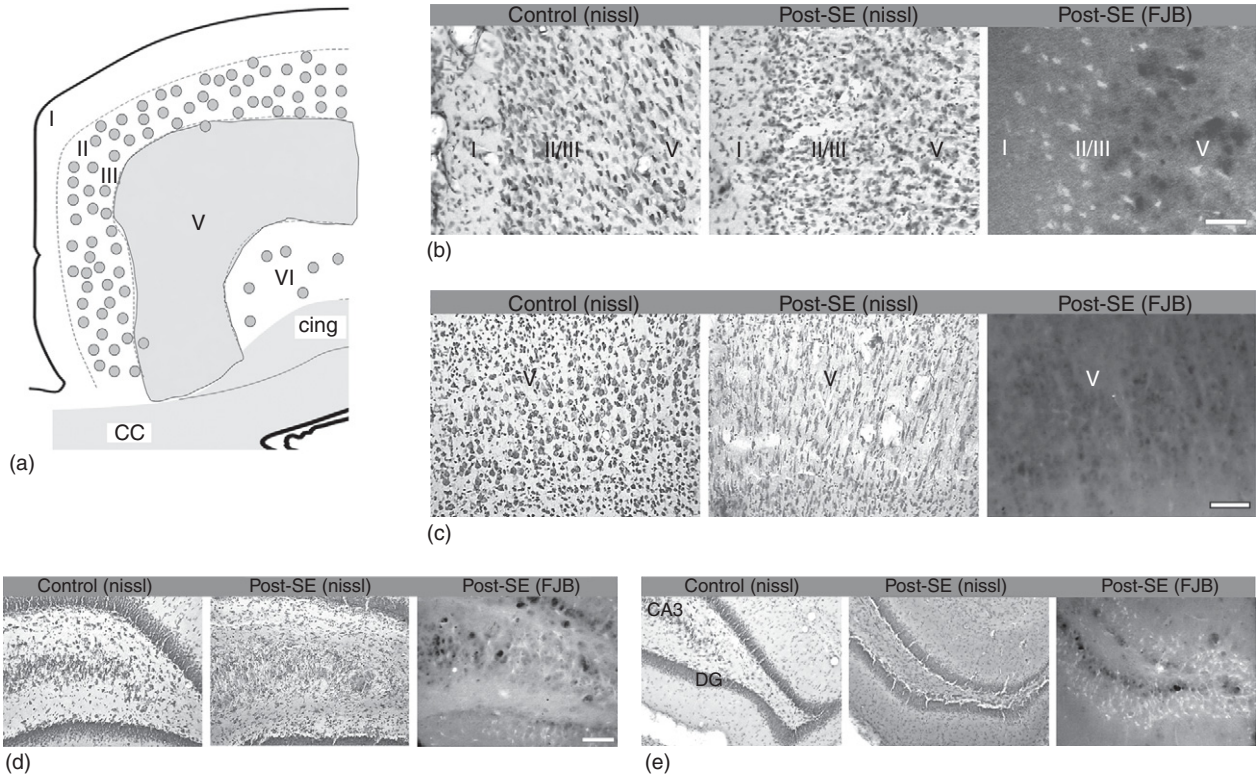


Figure 2 (a) Schematic representation summarizing the histopathological data: the circles refer to FJB-stained cells and the gray area to the edematous changes. The numbers refer to the cortical layers. (b) Histopathological features observed in the cingulate cortex. Nissl staining and FluoroJade B (FJB) histochemistry (revealing degenerating neurons) in the upper layers of control and post-SE animals. (c) Nissl staining and FJB histochemistry in layer V of control and post status epilepticus (SE) animals. (d) Nissl staining of the CA3 pyramidal layer of control animal and post-SE animals and FJB histochemistry in the adjacent section. (e) Nissl staining of the dentate gyrus (hilus) of a control animal and a post-SE animal and FJB histochemistry in the adjacent section of the post-SE animal. Scale bars: (b, c, e) = 35 μm, (d) = 50 μm. Modified from Fabene PF, Marzola P, Sbarbati A, and Bentivoglio M (2003) Magnetic resonance imaging of changes elicited by status epilepticus in the rat brain: diffusion-weighted and T2-weighted images, regional blood volume maps, and direct correlation with tissue and cell damage. *Neuroimage* 18: 375–389.

associated with albumin uptake into astrocytes, followed by downregulation of inward-rectifying potassium (Kir 4.1) channels, resulting in reduced buffering of extracellular potassium in astrocytes. This process, in turn, led to an activity-dependent increased accumulation of extracellular potassium, resulting in facilitated *N*-methyl-D-aspartate-receptor-mediated neuronal hyperexcitability.

Mechanisms of BBB Permeability and Leukocyte Recruitment

Recent studies have shown that BBB opening leads, by itself, to neuronal hypersynchronization and epileptiform activity, which is mediated by exposure of neuronal cells to blood albumin or potassium ions. Moreover, as indicated earlier, chronic BBB leakage during epilepsy contributes to its progression. Changes in BBB integrity may be caused by increased local blood pressure that occurs during seizures, free radical formation, and inflammatory responses such as leukocyte recruitment, cytokine and chemokine production, and/or loss of tight junction molecules.

Leukocyte migration through the endothelium represents a critical event in the pathogenesis of inflammatory responses and involves complex, sequential events. Leukocyte tethering and rolling represent the first step of leukocyte recruitment in blood vessels. Leukocyte migration from bloodstream to tissue requires rapid coordinated regulation of integrin-dependent adhesion and deadhesion. The immunoglobulin superfamily is in part composed by two adhesion molecules: intercellular adhesion molecule type 1 (ICAM-1) on the superficial part of vascular endothelial cells, and vascular cells adhesion molecules type 1 (VCAM-1). The tie between leukocyte function-associated antigen (LFA)-1 and ICAM-1 is the adhesion mechanism stabilizing the contact between the cytolytic cell and the endothelial cell.

Cytokines and Chemokines

Cytokines are small (8–10 kDa) signaling proteins, which play a key role in cellular communication and are produced by a variety of hematopoietic and nonhematopoietic

cell types. Chemotactic cytokines, characterized by the ability to induce directed chemotaxis in nearby responsive cells, are called 'chemokines.' Cytokines and chemokines are involved in both innate and adaptive immune responses. They are usually secreted by immune cells after pathogen detection, in order to activate and recruit more immune cells and to increase the system's response to the pathogen, but they can also be secreted by glial cells. Besides their role in the functioning of the immune system and in a variety of inflammatory diseases, cytokines and chemokines are involved in neuroplasticity and glia–neuron communication. Numerous cytokines can have both proinflammatory and anti-inflammatory roles, depending on target cells and their state of responsiveness to the cytokine. Chemokines can be induced during an immune response to promote immune cell recruitment to a site of infection; their release is often stimulated by proinflammatory cytokines such as interleukin (IL)-1. Inflammatory chemokines function mainly as chemoattractants for leukocytes, recruiting monocytes and neutrophils from the bloodstream to sites of infection or tissue damage. Others chemokines ('homeostatic chemokines') are involved in controlling physiological cell migration. Chemokines can also be involved in angiogenesis.

Methodological Approaches

In our laboratory, seizures are usually induced in Wistar rats and C57BL6/J mice by systemic administration of pilocarpine. Limbic seizures provoked in rodents by systemic administration of this muscarinic cholinergic agent provide a widely used model of human epilepsy, in which brain injury consequent to SE leads to spontaneous recurrent seizures after a latent phase.

Seizure severity and duration and occurrence are assessed by an EEG acquisition system, using telemetric technology (Dataquest® A.R.T. Data Acquisition 3.0 for telemetry systems, Data Sciences International, Arden Hills, MN, USA). With this system, EEG, body temperature, blood pressure, and movements can be recorded in each animal for 24 h day^{-1}, for up to 9–12 consecutive months in rats and 4–5 months in mice.

Magnetic resonance imaging (MRI) data are acquired using a 4.7-T Biospec Tomograph (Bruker, Karlsruhe, Germany) equipped with an Oxford 33-cm bore. Structural, functional, and perfusion data are obtained to analyze neurodegenerative and inflammatory processes in the epileptic brain, and to assess, quantitatively and qualitatively, the effects of pharmacological treatments. Relative alterations in the cerebral blood volume and flow (rCBV and rCBF) in given brain areas, relative to other brain regions, are assessed by acquiring T2*-weighted images during the first passage of a superparamagnetic contrast agent (USPIO) and at the equilibrium distribution.

Histochemistry is routinely used to evaluate cell density or morphology (Nissl staining) and neurodegeneration (Flouro Jade B and/or Dark Neuron stains), and immunohistochemical procedures are used to identify cell types and analyze the cellular expression of different proteins. Sections are studied on a Zeiss LSM 510 confocal microscope (Zeiss, Oberkochen, Germany), equipped with argon (488 nm) and helium/neon (543 nm) excitation beams. Sections treated as earlier, but in the absence of the primary or secondary antibody, are always used as control. For ultrastructural analysis, dissected tissue blocks are fixed, and 1-μm-thick semithin sections are stained with toluidine blue. Ultrathin sections are cut at 70-nm thickness on an Ultracut-E ultramicrotome (Reichert-Jung), stained with lead citrate and uranyl acetate, and observed on a Zeiss EM 10 electron microscope.

To make vascular cast preparations, animals are anesthetized with sodium pentobarbital and then thoracotomized. A catheter is introduced into the arch of the aorta through the left ventricle, and the right atrium is opened. The circulatory system is rinsed with phosphate-buffered saline, and a freshly prepared solution of a low-viscosity resin, Mercox CL-2B (Dainippon Ink & Chemicals, Tokyo, Japan), is injected along the same route. Maceration of the brain tissue is performed using a 10% solution of potassium hydroxide until only the resin casts of blood vessels remain. These specimens are rinsed by several passages in distilled water, and then frozen. The casts are freeze-dried (Modulyo, Edwards-Kniese, Marburg, Germany), fixed to stubs with colloidal silver, sputter-coated with gold (MED 010, Balzers), and examined under a scanning electron microscope (DSM 690, Zeiss).

For microarray analysis, mRNA is isolated from different brain areas. mRNA is used as template for generating a cDNA library. cRNA labeled with dUDP-biotin (Enzo Roche Molecular Biochemicals, Mannhein, Germany) is retrotranscribed. dUDP-biotin-cRNA is purified and hybridized on different OligoGEArrays containing probes specific for genes implicated in signal transduction pathways, apoptosis, DNA-damage response, chemokines, cytokines, or adhesion molecules. The hybridization pattern is revealed by CDP-Star® substrate fluorescence using Chemiluminescent Detection Kit and recorded on an X-ray film. Data from arrays are analyzed using GEArray Analyzer software (Superarray Bioscience Corporation).

Recent Results

As stated earlier, we recently showed SE-induced vascular alteration in cortical areas of Wistar rats (**Figures 1** and **2**), demonstrating that degenerating neurons are related to brain sites characterized by hyperemia. We decided to

better elucidate such pathological modifications of brain perfusion by assessing changes in the vascular structure, gene expression, and time course of neuronal degeneration in the cerebral cortex during the postacute phase of pilocarpine-induced SE. We showed that after 2 h, the infragranular layers of the somatosensory cortex exhibit reduced vascular perfusion indicative of ischemia, whereas the immediately adjacent supragranular layers exhibit increased perfusion (**Figure 3**). Infragranular layers exhibit necrotic pathology, whereas the supragranular layers are characterized by a delayed (24 h after SE) degeneration, apparently via programmed cell death. These results indicate that pilocarpine-induced SE elicits both excitotoxic and ischemic injury, and that vascular alterations play a major role in epileptogenesis.

One of the consequences of vascular alterations and consequent increased BBB permeability is the accumulation of serum proteins that enter the brain, which may contribute to increased excitability. It has been shown recently that epileptiform activity can be induced in rats by direct cortical application of an albumin-containing solution, suggesting that serum proteins play a role in

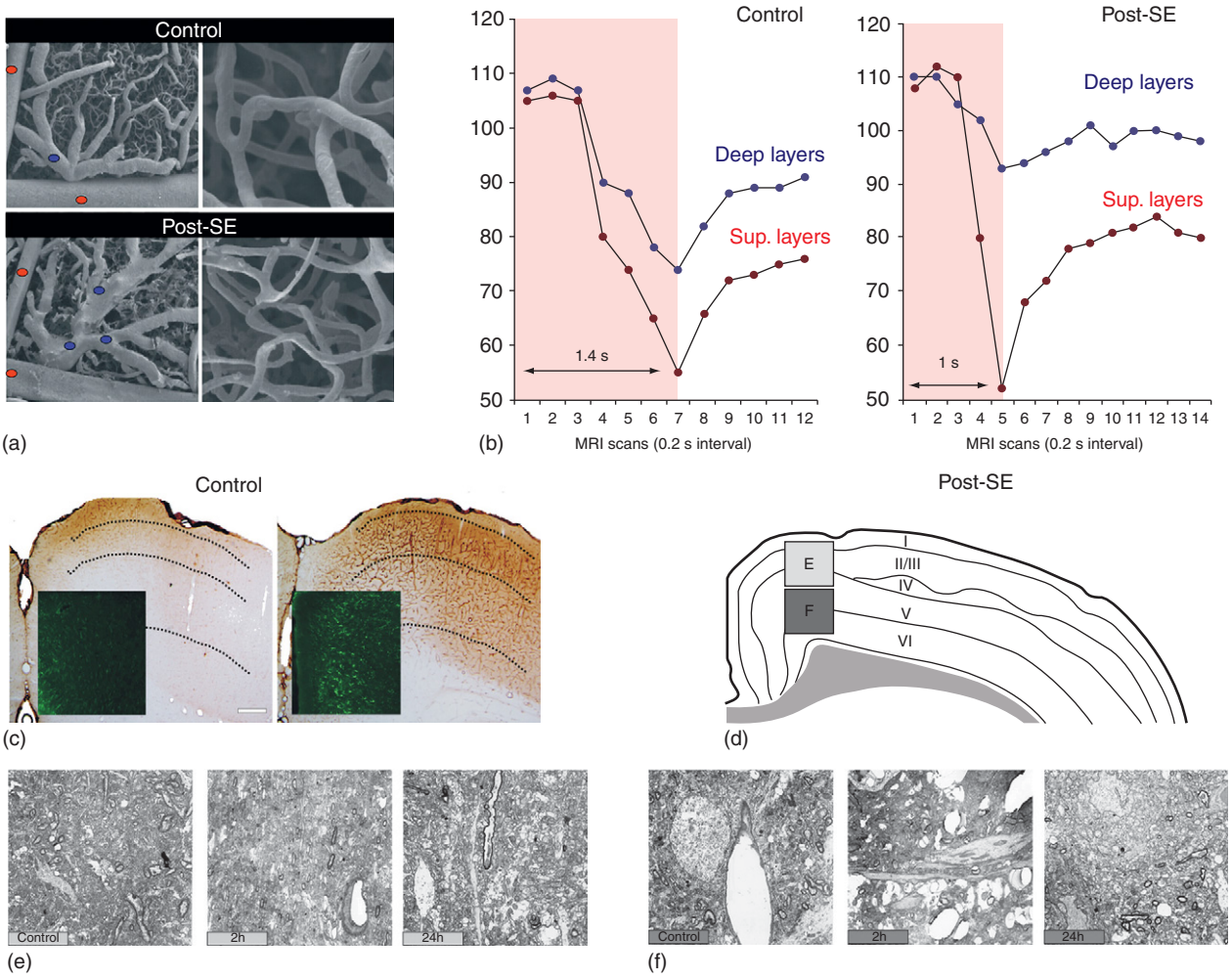


Figure 3 (a) Vascular casts reveal structural alterations in brain vessels 2 h after the onset of status epilepticus (SE). In the superficial, hyperperfused zone, veins are increased in diameter (right column) whereas in the hypoperfused, edematous subgranular region, veins appear collapsed (left column). (b) Peak enhancement in control and pilocarpine-treated animals. Compared with controls, in rats during SE infragranular layers show a decreased contrast medium peak concentration, indicating a relative ischemic core, whereas supragranular layers are characterized by hyperemia (red dots). (c) Agrin expression in control and pilocarpine-treated animals. Increased agrin expression in the endothelial cells is evident in both superficial and deep layers 2 h after SE onset. This increase is more evident in the supragranular than in the infragranular layers. Localization of glial fibrillary acidic protein (GFAP)-like immunoreactivity reveals selective increase in astrocytic GFAP expression in the less acutely damaged superficial layers (square). (d) Schematic drawing of the two different brain areas analyzed by electron microscopy (EM): (e) EM images in control animals, and 2 and 24 h after SE in both supragranular (e) and infragranular layers (f). Infragranular layers are characterized by severe tissue damage 2 h after SE whereas supragranular layers at this time point appear unaffected except for the perivascular edema. Twenty-four hour after SE, damage is evident in both regions. Modified from Fabene PF, Navarro Mora GN, Martinello M, *et al*. (2008) A role for leukocyte – endothelial adhesion mechanisms in epilepsy. *Natural Medicines* 14(12): 1377–1383.

the pathogenesis of focal epilepsies. Interestingly, albumin is found specifically in regions with more EEG spiking activity in human epileptic brain, and albumin can induce calcium waves in rat cortical astrocytes. A positive correlation between vascular alteration, BBB permeability to serum proteins such as albumin, and the occurrence of spontaneous seizures in epileptic rats has thus been demonstrated.

Recently, the involvement of cytokines in the pathogenesis of epilepsy has been suggested by a wealth of evidence. Limbic seizures increase mRNA of inflammatory cytokines in rodent forebrain. Proinflammatory cytokines (IL-1beta, tumor-necrosis factor-alpha, and IL-6) are rapidly upregulated by seizures in a reversible manner; IL-1beta is upregulated in rodent brain up to 60 days after SE during the occurrence of spontaneous and recurrent seizures. Microglia and astrocytes appear to be the first and main source of cytokine production in brain. Importantly, brain inflammation following seizures contributes to epileptic activity and to the associated neuronal cell loss.

In summary, the aforementioned data indicate that (i) pilocarpine-induced SE involves vascular alterations; (ii) these alterations can take the form of hyper- or hypoperfusion at different brain sites, even sites very close to each other; (iii) delayed neurodegeneration following SE is related to hyperemia and vascular lumen dilatation; (iv) during acute epileptic insults resulting in a vascular alteration, there is a considerable active and passive escape of plasma proteins from the bloodstream to the brain parenchyma; and (v) proinflammatory cytokines and chemokines are involved in the epileptogenic process.

We hypothesized that the inflammatory response involves three major sequential events: dilatation of capillaries to increase blood flow, microvascular structural changes and escape of plasma proteins from the bloodstream, and leukocyte transmigration through the endothelium and accumulation at the site of injury. The first two steps have been extensively investigated, whereas leukocyte recruitment and transmigration have not received significant attention. We, therefore, decided to examine whether SE can induce leukocyte recruitment. We are currently analyzing whether, by interfering with leukocyte recruitment, we can prevent the BBB breakdown and related cascade of inflammatory processes that appear to be responsible for, or at least contribute to, epileptogenesis.

Our data show a direct correlation between endothelial activation, leukocyte adhesion, BBB opening, and seizure generation. These mechanisms could be involved in inflammatory processes causing seizures and epilepsy by different causes. Our results suggest that leukocyte–vascular interactions can contribute to seizure generation, amplifying existing signals and reducing the threshold for seizures by acting synergistically with other pathogenetic events. Leukocyte adhesion to the BBB endothelium, BBB leakage, and neuronal hyperactivity may 'synergize' with each other, amplifying the vascular and neuronal changes that ultimately initiate a full-blown seizure. Thus, endothelial activation that allows leukocyte adhesion and BBB damage may represent a key event, not only in seizure induction but also in the progression toward chronic seizure generation.

We thus hypothesize that a cycle of seizure-induced inflammation and inflammation-mediated stimulation may amplify initial effects, leading to recurrent seizure activity (**Figure 4**). BBB activation, with upregulation of adhesion molecules, is provoked by a convulsant agent (pilocarpine in our experiments) or can be provoked by inflammatory pathology, such as CNS infections or head trauma. Moreover, seizure activity, generated in experimental models of

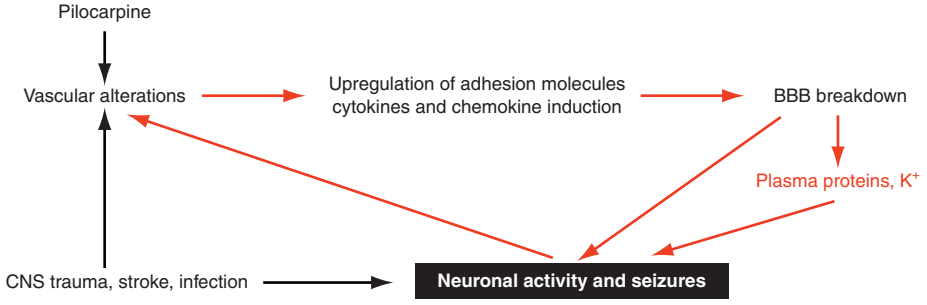


Figure 4 Proposed model for the contribution of vascular inflammation to seizure pathogenesis. CNS vascular activation with upregulation of adhesion molecules, cytokines, and chemokines can be provoked by the convulsant agent (pilocarpine) or by inflammatory pathologies such as CNS infection or injury following head trauma. Activation of brain endothelium can alter endothelial barrier function resulting in leakage of plasma constituents into the brain parenchyma. Exposure of neuronal cells to plasma proteins such as albumin and K^+ accumulation contribute to neuronal electrical hyperactivity, lowering the threshold for seizures. Seizure activity, whether induced by inflammatory processes or by primary neuronal or developmental defects (such as heterotopic gray matter), exacerbates and may itself induce vascular activation. The 'triggering' event(s) in the model could thus be primarily neuronal, vascular, or inflammatory. Neuroinflammation, BBB leakage, and neuronal hyperactivity may synergize with each other, amplifying the vascular and neuronal changes that ultimately lead to seizures. Ongoing positive feedback between adhesion molecule upregulation, altered vascular function, and CNS electrical hyperactivity may contribute to chronic pathology and recurrent seizures (red arrows).

epilepsy or in humans suffering from developmental abnormalities such as cortical heterotopias, can lead to upregulation of adhesion molecules in brain endothelium. Activation of brain endothelium leads, in turn, to leukocyte adhesion, which contributes to BBB leakage; this sequence further compromises barrier function, resulting in lowered threshold to seizures. Exposure of neuronal cells to plasma proteins such as albumin and K^+ accumulation contribute to neuronal electrical hyperactivity.

Leukocytes may contribute to seizure pathogenesis acutely through effects on the BBB, and chronically through several mechanisms, including occlusion of microvasculature, generation of oxygen free radicals, release of cytotoxic enzymes, vascular alterations, and increase in the release of cytokine and chemoattractant molecules. Importantly, even leukocyte adhesion to the vessel wall alone – triggered rapidly after an in vivo stimulus and occurring normally at low stimulation frequency – induces signaling events in the endothelium. These events include calcium signaling, and modifications of small GTPases involved in cytoskeletal organization and c-Jun-N-terminal kinase. Moreover, neutrophil adhesion is known to induce alterations in vascular permeability. BBB leakage in the cortex supports the occurrence of leukocyte–endothelium interactions and upregulation of adhesion molecules in cortical vessels.

Consistent with this scenario, pilocarpine-induced vascular adhesion molecule expression is reduced if seizures are prevented pharmacologically with diazepam, suggesting that electrical activity amplifies the vascular response. Moreover, electrical activity is reduced if leukocytes are depleted or vascular interactions are inhibited. It has also been shown that BBB leakage per se may result in the development of seizures, while drugs able to reduce vascular damage reduce seizure activity.

In conclusion, the earlier discussed data show that vascular alterations and BBB leakage induced by protracted seizures are not merely epiphenomena detected in animal models of chemically induced epilepsy, but they may represent crucial steps of a cascade of events responsible for epileptogenic mechanisms. Furthermore, we suggest that such events can be followed by leukocyte recruitment and by cytokine and chemokine release.

Identify the Challenges for the Future

Future challenges concern the identification of the adhesion molecules involved in leukocyte recruitment in the brain after experimental seizures. Characterizing the effect of adhesion molecule blockade on the clinical course of disease is another major challenge. By means of blocking monoclonal antibodies, we hope to identify the adhesion molecules involved in the process of sticking leukocytes to inflamed brain endothelium at various time points in experimental rodents after protracted seizures. Investigation of the effects of anti-integrins and anti-immunoglobulins blocking antibodies, and the consequences of gene deficiency of integrins and immunoglobulins on the induction and clinical course of experimental limbic epilepsy, are likely to open interesting new perspectives.

See also: **Inflammation**: Cerebrovascular Diseases, Seizures and Epilepsy; Connections between Infections and Seizures; Inflammatory Responses and Cytokines; Neurodegeneration, Neuroprotection and Inflammation in the Brain.

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Neurodegeneration, Neuroprotection and Inflammation in the Brain

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Introduction

Brain insults, such as infection, febrile seizures, inflammation, may result in a sequence of pathological events, leading eventually to the occurrence of seizures. This long-lasting process of epileptogenesis can take years, and may be associated with progressive cell loss initiated by the insult.

Neural Degeneration

There is no doubt that even a single seizure can be a reason for neuronal cell loss in vulnerable brain areas. Status epilepticus (SE), a neurological condition that may last for hours, often produces diffuse brain lesions, as is evident from diverse animal models. The more quickly SE is stopped, the fewer neurons die. Excitotoxic damage to neurons occurring during seizure activity is mainly due to the excessive release of glutamate, which acts on ionotropic glutamate receptors – for *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), and kainate (KA). The neurotoxic potential of glutamate analogs in vitro and in vivo has been broadly documented. The same effect was later reproduced by prolonged electrical stimulation of the glutamatergic perforant path, resulting in damage to hippocampal neurons. These results suggest the possibility that glutamate antagonists, or antiepileptic drugs (AEDs) with anti-glutamatergic potential, may limit seizure-related brain damage – and thus be 'antiepileptogenic.'

Neuronal death is caused by two separate processes – necrosis and apoptosis. The former is a direct result of the excitotoxic activity of glutamate and the latter is dependent on the activation of apoptotic enzymes – caspases. Excessive glutamate release (caused by seizure activity) acts on AMPA receptors to cause massive sodium ion and subsequent water influx into neurons, which leads to the first phase of neurodegeneration – neuronal swelling. Initial depolarization mediated by AMPA receptors alleviates the magnesium block of NMDA receptors, and the neurodegenerative process enters its second phase, involving neuronal calcium ion influx. Calcium influx in turn activates proteases (e.g., calpain I and cathepsin D) and phospholipases, eventually destroying the cytoskeleton and damaging microtubular functions. Also, nitric oxide synthase is activated, which eventually generates free radicals (peroxynitrite), causing damage to DNA. Damaged DNA stimulates the repair enzyme, poly (ADP-ribose)polymerase-1, a process responsible for profound reductions in neuronal ATP and nicotinamide adenine dinucleotide, causing total energy failure. Intraneuronal increase in calcium ion concentration seems to be the main factor of necrosis. In this regard, it is noteworthy that hippocampal granulae neurons are relatively resistant to glutamate excitoxicity, probably because of their high content of the calcium binding protein, calbindin.

Mitochondria are assumed to play an important role in both necrosis and apoptosis. In trying to maintain neuronal calcium homeostatsis, mitochondria become loaded with calcium ions upon glutamate-induced excitation. This excessive mitochondrial calcium load is associated with increased mitochondrial permeability through mitochondrial permeability transition pore or enhanced permeability of the outer mitochondrial membrane. Activation of the transition pore by excess of calcium causes mitochondrial swelling, followed by impaired ATP production and increased synthesis of free radicals. This sequence of events leads to further opening of pore, and eventually causes necrosis. In contrast, increased mitochondrial permeability also triggers apoptosis via release of pro-apoptotic proteins – apoptosis-inducing factor and cytochrome c – leading to activation of caspases.

Inflammation

Experimental convulsions as well as clinical epilepsy generate pro- and anti-inflammatory cytokines in the central nervous system. Data from experimental models indicate that inflammation in the brain may elevate the neuronal excitability, promote neuronal death, and decrease the integrity of the blood-brain barrier.

Background

Neural Degeneration

Pilocarpine-induced SE in rats results in diffuse brain damage, including the substantia nigra, amygdala, hippocampus, septum, thalamus, olfactory and temporal cortex. Also, in epileptic patients who have died within a couple

of days after SE, massive neurodegeneration was evident in the cerebellum, hippocampus, and neocortex. Studies of seizure-related brain damage have developed, therefore, aimed at finding effective neuroprotective agents. AEDs, used as seizure-preventing drugs, have been widely tested for their potential neuroprotective properties.

The benzodiazepine, diazepam (which enhances GABAergic inhibitory function via the $GABA_A$ receptor complex), has been shown to provide essential protection against pilocarpine-induced brain damage in adult rats. However, benzodiazepines (diazepam, clonazepam) themselves produced diffuse apoptosis in infant rat brains. Similarly to benzodizepines, administration of phenobarbital to young rats resulted in neurodegenration which was apoptotic in nature. The barbiturate, phenobarbital (also mainly a GABA enhancer), proved effective against KA-induced neurodegeneration in the dentate gyrus but not in other hippocampal regions. Carbamazepine (a voltage-gated sodium channel blocker) was also only partially effective against SE-induced hippocampal lesions, in that it protected only CA_1 neurons but was ineffective in the remaining hippocampal regions. In a perinatal rat model of NMDA-induced neurodegeneration, carbamazepine conferred a partial (less than 40%) protection. However, no neuroprotection was offered by carbamazepine against 4-aminopyridine-induced hippocampal neuronal death. Phenytoin (another sodium channel blocker) was shown to afford some protective effects in rat cortical or hippocampal cultures exposed to glutamate, although it was without effect in cortical cultures exposed to NMDA. When added to neonatal mouse cerebellar cultures, phenytoin was clearly neurotoxic, this effect being concentration-dependent. In contrast, mature cerebellar cultures were resistant to phenytoin neurotoxicity. Phenytoin was also shown to be an apoptosis-inducer in vivo. Valproate (a sodium channel inhibitor, GABA enhancer, and probably T-type calcium channel blocker) protected cultured cerebellar neurons against neurotoxic effects of glutamate but was ineffective against 4-aminopyridine-induced hippocampal damage in vivo. However, valproate afforded potent neuroprotection to the hippocampus in a rat model of SE, induced by electrical stimulation of amygdala. Similarly to benzodiazepines, phenobarbital, and phenytoin, valproate was an apoptotic agent in young rats in vivo.

Inflammatory Reactions and Epileptogenesis

There is now considerable interest in the question of whether inflammatory reactions are in some way related to epileptogenesis and the progressive nature of epilepsy. The answer is probably 'yes,' as chronic inflammation in the brain contributes to neuronal death, seizure activity, and increased permeability of the blood-brain barrier – which in turn affect both, neuronal plasticity and neurogenesis.

Methods

Experimental seizures may be induced by means of physical stimuli (for example, electroconvulsions) or chemical stimuli (for example, bicuculline, KA, NMDA, pentylenetetrazol, pilocarpine, or picrotoxin – given systemically or intracerebrally). Electroshocks may be delivered via auricular electrodes or via electrodes implanted into the brain (amygdala, hippocampus). With brain electrodes, it is possible to induce SE by prolonged suprathreshold stimulations or kindled seizures, by subthreshold stimulations every 24–48 h. SE may be also produced by chemical convulsants – most frequently by KA and pilocarpine. It is noteworthy that SE results in massive neurodegeneration which may be modified by, for instance, AEDs; in this way, neuroprotection by various treatments may be evaluated. Following SE, after a latent period, spontaneous seizures may develop, enabling the investigators to study epileptogenesis.

Inflammation in the brain can be triggered by administration of bacterial lipopolysaccharide in order to mimic infection. The lipopolysaccharide is recognized by so-called TLR receptors, the activation of which stimulates antigen-presenting cells. This process eventually leads to the proliferation of microglia, lymphocytes, monocytes and neutrophils. Experimental brain inflammation can also be induced by viral infections (for example, measles virus- or mouse-hepatitis-virus-induced encephalitis).

Recent Results

Neuroprotection Provided by Newer AEDs

Some of the newer AEDs have now been studied for their neuroprotective potential. Gabapentin (binds to the $alpha_2\delta$ subunit of calcium channels) afforded a significant protection to hippocampal neurons in rats with KA-induced SE. Interestingly, the drug was injected 24 h *after* induction of SE and administered for one month. Lamotrigine (a sodium and calcium channel blocker) also protected hippocampal neurons in rats with KA-induced SE. Levetiracetam, which has a unique mechanism of action (binds to the vesicle synaptic protein, SV2A, and blocks N calcium currents), has been shown to provide neuroprotection in a model of SE (self-sustaining SE) in rats; in contrast, it has been found ineffective against pilocarpine-induced hippocampal damage. Pregabalin, (similarly to gabapentin, blocks the $alpha_2\delta$ subunit of voltage-dependent calcium channels), potently protects against lithium/pilocarpine-induced neurodegeneration. Interestingly, this neuroprotective effect was associated with the subsequent inhibition of spontaneous seizure activity. Tiagabine (an inhibitor of neuronal and

glial GABA uptake), in a model of rat SE induced by stimulation of perforant path, considerably reduced hippocampal cell loss, as well as impairment of spatial memory. In an in vitro study, tiagabine, however, was toxic to developing hippocampal neurons. Topiramate (an AED with multiple mechanisms of action – blockade of AMPA receptors, enhancement of GABA-mediated inhibition, blockade of sodium and L-calcium channels, enhancement of potassium currents) significantly and dose-dependently reduced the extent of hippocampal damage caused by SE in rats, evoked by unilateral hippocampal electrical stimulation. The drug was given to animals seizing for 140 min; at 40 and 80 mg/kg, the neuroprotective effect was evident for the whole hippocampus, while at 20 mg/kg, only neurons in the CA_3 region and hilus were protected. In a pilocarpine (380 mg/kg) model of SE (lasting for 40 min) in rats, topiramate was given at 20, 40, and 100 mg/kg and SE was stopped after 3 hrs with diazepam (4 mg/kg); topiramate was protective in a dose-dependent manner. At 100 mg/kg the protection was full in the whole hippocampus, and spontaneous seizure activity was reduced (suggesting an antiepileptogenic effect of topiramate). However, in another study using the lithium/pilocarpine model of SE in rats, while diazepam-topiramate treatment produced clear-cut neuroprotection in the hippocampus and ventral entorhinal cortex, the occurrence of spontaneous seizure activity was not modified. Vigabatrin (elevating GABA concentration in the synaptic cleft by irreversible inhibition of GABA transaminase) has been found neuroprotective in the perforant path stimulation model and pilocarpine model of SE, but not in the electro-stimulation of hippocampus. A summary of the neuroprotective and apoptotic potential of AEDs is shown in **Table 1**.

Table 1 Neuroprotective effects of conventional and newer AEDs and induction of apoptosis (studied in young animlas) by AEDs in vivo

AEDs	*Neuroprotection*	*Induction of apoptosis*
Benzodiazepines	+	+
Carbamazepine	+/−	NT
Phenobarbital	+/−	+
Phenytoin	−	+
Valproate	+/−	+
Gabapentin	+	NT
Lamotrigine	+	NT
Levetiracetam	+/−	NT
Pregabalin	+	NT
Tiagabine	+	NT
Topiramate	+	+[a]
Vigabatrin	+/−	+

[a]Effect observed at supramaximal doses.
+, neuroprotective effect; +/−, inconsistent data or partial protection; NT – not tested in vivo.

Inflammation, Seizure Activity, and AEDs

Recent experimental studies clearly indicate that seizure activity may induce inflammation in the brain, that seizure-accompanying neurodegeneration promotes inflammatory reactions, and that anti-inflammatory drugs may provide protection against seizure activity (as has also been shown in clinical studies of epilepsy). Increased concentration of pro-inflammatory markers has been found in the serum of children suffering from the West syndrome; since these markers are seen in both cryptogenic and symptomatic forms, it appears that elevated inetrleukin-2 is related to the epilepsy per se. Interestingly, anti-inflammatory drugs (e.g., ACTH and steroids) are therapeutically effective in this syndrome, and their positive effect lasts beyond their discontinuation. This effect speaks not only to their acute anticonvulsant effects, but also to the likelihood that they 'reset' some disturbed central mechanisms – possibly associated with inflammation. Interestingly, among conventional AEDs, carbamazepine and valproate share some anti-inflammatory properties; they reduce lipopolysaccharide-induced activation of nuclear factor κB and inhibit lipopolysaccharide-stimulated production of prostaglandins and nitric oxide. However, it is not clear to what extent these effects contribute to the anticonvulsant action of these AEDs.

Future Goals

Taking into consideration that even a single seizure episode may result in significant neuronal loss, the neuroprotective activity of AEDs (as seen in models of SE) offer clinically relevant potential. A substantial body of evidence indicates that several conventional and newer AEDs provide significant neuroprotection in models of SE in which diffuse brain damage is generally encountered (i.e., without any neuroprotective treatment). Interestingly, in mice with intracerebroventricular KA-induced lesions of the hippocampus, the protective action of benzodiazepines and barbiturates against electroconvulsions, when evaluated five days after the lesion, is markedly reduced. In this context, it would be of interest to find out whether the anticonvulsant activity of newer AEDs is reduced – using this experimental approach. Moreover, it would be crucial to find out if lesions to other than hippocampal brain areas may result in the impairment of the anticonvulsant action of some AEDs.

Since epileptogenesis may be associated with neurodegeneration, neuroprotective strategies may stop or retard the conversion of normal brain into epileptic brain. However, some experimental data do not support this assumption. For instance, dizocilpine (MK-801; un

uncompetitve NMDA receptor antagonist) was found to exert a potent neuroprotective effect in the hippocampus in rats surviving SE induced by KA, and yet the spontaneous seizures were not inhibited. This relationship between neuroprotection and epileptogenesis needs further study. Indeed, there are also studies indicating that neuroprotection may be associated with *increased* epileptogenesis, although cognitive functions may be improved. Generally, individual AEDs have been examined for their neuroprotective effects, as well as their effects on subsequent spontaneous seizures and cognitive performance. Considering that many diverse combinations of AEDs have been evaluated for their anticonvulsant activities, and that some of them exert potent synergistic effects with other drugs, it is possible that such combinations may provide qualitatively better neuroprotection, not only resulting in more beneficial cognitive functions but also in impaired epileptogenesis.

A possible use of AEDs as neuroprotective agents in other conditions than epilepsy (Alzheimer's disease, Parkinson's disease, Huntington's chorea) also needs experimental and clinical evaluation. Further, the search for neuroprotective agents should expand beyond AEDs, and focus on agents that are known to inhibit the processes involved in neurodegeneration – for example, immunophilins (affecting transition pores of mitochondria and stabilizing ion channels), antioxidants, and caspase inhibitors. Initial results of such studies are encouraging, and further research on this issue may provide valuable neuroprotective agents. Studying the association between inflammatory processes and epileptogenesis may also open new therapeutic approaches to delay the progression of epilepsy.

Genetic background is also an important factor in regulating the neuronal susceptibility to neurodegenerative insults. For instance, KA has been shown to produce similar seizure activity in two different strains of mice, although clear-cut neurodegeneration was evident in one strain only. With recently emerging molecular techniques, it will be possible to conduct detailed studies on the relationship between the genetic background and neuroprotection or epileptogenesis.

Finally, a considerable number of AEDs (benzodiazepines, phenobarbital, phenytoin, valproate, and vigabatrin) may induce massive apoptosis in immature rodent brain upon administration at pharmacologically relevant doses (anticonvulsant levels used in young or adult animals). Topiramate has been documented to produce a comparable effect, but at supramaximal doses, several folds higher than its respective anticonvulsant ED_{50} in infant rodents. It is thus necessary to evaluate other newer AEDs in immature animals, and to find out whether there is clinical relevance of this hazardous action for treatment in pediatric patients.

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; Excitotoxicity and Neurodegenerative Disease; Molecular Mechanisms of Cell Death After Seizures; **Inflammation**: Cerebrovascular Diseases, Seizures and Epilepsy; Inflammatory Responses and Cytokines; Modulation of Neuronal Excitability: Vascular Changes and Inflammation; **Neurotrophic Factors**: Neuroprotective Strategies in Epilepsy.

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INTERNEURONS

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GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex
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Introduction

The most widely accepted dogma explaining hyperexcitability of cortical networks in epilepsy is the imbalance of glutamatergic excitation and GABAergic inhibition. This view is supported by well known observations on the proconvulsant effects of glutamate receptor agonists and $GABA_A$ receptor antagonists, and the anticonvulsant effects of glutamate receptor antagonists and $GABA_A$ receptor agonists. However, the precise mechanisms and sequences of events that ultimately result in reorganized cortical networks generating spontaneous recurrent seizures are still debated. The search for elucidation of these mechanisms leads to numerous questions for future research: Is the key (and initiating) change an enhanced excitation or a decreased inhibition, or do they both change together? Are these changes interrelated? – that is, does the potentiated excitation result from a loss of inhibitory control of glutamatergic synaptic plasticity, or is the loss of inhibition caused by potentiated excitatory input and excitotoxic death of interneurons? – Are these changes and causal relations constant throughout the various stages of epileptogenesis – that is, during the initiating trauma, the latent phase, and the chronic recurrent seizure phase – or do the changes differ profoundly across stages? If the inhibitory and/or excitatory networks are permanently reorganized, why are seizures sporadic, and separated by normal activity patterns? Do interictal events develop into full-blown seizures as a result of an impaired homeostasis of GABAergic and/or glutamatergic transmission, which will only surface when challenged?

Answers to at least some of these questions can be approached, if we better understand the organizational principles of local cortical microcircuits, particularly their inhibitory components. GABAergic inhibition in cortical networks is supplied by a wide range of interneuron types that subserve different functions, and possess specific

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connectivity features – morphological, electrophysiological, and neurochemical characteristics that enable them to fulfil these diverse roles. The major transmitter of these interneurons is invariably GABA, but several types may contain and corelease neuropeptides as well. In addition, interneurons considerably differ in their vulnerability to damage in epilepsy (also in ischemia), and therefore differentially contribute to epilepsy-related synaptic reorganization of cortical networks. Since vulnerability correlates with cell type and function, certain well-defined inhibitory operations are expected to be impared, others unchanged, and yet another aspect of inhibitory control may even be enhanced during epileptogenesis. In the following article, a functional classification scheme of interneuron types will be described together with the sensitivity/plasticity profile of these cell populations. In addition, the potential contribution of these different interneuron populations to epileptogenesis and seizure activity will be examined. However, it should be emphasized that none of the pathophysiological events associated with epileptogenesis can be explained by changes in inhibition alone. Abnormalities in glutamatergic transmission is crucially involved at every stage of the process, and these changes are described in other articles in this encyclopedia.

Background – Inhibitory Cell Types of the Hippocampus

Detailed reviews have been published by the author and several other investigators on this subject (see Further Reading), and therefore only an updated brief account is given here. The present classification scheme is based mostly on rodent data, but the correspondence to major interneuron types in humans is rather close, although there are variations particularly in the neurochemical characteristics. The majority of in vivo and in vitro electrophysiological features also derive from rodents, their correspondence to the behavior of human interneurons can only be estimated based on indirect lines of evidence.

For a functional classification of interneurons, the most important features are their input–output properties – i.e., identification of afferents driving the particular cell type, and the targets at which these cells exert their inhibitory effects. Postsynaptic targets of cortical interneurons in most cases are principal cells (pyramidal or dentate granule cells), and in a few special cases, other interneurons. This difference in targets represents the first dichotomy in the classification scheme, as there are subsets of interneurons containing the calcium binding protein calretinin (CR) or the neuropeptide vasoactive intestinal polypeptide (VIP), that were shown to form synapses mostly on other GABAergic cells (particularly those inhibitory neurons that innervate pyramidal cell dendrites); these interneurons were therefore termed interneuron-selective interneurons (or IS cells). These IS cells occur in all subfields and layers of the hippocampus, often form dendrodendritic contacts with each other, and are ideally suited to synchronize inhibition (arriving to pyramidal cell dendrites) throughout the hippocampus. IS cells represent only a small proportion of interneurons, as the vast majority selectively innervates different compartments of principal cells.

Selection of the principal cell compartment results in the next major branching in the classification scheme. Inhibitory input to pyramidal cell dendrites is functionally distinct from that to the perisomatic region; the latter domain is responsible for the summation of postsynaptic potentials arriving from all dendritic branches, and determines whether cell depolarization will reach threshold for action potential generation. The perisomatic region of pyramidal cells, in particular the axon initial segment which has the highest concentration of voltage-gated sodium channels, plays a crucial role in the generation of the cell's output. Thus, interneurons (the perisomatic inhibitory cells) innervating somata, proximal dendrites or axon initial segments are specialized to control output from large principal cell populations. Their likely function is to synchronize principal cell activity rhythmically at theta (4–8 Hz) and gamma (30–100 Hz) frequencies. In contrast, the dendritic tree is the site of termination of most (if not all) excitatory (largely glutamatergic) afferents onto principal cells, as well as the site of short- or long-term plastic changes in synaptic efficacy resulting from various dendritic active conductances, calcium spikes and backpropagating action potentials. Thus, interneurons (the dendritic inhibitory cells) innervating principal cell dendrites are designed to control the efficacy, and most notably the plasticity, of excitatory inputs to principal cells. Finally, there are a number of GABAergic cell types in the hippocampus that, in addition to their local axon collaterals, send major projections outside their own subfield, or even outside the hippocampus (e.g., to the medial septum), and are therefore termed long-range interneurons.

Perisomatic Inhibitory Cells

The precise site of their termination, as well as their specific input characteristics, makes it possible to dissect this cell group into three major types. The axo-axonic, or chandelier cells innervate exclusively the axon initial segments of principal cells. Each axo-axonic cell forms radially running rows of boutons that climb along the axon initial segments of 1000–1500 pyramidal cells. Due to the strategic location of their axon terminals, axo-axonic cells were previously thought to simultaneously inhibit output from large principal cell populations. However, recent evidence suggests that, via a depolarizing GABA effect,

these interneurons may be able to discharge the entire pyramidal cell population they innervate, thus synchronizing the pyramidal cell output and/or resetting conductances in their dendritic trees. Axo-axonic cell bodies are located within or near the pyramidal cell layer, and have radially running dendritic trees that span all layers (although a few exceptions have dendrites confined to stratum oriens). They can be recruited by both feed-forward and feed-back inputs, and fire characteristic bursts at the initiation of hippocampal sharp wave-associated ripple oscillations (synchronous discharges of CA3 pyramidal cells). Their activity is phase-coupled to the positive peak of the theta waves recorded extracellularly in stratum pyramidale.

The remaining two types of perisomatic inhibitory cells are basket cells that form multiple synaptic contacts on the somata and proximal dendrites of principal cells, each basket cell targeting 1500–2000 principal cells. The two types can be distinguished most easily on the basis of their neuropeptide/calcium binding protein content. This neurochemical difference is coupled to functionally meaningful unique features related to synaptic inputs and receptor expression patterns. The parvalbumin (PV)-containing basket cell bodies are located within or near stratum pyramidale, and have radially running dendrites that span all layers. They are driven mostly, and very efficiently, by local principal cells in a feed-forward manner, and form synapses on pyramidal cells via α1 subunit-containing $GABA_A$ receptors. The cholecystokinin (CCK)-containing basket cell bodies can be located in all layers, but typically in stratum radiatum, and have radially running dendritic trees spanning all layers. In addition to CCK, one subset of these interneurons may also contain VIP, and another subset the vesicular glutamate transporter vGluT3. CCK basket cells generally receive far fewer local glutamatergic inputs than PV cells, but express $5HT_3$ receptors and are selectively innervated by serotonergic afferents from the median raphe nucleus. They also express nicotinic α7 and α4 receptor subunits, as well as presynaptic cannabinoid (CB_1), $GABA_B$ and estrogen1 receptors, and form synapses on pyramidal cell bodies and dendrites largely via α2 subunit-containing $GABA_A$ receptors. In contrast to PV basket cells, feed-forward input alone can hardly activate them. However, due to their large membrane time constants, they are uniquely capable of summating feed-forward and feed-back drives over long time windows. Thus, they appear to come into play, when external excitatory inputs are strong enough to discharge local principal cells, leading to a summation of local and external excitation on CCK basket cells. During theta activity, both basket cell types fire at the positive peak of extracellularly recorded field EEG waves (out of phase with pyramidal cells, which fire at the trough), with PV cell firing shifted slightly towards the descending, and CCK cell firing towards the ascending phase. While PV cells strongly increase their firing during sharp waves, CCK cell discharges remain unchanged. This difference is consistent with the fact that ripples in CA1 are driven by CA3 in a feed-forward manner, which is insufficient to recruit CCK cells, as they can be activated only by a combination of feed-back and feed-forward drives. Recent data indicate that CCK massively enhances the firing of PV basket cells, and reduces IPSCs derived from CCK basket terminals via triggering the release of endocannabinoids as retrograde signaling molecules from postsynaptic pyramidal cells. Thus, CCK – via acting on different CCK receptors expressed by PV basket and pyramidal cells – can change the relative weight of inhibition derived from the two basket cell types.

Although both basket cell types are primarily responsible for rhythmic synchronization of the activity of large principal cell populations at theta and gamma frequencies, there is a considerable division of labour between them associated with their differential behaviour during sharp waves, and with different excitatory drives. A synthesis of the above features led to our proposal that the electrically- and synaptically-coupled, and mostly locally driven, ensembles of PV-containing basket cells are indispensable components of the oscillating cortical hardware; they represent a precision clockwork without which no cortical operations are possible. The activity of a similar syncytium of CCK-containing basket cells is superimposed on the PV basket cell-entrained network, conveying emotional and motivational effects carried by serotonergic and cholinergic pathways. In addition, actions of the CCK cell ensemble are highly modifiable by local neuromodulators and retrograde signaling molecules, which may allow further fine tuning of principal cell cooperation. Impairment of this tuning system is likely to result in mood disorders such as anxiety. Interestingly, most if not all of the inputs and receptor expression patterns that distinguish CCK cells from PV cells are strongly implicated in anxiety. For more details about this hypothesis, see Further Reading.

Dendritic Inhibitory Cells

This group of interneurons is the most diverse, both morphologically and functionally. The functionally relevant classification – based on afferent and efferent connectivity – is easy to determine in the hippocampus, where afferent pathways and cellular compartments are confined to different laminae. Distinct types of dendritic interneurons have evolved to control glutamatergic inputs to principal cells from different sources. The so called oriens–lacunosum-moleculare (O-LM) interneurons selectively terminate in stratum lacunosum-moleculare in conjunction with exitatory input coming from the entorhinal cortex. They have a cell body with

horizontally oriented dendritic tree confined to the stratum oriens in the CA1 subfield; in the CA3 subfield, the dendritic tree reaches all layers but stratum lacunosum-moleculare. In the dentate gyrus, these cells have been named HIPP (hilar perforant path associated) cells, as their somata and dendrites are confined to the hilus, and the axon extensively arborizes in the outer two-thirds of the molecular layer, in perfect overlap with the entorhinal input to granule cells. These HIPP cells are driven exclusively in a feed-back manner by recurrent collaterals of local principal cells. Their distinctive neurochemical marker is somatostatin (SOM), but some of them also contain neuropeptide Y (NPY) or low concentrations of PV only in the cell body. Their axon arbour is rather focused in the transverse plane but elongated in the longitudinal direction, covering an area of about 400 by 800 μm. During theta activity, O-LM cells fire together with the pyramidal cells at the trough of extracellularly recorded field EEG waves, and are silenced during sharp wave-associated ripples. Their function is likely to be the blockade of synaptic plasticity of entorhinal inputs in time windows, when the largest number of local pyramidal cells discharge – i.e., during the trough of extracellularly recorded theta – as it is in this phase when local pyramidal cell activity is sufficient to provide feed-back activation of O-LM cells. However, in time windows, when only some pyramidal cells fire in phase precession mode (i.e., ahead of the population – which happens when they are conveying specific signals such as place field-dependent activity), their chance of activating O-LM cells is much lower; thus, they will escape feed-back inhibition and their entorhinal input synapses can be potentiated.

The other major excitatory pathway innervating hippocampal principal cells derives from pyramidal cells of the CA3 subfield, which give rise to Schaffer collaterals that terminate in strata radiatum and oriens. The interneurons that specialize to control this input are called Bistratified cells. This cell type has an axon that arborizes in perfect overlap with Schaffer collaterals – i.e., in strata radiatum and oriens, skipping stratum pyramidale – and form synaptic contacts with second or third order thin dendrites of pyramidal cells, in the vicinity of spines that receive the excitatory input. The axon of a subtype of Bistratified cells is confined to stratum radiatum only, and there seems to be a continuum between this subtype and the classical Bistratified arbors, with a varying degree of a stratum oriens component. The Bistratified cell bodies can be found in all layers, and possess radially oriented dendritic trees. The molecular characteristics of this cell type typically include expression of calbindin (CB), and occasionally CCK, PV and SOM.

There is also a CCK-containing interneuron subset that has considerable axon collateralization entering str. lacunosum-moleculare, and is therefore not considered as a classical Bistratified cell. It is probably closer to the type of CCK-containing cells that selectively innervates str. lacunosum-moleculare, and is called LM-perforant path associated (LM-PP) cell. The dendritic tree of both Bistratified and LM-PP interneurons arborizes most extensively in str. radiatum or lacunosum-moleculare, which explains their predominantly feed-forward drive.

Bistratified cells fire in phase with O-LM and pyramidal cells during theta activity (i.e., at the trough of the extracellularly recorded EEG waves), whereas they are strongly excited during sharp wave-associated ripples, consistent with a strong feed-forward input from Schaffer collaterals. Their likely roles include feed-forward gain control of the efficacy of Schaffer collateral synapses, as well as the regulation of synaptic plasticity via inhibiting active conductances, calcium spikes and backpropagating action potentials in the dendrites.

The recently discovered Ivy cells also belong to the group of dendritic inhibitory interneurons. They were named after their dense and fine axonal meshwork that targets predominantly the basal and apical tufts of the pyramidal cell dendritic tree. Cells of this type are suggested to be more numerous than the parvalbumin-expressing neurons, and are characterized by the expression of nitric oxide synthase together with neuropeptide Y. They fire rather slowly, but phase-locked to network patterns such as theta or gamma oscillations. The slow rise and decay of the IPSCs they produce in their target pyramidal cells and the similarity to the neocortical neurogliaform cells suggest that their IPSCs may be mediated, at least in part, by $GABA_B$ receptors.

Individual dendritic inhibitory cells of any type provide 2–20 synapses onto a single target pyramidal cell, which are scattered all over the dendritic tree. Thus, the contribution of a single dendritic inhibitory cell to the control of dendritic functions of principal cells is likely to be negligible. They must fire in synchrony. The interneuron-selective interneurons (the IS cells described above) are designed to synchronize the dendritic inhibitory cells, thereby enabling them to exert a powerful effect on dendritic electrogenesis and associated synaptic plasticity.

Long-Range Interneurons

Commissurally projecting GAD/GABA-positive neurons have been described in the hilus and stratum granulosum of the dentate gyrus, as well as in the rat somatosensory cortex decades ago. With the advent of more sensitive tracing techniques several GABAergic projections have been revealed in the hippocampal formation, neocortex and between the two regions, challenging the traditional view of cortical inhibition in which all trans-areal inhibition was thought to be disynaptic. A reciprocal long-range GABAergic projection has been described between the medial septum and the hippocampus. The hippocampal

sources of this projection are the so called hippocampo-septal (HS) cells, which are the best characterized of all long-range GABAergic neurons. They express SOM and CB, and have a cell body in stratum oriens of CA1–3, in str. lucidum and radiatum of CA3, and in the hilus of the dentate gyrus. HS cells have horizontal dendrites that remain in str. oriens in CA1, and are restricted to the dentate hilus, which is consistent with a predominantly feed-back drive. The dendrites are extremely long, suggesting that the neuron is sampling input from a large population of local principal cells, apparently monitoring the level of global population synchrony. This information is then fed back to the medial septum, where their targets are the GABAergic pacemaker cells that project back to the hippocampus to selectively innervate all interneuron types. The local axon collaterals of HS cells span very large distances; they can cross the entire hippocampus from the rostral to the temporal pole, and one subset appears to innervate predominantly other interneurons (including other HS cells). Thus, these HS cells are ideally suited to synchronize inhibition, and thereby the entire principal cell population, along the hippocampo-septo–hippocampal loop that is crucial for the generation of theta activity. There are HS cells that innervate mostly principal cell dendrites, and rarely interneurons; thus, they are functionally different from the interneuron-selective HS cells. There are reports of so-called 'back-projection' interneurons, which innervate all hippocampal subfields, form synapses primarily with principal cells, and are very similar to the HS cells. They may in fact also be HS cells, but with their septally projecting axon not labelled (due to technical accident). Connectivity features of the trilaminar cells are also remarkably similar to HS (or backprojection) cells with no (or limited) preference for interneuron targets; however, trilaminar cells are without extrahippocampal or distant projections. Thus, they too may reflect incompletely filled HS cells. Another variation of HS cells may include a population that, besides participating in the septal pathway, also projects to the subiculum and other cortical areas.

Methods

Temporal lobe samples, surgically removed from epileptic patients, have been examined by light and electron microscopy following immunostaining for several different antigens. Patients with intractable temporal lobe epilepsy were operated on in the National Institute of Neurosurgery, Budapest, within the framework of the Hungarian Epilepsy Surgery Program. The anterior third of the temporal lobe was removed together with the temporomedial structures. Control autopsy brain samples were obtained from six individuals (3 women, 3 men; ages, 37, 47, 48, 51, 53, and 56) who died from nonepilepsy related causes; none of the control subjects had a record of any neurological disorders. Brains were removed 2–4 h after death. Autopsies were performed in the Department of Forensic Medicine of the Semmelweis University Medical School, Budapest; harvesting of tissues was approved by the local ethics committee, and informed consent was obtained from the next of kin.

After surgical removal, the epileptic tissue was immediately dissected into 3–4 mm thick blocks, and immersed into a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 g mol^{-1} phosphate buffer (PB, pH = 7,4). Two of the control brains were removed from the skull 2 h after death, both internal carotid and vertebral arteries were cannulated, and the brains were perfused first with physiological saline (2 l in 30 min) followed by a fixative solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 g mol^{-1} PB (6 l in 1.5 h). The hippocampus was removed after perfusion and cut into 3–4 mm thick blocks, which were postfixed in the same fixative solution overnight.

Vibratome sections (60 μm thick) were cut from the blocks, and processed for immunostaining as follows: They were transferred to TRIS buffered saline (TBS, pH 7.4), then endogenous peroxidase activity was blocked by 1% H_2O_2 in TBS for 10 min. TBS was used for all the washes (3 × 10 min between each antiserum) and for dilution of the antisera. Nonspecific immunostaining was blocked by 5% milk powder and 2% bovine serum albumin. The sections were incubated in the primary antisera, usually for two days, at 4 °C. For the visualization of immunopositive elements, biotinylated anti-rabbit IgG (1:300, Vector) was applied as secondary serum followed by avidin-biotinylated horseradish peroxidase complex (ABC, 1:300, Vector). The immunoperoxidase reaction was developed by 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) as a chromogen dissolved in TRIS buffer (TB, pH = 7.6). Sections were then treated with 1% OsO_4 in PB for 40 min, dehydrated in ethanol (1% uranyl acetate was added at the 70% ethanol stage for 40 min.) and mounted in Durcupan (ACM, Fluka). Pre-embedding immunogold reaction was carried out in the same way, but after 48 h incubation in the primary antiserum, sections were washed extensively and blocked in 0.8% BSA, 0.1% IGGS gelatin (Amersham Life Science) and 5% normal goat serum in TBS for 30 min. This step was followed by incubation with 1 nm gold-labeled goat anti-rabbit IgG (Amersham Life Science) diluted 1:50 in 0.8% BSA, 0.1% IGGS gelatin and 1% normal goat serum in TBS for 6 h. After incubation, sections were washed and postfixed with 1% glutaraldehyde in TBS for 10 min. The 1 nm gold particles were silver-enhanced by IntenSE™ M (Amersham Life Science) for 5–10 min.

After light microscopic examination, areas of interest were reembedded and sectioned for electron microscopy. Ultrathin serial sections were collected on Formvar-coated

single slot grids, stained with lead citrate, and examined with a Hitachi 7100 electron microscope.

In a previous study, we examined 12 control brains – with different post mortem delays, different ages, of both genders – for several antigens. The progression of age influences the quality and quantity of immunostaining; i.e., most of the antisera showed weaker staining and lower number of cells in aged subjects. Therefore, subjects older than 60 years have been excluded from the detailed analysis in our current studies. For electron microscopic studies, subjects were chosen with a post mortem delay of less than 4 h, because longer post mortem delays (6–24 h) resulted in poor ultrastructural preservation, and a decrease in immunoreactivity. The electron microscopic analysis of these tissues revealed acceptable ultrastructural preservation even in the immersion-fixed controls, although preservation was inferior to the perfused tissue.

Mice and rats (control animals or epileptic animals at various times after status epilepticus or spontaneous seizures, induced by kainate or pilocarpine) have been perfused with the same fixatives as the human samples, and the brains were processed in the same way for light and electron microscopic immunohistochemistry. For details see Further Reading.

Results – Differential Vulnerability of Interneurons

The efficacy of GABAergic inhibition in cortical networks can be modified in various ways. Proconvulsive changes may affect GABA receptor numbers or their subunit composition. Alternatively, changes in GABAergic inhibition can be associated with changes in intrinsic features of interneurons which influence their firing properties, release probability, presynaptic control machineries, the direction of chloride gradient through the $GABA_A$-chloride channel, etc. The focus of this article is on the most widely investigated proconvulsive alteration in cortical networks: the damage of interneuron types (described above) and plastic changes in their afferent or efferent connectivity. Numerous studies reported a decrease in spontaneous or evoked inhibitory events, and/or a decline in the underlying molecular and anatomical substrate, associated with seizure activity. The latter changes include a decrease in the number of neurons and/or axon terminals labelled with markers of GABAergic transmission (e.g., GABA itself, or its synthesizing enzyme, glutamic acid decarboxylase (GAD), GABA transporters, coexpressed neuropeptides, calcium binding proteins), and/or a change in the level of $GABA_A$ receptor binding, immunoreactivity, or subunit composition. All these changes have been seen in identified epileptic foci, both in animal models and human patients. However, other reports – using different experimental models, markers, or paradigms – have not found any preferential loss of GABAergic neurons, and some human studies support their preservation in both the sclerotic and nonsclerotic hippocampus. Indeed, some studies even report hypertrophy or sprouting of certain interneuron types, which may be induced by excessive release of neurotrophins by principal cells following seizures. Moreover, evidence has emerged that supports the view that the hypersynchrony required for the propagation of seizure activity depends on highly efficient GABAergic inhibition. Many of these controversial findings can only be resolved by a differential investigation of the funcionally distinct interneuron types in epileptic brains. A synthesis of data from such studies will be presented in the following sections. Most of the studies reviewed here rely on immunocytochemical localization of neurochemical markers selectively expressed by different cell types; in this way the entire population is simultaneously visualized. In contrast, single cell labeling data are difficult to obtain (especially in human epileptic tissue), and it is hardly possible to generalize such data to the entire population due to the generally very small sample size. However, the immunostaining approach also has its major disadvantages. For example, it is difficult to establish whether a loss of immunostaining is due to the actual loss of the particular cell type that used to express this marker, or simply the expression of this marker has diminished below detection threshold (while the cell is still functioning normally). Wherever animal data are available, the changes in interneuron types will be described separately for the three phases of epileptogenesis: the acute initiation phase, the latent period, and the chronic phase with spontaneous recurrent seizures. In human surgical samples, all available data obviously reflect the chronic stage – and only of medically intractable cases, as the other cases don't proceed to surgery. Nevertheless, even in this limited sample of human epileptic tissue, we can distinguish between the non-sclerotic and sclerotic cases.

Perisomatic Inhibition

The most widely used marker for this cell group is PV expressed by both axo-axonic and basket cells, as well as CCK that is present in another subset of basket cells. Most of the reports, in both human tissue and animal models, are consistent with the survival of perisomatic interneurons, and the preservation of IPSCs arriving at the somata of principal cells in all hippocampal subfields. Only a transient downregulation of perisomatic IPSCs occurs during the latent phase, which recovers by the chronic stage, as perisomatic interneurons were shown to increase their exitability and firing. This plasticity may partly be explained by the reported increase in excitatory inputs to PV-containing dendrites in the dentate gyrus, mostly

as a result of mossy fiber sprouting. A decrease in PV-immunoreactive cell numbers has been repeatedly reported in humans and in all phases of epilepsy in animal models, but it is likely to be due to a loss of PV from surviving interneurons, as suggested by the electron microscopic demonstration of the normal synaptic coverage of the soma membrane and significant increase in the synaptic coverage of axon initial segments of dentate granule cells in human samples. These data suggest that chandelier cell axons may even sprout (in spite of loosing their PV content).

Basket and chandelier cells remain intact also in the CA1 region whenever/wherever their target pyramidal cells survive. In the human nonsclerotic hippocampus, there is no change in the numbers of these interneurons, but in the sclerotic CA1 there is a profound decrease both in cell and terminal numbers – in parallel with the disappearance of pyramidal cells. There is evidence for the formation of highly complex chandelier cartridges around the axon initial segments, and unusually dense basket-like arrays of boutons around the somata of neurons at the border of extensively damaged areas. In the pilocarpine model, there seems to be a small reduction in axon initial segment synapses on CA1 pyramidal cells in the chronic phase of epileptogenesis.

The question arises whether the frequent enhancement of perisomatic inhibition in the chronic phase – due to hyperactivity of basket cells and/or sprouting of axon initial segment synapses – is: (a) a compensatory change with a neuroprotective role, or (b) has a proconvulsant effect by generating abnormal degrees of population synchrony. The former interpretation is supported by recent data on the voltage-gated sodium channel, $Na_v1.1$, in the cortex; a loss-of-function mutation of the gene for this channel is known to lead to a disorder called severe myoclonic epilepsy in infancy (SMEI). This channel is clustered predominantly on the axon initial segments of PV-containing cells. A mouse knock-in model with non-functional $Na_v1.1$ shows an epileptic phenotype, and action potential amplitudes of fast-firing PV cells exhibit a strong decrement in bursts. Thus, impaired firing of PV-positive interneurons seems to be causally linked to a form of epilepsy, suggesting that enhancement of perisomatic inhibition found in animal models or in human TLE is likely to be a compensatory neuroprotective change, rather than a primary cause of hyperactivity or hypersynchrony.

CCK-containing interneurons are also resistant in epileptic tissue, with only a transient downregulation of CCK observed in some interneurons during the initiation phase of epileptogenesis in animal models. A seizure-induced loss of CCK cells has been observed in the piriform cortex. However, CCK cells are functionally heterogenous in cortex (e.g., only 60% express CB1 receptors, and even fewer can be considered basket cells), and the identity of the sensitive cortical CCK cell type has not yet been determined. In hippocampus, CCK cells were found to be damaged only in models with extensive, nonselective cell loss due to intense seizure load (e.g., following over 90 seizures in a kindling model). CCK-positive axons may even sprout, as suggested by the increased density of the CCK-immunoreactive fiber meshwork in the dentate inner molecular layer, as well as in the pyramidal cell layer of rodents. However, as there is no significant enhancement in perisomatic synapse density either in human or in rodent hippocampi, this observation is unlikely to be a reflection of real sprouting, but is rather an increase of CCK content above detection threshold in a larger number of boutons.

Dendritic Inhibition

The least debated observation both in anatomical and physiologcal studies is that dendritic inhibition is impaired in epileptogenesis, from the initiation phase through to the chronic phase. In animal models, SOM and/or NPY-containing neurons were found to be damaged 6 h after initiation (e.g., by status epilepticus); the damage persisted throughout the latent period, and was maintained at an even more decreased level during the phase with spontanous recurrent seizures. The loss of SOM cells accounted for over 80% of GABAergic cell loss in the hilus, and for about half of that in stratum oriens of CA1–3. The number of SOM cells is profoundly decreased also in the human epileptic dentate hilus, both in nonsclerotic and sclerotic cases, the latter being more severe and accompanied by a nearly complete damage of all hilar cell types. Similar to the case of PV-containing cells, it is important to ask whether the SOM cells indeed die or only lose their SOM content. As the damaged SOM cells are positive for the TUNEL stain (visualizing fragmented DNA), for active caspase-3 (the apoptotic enzyme), as well as for the degeneration marker Fluoro-Jade B at 6 h after the insult, and as a marked decrease in total cell number is seen in the hilus of the dentate gyrus, we can conclude that the majority of cells of this type indeed disappear from the hilus. A similar conclusion can be drawn about SOM cells in stratum oriens of CA1–3, although on a smaller scale. There is some evidence for a possible SOM and/or NPY axon sprouting, which may be just an enhanced peptide expression or staining intensitiy of the axons of remaining SOM/NPY cells. Even if there is real sprouting, these fibers are unlikely to be able to compensate for the loss of the majority of cells of this type. GABAergic synapses are decreased by about one third in the termination zones of SOM cells (i.e., in stratum lacunosum-moleculare of CA1 and in the outer two thirds of the dentate molecular layer), which again confirms the loss of considerable

numbers of this type of dendritic inhibitory cells, and the lack of compensation by sprouting.

Electrophysiological experiments in rodent models lead to the same conclusions: Recordings of spontaneous and miniature IPSCs from pyramidal cell dendrites in CA1 stratum radiatum revealed that there was a significant reduction in the frequency of distal dendritic IPSCs, and demonstrated that the likely explanation was a loss of the presynaptic interneurons. A similar conclusion has been drawn from whole-cell recordings from dentate granule cells, in which the typical dendritic IPSCs with slow rise times were selectively reduced one day after status epilepticus. These acute changes are maintained throughout the latent period, and the frequency of spontaneous dendritic IPSCs tends to decrease even further during the chronic phase.

The reason for this exceptional vulnerability of SOM cells is still unclear. The uniquely homogenous excitatory input could be one reason. These cells are exclusively driven in a feed-back manner – i.e., they receive glutamatergic synapses only from recurrent collaterals of local principal cells. In the dentate hilus, this pattern means extensive input from zinc-containing mossy fibers largely onto kainate receptors which the SOM cells share with all other hilar cell types. And indeed, all other hilar cell types, whether glutamatergic (mossy cells) or GABAergic, are highly vulnerable both in epilepsy and ischemia. An alternative explanation may be the presence of a unique molecular machinery in SOM cells. In particular, they express large amounts of a potent negative regulator of the neuroprotective ERK/MAPK pathway (the striatal enriched protein tyrosine phosphatase, STEP). The inhibition of STEP is able to rescue hilar SOM cells from excitotoxic death.

The transcription factor Dlx1 is required for the development and adult viability of SOM- and calretinin-containing interneurons; hence, these cells undergo apoptotic death from early adulthood in the Dlx1 knockouts. Importantly, behavioral and electrographic seizures begin only 3–4 weeks after the loss of these interneurons, providing evidence that the loss of SOM cells is the cause, and not the consequence, of epileptic activity. In epilepsy models, dendritic interneuron damage happens only a few hours after the induction insult, and persists throughout the latent period without resulting in recurrent spontaneous seizures. This period can range from a few weeks (as in animal models involving status epilepticus, or in Dlx1 KOs), to a couple of years (as in epilepsies in humans following head trauma or transient ischemia). The only abnormality observed during this latent phase is the occurrence of interictal spikes at various frequencies, as observed in the pilocarpine model. Thus, the loss of dendritic inhibition alone does not cause spontaneous seizures, but – in conjunction with the interictal spikes – provides the necessary conditions for abnormal degrees of plastic changes in the excitatory network. Impaired feed-back inhibition in the distal dendrites facilitates dendritic electrogenesis and backpropagating spikes, and enhances the NMDA component of EPSCs. The most consistent loss of inhibition is from the termination zones of the entorhinal cortex, where the role of this inhibitory input is normally to regulate the efficacy and plasticity of these glutamatergic afferents. Therefore, the most likely gradual change of the hippocampal network that takes place during the latent period is the abnormal potentiation of entorhinal inputs either to the dentate gyrus, or directly to the CA1 subfield through the temporo-ammonic pathway, as convincingly demonstrated in animal models. In the dentate gyrus, this process is accompanied by sprouting of granule cell axons, providing excessive recurrent excitation, which further distorts the excitation/inhibition balance.

Much less is known about the functional consequences of changes in calbindin-containing cells, which represent, at least in rodents, another type of dendritic interneurons – those that innervate apical and basal dendrites of pyramidal cells in conjunction with the Schaffer collateral input (Bistratified, or stratum radiatum interneurons). CB-positive cells appear to be highly resistant in the epileptic human hippocampus, at least in regions where most of their targets survive (i.e., in the CA1–3 subfields of nonsclerotic cases), and in the dentate gyrus of both sclerotic and nonsclerotic cases. In many cases, these cells not only survive but even become hypertrophic, with a considerably enhanced soma size (suggesting the maintenance of a more extensive axon arbor), elongated dendrites, and the appearance of spines on their somata and dendrites. This alteration is more typical of the dentate CB cells, but it can be observed also in the sclerotic CA1. In Ammon's horn, their postsynaptic target selection also changes; they tend to innervate significantly more interneurons, including other CB cells, than in controls. Since these changes can be found both in sclerotic and nonsclerotic cases (more pronounced in the former), they likely precede pyramidal cell loss. CB cells are also largely preserved in animal models. Interneurons in stratum oriens that colocalize SOM and CB are known to correspond to the hippocampo-septal (HS) cells. They were found to be resistant, in contrast to cells in the same layer containing SOM alone (O-LM cells). However, a specific subset of HS cells, the spiny CR-containing neurons found exclusively in areas where mossy fibers arborize, are particularly vulnerable (see below).

Recordings from feed-forward driven interneurons in stratum radiatum, which likely correspond to CB-positive Bistratified or stratum radiatum interneurons, showed cell firing frequency to increase twofold. This increase may be due to potentiation or sprouting of their excitatory

inputs, or an impairment of their inhibitory input (e.g., from calretinin-positive cells). Evidence for the latter will be described in the following section.

Interneuron-Selective Interneurons

The routinely used marker of these cells is calretinin (CR), although in the human hippocampus CR-containing cells are heterogenous with regard to target selectivity. In addition to the types characteristic of rodents, there are also others that innervate predominantly pyramidal cell dendrites. In rodents, a subset of CR cells also contains VIP. However, as VIP is present also in some of the CCK-containing basket cells, this peptide is rarely used for the visualization of IS cells. IS cells have recently begun to gain more attention in physiology and pathophysiology due to their unique function in normal network activity patterns and their selective vulnerability in epilepsy and ischemia (**Figure 1**). They were found to show the first signs of exitotoxic damage a few hours after the initial insult, either status epilepticus or transient ischemia. CR-containing IS-like cells decrease in number in the epileptic human dentate gyrus, both in sclerotic and nonsclerotic cases, whereas the small, CR-positive Cajal–Retzius cells near the hippocampal fissure survive. A specific subset of CR-containing neurons – located in stratum lucidum of CA3 and the dentate hilus where mossy fibers arborize – colocalize SOM, and are particularly vulnerable both in ischemia and epilepsy. They receive extremely sparse GABAergic input, but are densely covered by long hair-like spines, each of which receives several synapses from mossy fiber filopodia. This profound imbalance in favour of excitatory input may be related to their exceptional vulnerablity. In addition to SOM cells, CR-containing neurons are also lost in epileptic Dlx1 knockout animals. The lost neurons may include the spiny CR/SOM cells in the hilus and CA3 stratum lucidum, which are unlikely to belong to the IS type. However, several CR cells in CA1, which do not colocalize SOM and are definitely of the IS type, also degenerate, which confirms the conclusion that damage to IS interneurons contributes to epileptogenesis.

One study reported an increase in CR cell numbers in the epileptic human hippocampus, but in this study the control tissue was from a long-delayed postmorten sample in which most of the CR cells remained unstained or were disintegrated. However, lobectomy tissue is always fixed immediately after surgical removal, so that CR immunoreactivity is preserved much better. This observation highlights the importance of using adequate control tissue that is not only age-matched, but also fixed in the same way at a similar postmortem delay.

As mentioned above, the major function of IS cells is likely to be the synchronization of dendritic inhibitory cells, which are individually unable to exert significant effects on dendritic electrogenesis or other influences on glutamatergic input plasticity of principal cells. However, when acting in concert, they have been shown to block, for example, dendritic calcium spikes efficiently. Taken together, one may speculate that in the epileptic hippocampus, where dendritic inhibitory cells are reduced in number, dendritic inhibition is further impaired by the loss of a synchronizing element, the CR-positive IS cells. Thus, even if axonal sprouting of surviving SOM and NPY cells compensates for the loss of GABAergic synapses terminating on pricipal cell dendrites, the efficacy of dendritic inhibition is unlikely to recover if the synchronizing mechanism is impaired.

Directions for Future Research

The most vulnerable GABAergic cell types in the hippocampus are those that inhibit principal cell dendries, and thereby control the potentiation of their glutamatergic inputs. The IS interneurons, likely responsible (at least in rodents) for the synchronization of dendritic inhibitory cells, are also damaged, which further impairs the control of glutamatergic synaptic plasticity. Damage of these interneuron types precedes, and is causally related to, the development of the chronic phase of epileptogenesis (with spontaneous recurrent seizures). The loss of these interneuron types alone manifests as interictal spiking, which cooperates with reduced dendritic inhibition to produce a reorganized circuit capable of generating ictal activity. This 'maturation' or latent phase can last from weeks to years, depending on the species and the type or severity of the induction insult. In contrast, perisomatic inhibitory cells are resistant to damage, and their occasional sprouting serves to compensate for the loss of dendritic inhibition. However, due to their specific termination zone and afferent inputs, they are often unable to succeed with this compensatory task, and may even contribute to hypersynchrony of principal cells that characterize interictal and ictal bursts.

A major challenge for future research is to further elucidate the correspondence of interneuron types and functions between the rodent and the human hippocampus, and to directly demonstrate a causal link between early interneuron loss and the abnormal potentiation of excitatory inputs in the latent phase of epileptogenesis. Insight into this relationship could provide the basis for the development of neuroprotective strategies for rescuing vulnerable interneurons in the human hippocampal formation, based on their unique molecular architecture and receptor expression pattern. It is important to emphasize that any future pharmacotherapy should take into account the functional and neurochemical heterogeneity, as well as the differential vulnerability, of GABAergic interneuron types. Such considerations could help achieve a site-specific alteration of inhibitory operations in microcircuits of the epileptic temporal lobe.

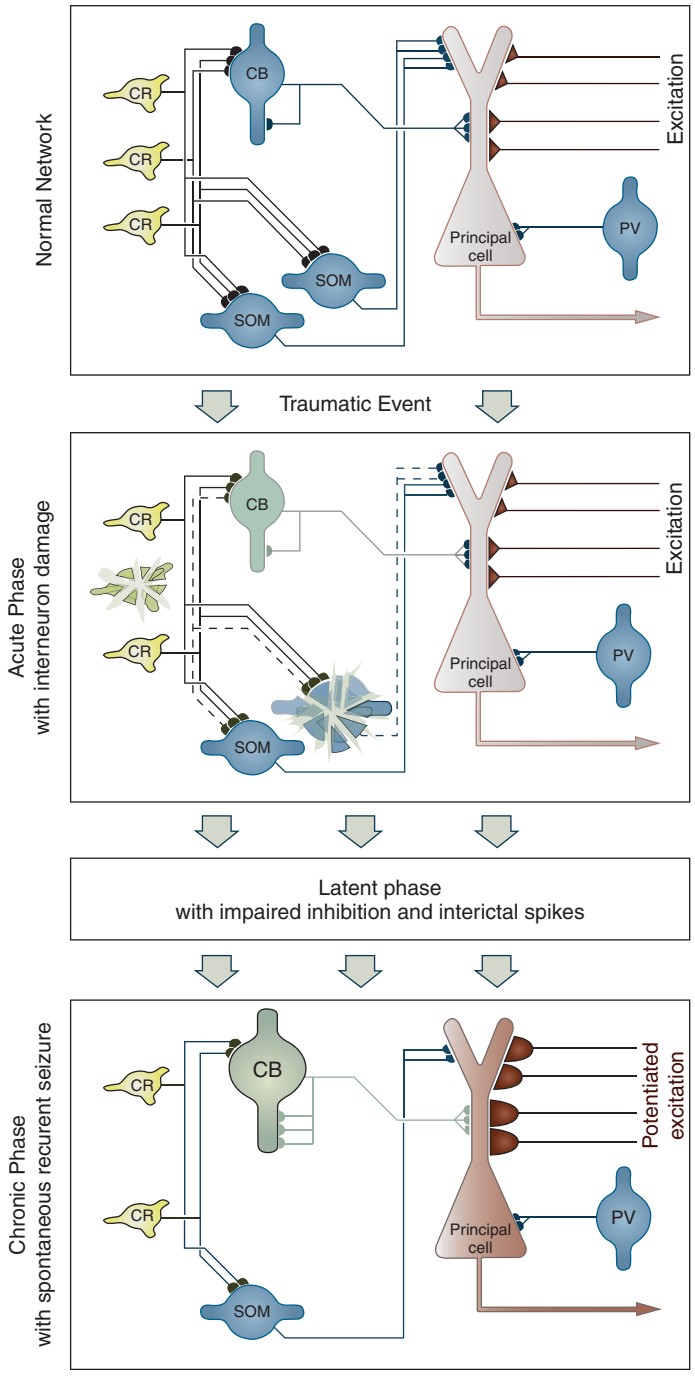


Figure 1 Schematic representation of changes in hippocampal interneuronal circuits in different phases of epileptogenesis. The top panel illustrates the Normal network. Principal cells, representing pyramidal cells of CA1–3 as well as dentate granule cells, receive excitatory input on their dendritic trees (distally from the entorhinal cortex, and more proximally from CA3 pyramids in the Ammons horn and from hilar mossy cells in the dentate gyrus). Their inhibitory input derives from two major sources, basket cells innervate the perisomatic region (only PV cells are represented here), and dendritic inhibitory cells terminate on different dendritic segments, the SOM cells in conjunction with the entorhinal input, and the CB cells in register with the Schaffer collateral input in CA1–3. The CR-containing interneuron-selective interneurons (IS cells) predominantly innervate dendritic inhibitory cells. A Traumatic event – which can range from a status epilepticus or perinatal ischemia to a tumor or acute head trauma – leads to the Acute phase in which, besides possible changes in other transmitter systems such as glutamate, GABAergic interneurons belonging to the SOM- and CR-containing populations are permanently damaged. These events are followed by the Latent phase, which can last from weeks to years. There are no spontaneous recurrent seizures in this phase, but impaired inhibition is accompanied by interictal spikes to generate the necessary conditions for an abnormal potentiation of glutamatergic excitatory circuits. This leads to the final stage of epileptogenesis, the Chronic phase with spontaneous recurrent seizures.

Acknowledgements

The author is grateful to Drs. Z. Maglóczky and N. Hájos for helpful comments on the manuscript, and to Dr. Gábor Nyíri for the artwork of figure 1. Our own studies that led up to this review were supported by the Howard Hughes Medical Institute, grants from FP6 of the EU (EPICURE LSH-CT-2006-037315), NIH (MH54671) and the Szentágothai Knowledge Center 05/2004.

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GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex

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Introduction

Temporal lobe epilepsy is the most common type of epilepsy in adults. It usually involves a preceding brain injury. Complex partial seizures that initiate in the mesial temporal lobe may secondarily generalize, and frequently cannot be controlled adequately by medication. Developing better treatments and preventative strategies depends critically on understanding pathophysiological mechanisms. In patients with temporal lobe epilepsy, neuron loss and tissue shrinkage has been reported for several forebrain regions – including amygdala, thalamus, neocortex, and entorhinal cortex – but the hippocampus is affected most consistently and severely. Tissue shrinkage is associated with neuron loss, and the most vulnerable neurons are in the hippocampal CA1 region and hilus of the dentate gyrus. Hilar neuron loss is the most consistent neuropathological abnormality in patients with temporal lobe epilepsy. Many patients also display preferential loss of layer III neurons in the medial entorhinal cortex. Similar patterns of neuron loss occur in laboratory animal models of temporal lobe epilepsy (**Figure 1**). How might these neuropathological changes contribute to seizure activity?

EEG recordings in patients and models of temporal lobe epilepsy reveal that seizures frequently appear to

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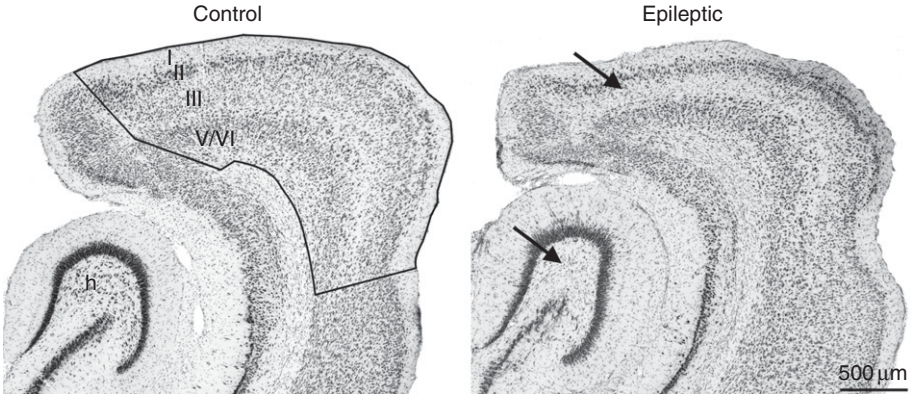


Figure 1 Neuron loss in the hilus and layer III medial entorhinal cortex in an epileptic rat. Nissl-stained 40-μm-thick horizontal sections from 103–112-day old, male Sprague-Dawley rats. The epileptic rat was treated systemically with pilocarpine to induce status epilepticus, observed to have spontaneous recurrent seizures in subsequent weeks, and perfused 79 days after pilocarpine treatment. The control rat was not treated with pilocarpine. The hilus (h) and entorhinal cortex (outlined) are indicated in the section from the control rat, and layers are labeled in the medial entorhinal cortex (I, II, III, and V/VI). Arrows indicate loss of neurons in the hilus and layer III of the medial entorhinal cortex in the epileptic rat.

begin focally or simultaneously in amygdala, hippocampus, dentate gyrus, and entorhinal cortex. Within this group of structures, the dentate gyrus and entorhinal cortex are particularly important. Normally, sensory cortical information enters and exits the hippocampal formation through the entorhinal cortex. The major excitatory synaptic input to the dentate gyrus comes via the perforant path from principal cells in layer II of the entorhinal cortex. Layer II cells of the medial and lateral entorhinal cortex project to the middle and outer thirds of the dentate molecular layer, respectively, where their axon terminals account for at least 85% of the excitatory synapses formed with granule cell dendritic spines. Output of layer II cells, therefore, profoundly affects activity of dentate granule cells.

Work from several laboratories (e.g., Jones, Heinemann, Lothman, and others) suggests the dentate gyrus normally functions like a 'gate' that restricts seizure activity in the entorhinal cortex from entering the hippocampus and then spreading to other brain structures. However, in epileptic tissue, granule cells and layer II cells are hyperexcitable. Single-cell recordings show that granule cells discharge excessively in response to synaptic activation in tissue from patients and models of temporal lobe epilepsy. Several laboratories (e.g., Lothman, Scharfman, Lopes da Silva, and others) have reported that in models of temporal lobe epilepsy, layer II cells in medial entorhinal cortex display prolonged excitatory responses to synaptic activation. We have found that layer II cells, in turn, generate excessive, spontaneous, hypersynchronous input to dentate granule cells. In TLE patients, stimulation of entorhinal cortex evokes responses in hippocampus that resemble interictal spikes. What causes this hyperexcitability of layer II EC cells and dentate granule cells? Intrinsic electrophysiological properties of granule cells and layer II EC cells in epileptic tissue appear relatively normal. Changes in excitatory synaptic input are likely to contribute to hyperexcitability of granule cells (mossy fiber sprouting), but not layer II ECs cells. Whether changes in inhibitory synaptic input contribute to hyperexcitability of granule cells and layer II cells is the focus of this article.

Background

Dentate Gyrus

Different approaches have been used to measure inhibition in the dentate gyrus of patients and models of temporal lobe epilepsy, and results have been mixed. Some studies, including our own, reported normal or enhanced granule cell inhibition. Other studies, including our own, found significant reductions. The mixed results are attributable, in part, to different methods used to evaluate inhibition. Field potential responses to orthodromic stimulation provide an index of activity of a relatively large population of granule cells, can be obtained readily in slices in vivo, and are used commonly. They can be obtained readily in slices and in vivo and are used commonly. However, the stimulation technique is of questionable physiological relevance. It is unlikely that perforant path fibers are ever normally activated so synchronously and intensely. Furthermore, if baseline inhibition is reduced, similar levels of stimulation are likely to activate more granule cells, which might recruit more feed-back inhibition, thereby masking underlying reductions in inhibition (i.e., polysynaptic amplification of inhibition). Intracellular current-clamp recording with sharp electrodes has been used to obtain recordings of evoked inhibitory postsynaptic potentials (IPSPs) that have fast and slow components that represent $GABA_A$- and $GABA_B$-receptor activation, respectively, permitting analysis of these different inhibitory mechanisms. Glutamate

receptor antagonists can isolate monosynaptic IPSPs and avoid confounding effects of excitatory PSPs and polysynaptic amplification. Again, however, the stimulation technique is not very physiological. Whole-cell voltage-clamp recording with patch electrodes offers the best signal-to-noise ratio for measuring spontaneous inhibitory postsynaptic currents (IPSCs), events that most accurately reflect normal functioning of $GABA_A$ synapses. A miniature IPSC, recorded in the presence of glutamate-receptor antagonists and tetrodotoxin, represents release of GABA at a single synapse, independent of action potential discharge. Frequency of miniature IPSCs serves as a functional measure of the number of inhibitory synapses impinging on a recorded cell and the probability of synaptic release. In normal bathing medium, the recorded spontaneous IPSCs reflect a combination of both miniature IPSCs and IPSCs that occur when presynaptic interneurons discharge action potentials. The whole-cell voltage-clamp approach is powerful; however, it too has disadvantages. The recording electrode has a large diameter tip, and the recorded cell is dialyzed, producing wash-out effects. Spontaneous $GABA_B$-receptor-mediated events are not evaluated, because they do not normally occur. And, three-quarters of the GABAergic synapses on granule cells are located on the dendrites, at distances where it is impossible to achieve complete voltage control with somatic voltage-clamp. Because of this space-clamp limitation, synaptic events generated in dendrites will be filtered and attenuated, and their apparent amplitudes and time-courses distorted. Each recording technique, therefore, has advantages and limitations for measuring functional inhibition of granule cells and other neurons, including layer II cells in entorhinal cortex.

Like the electrophysiological data, anatomical findings are mixed with respect to interneuron loss in the dentate gyrus of patients and models of temporal lobe epilepsy. Some studies report little, if any, loss of GABAergic interneurons, despite clear loss of excitatory cells in the hilus of the dentate gyrus. However, de Lanerolle's laboratory has found substantial loss of somatostatin-immunoreactive hilar neurons in patients with temporal lobe epilepsy. Somatostatin is expressed by a large fraction of GABAergic (but not glutamatergic) neurons in the dentate gyrus. Subsequent studies with human tissue by several laboratories (e.g., Mathern, Sloviter, Freund, and others) report significant loss of somatostatin-positive interneurons and other interneuron subtypes, including NPY-, parvalbumin-, substance P receptor-, and calretinin-expressing cells in the dentate gyrus. However, it is not always clear whether these interneurons have actually died or simply have become immunocytochemically undetectable. Surprisingly, despite apparent significant losses of specific interneuron subtypes (based on the above immunocytochemical results), numbers of neurons immunoreactive for glutamic acid decarboxylase (GAD), an enzyme that synthesizes GABA and is expressed by all GABAergic interneurons, have been reported to be similar to controls in human studies. The apparent discrepancy across studies may be attributable to limited sensitivity of GAD-immunocytochemistry and seizure-related changes in GAD expression. Houser's laboratory used in situ hybridization for GAD mRNA, a more sensitive method for detecting interneurons, and found significant loss of hilar GABAergic neurons in a model of temporal lobe epilepsy. Subsequent studies have confirmed loss of GAD-positive dentate cells in models of temporal lobe epilepsy. Some surviving interneurons in human epileptic tissue display changes in somatic, dendritic, and axonal morphology and, therefore, are likely to function abnormally.

Entorhinal Cortex

Compared to granule cells, inhibition of layer II cells of the medial entorhinal cortex in epileptic tissue has received less attention. There is little slice electrophysiological information about layer II cells in entorhinal cortex obtained from patients with temporal lobe epilepsy. The limited data from layer II cells in models of temporal lobe epilepsy include evidence for hyperexcitability but normal monosynaptic IPSPs. Previous anatomical studies of entorhinal cortex, including one of our own, reported no epilepsy-related reductions in interneuron profile density, despite clear reductions in layer III Nissl-stained profiles. However, methodological issues affect detectability of interneuron loss. Interneuron labeling methods vary in their degrees of sensitivity and specificity. Seizure activity affects expression of some interneuron markers, causing increased labeling in some cases (e.g., GAD and somatostatin) and transient marker drop-out in others (e.g., parvalbumin). Furthermore, studies vary in tissue sampling and cell counting schemes. Most studies measure neuron profile densities, and do not employ modern design-based stereological analyses, which can be more sensitive.

In sum, uncertainty about whether the neuropathology of temporal lobe epilepsy involves loss of GABAergic interneurons and reduced inhibition of dentate granule cells and layer II cells of medial entorhinal cortex motivated our experiments to evaluate interneurons and inhibition in those regions.

Methods

Animals

We make rats epileptic by treating them systemically with kainic acid or pilocarpine. Subsequent development of spontaneous, recurrent seizures is verified by behavioral monitoring. Control groups consist of naïve rats and pilocarpine-treated rats that do not develop status epilepticus or spontaneous seizures. Both control groups appear similar to one another.

Anatomy

We use in situ hybridization for GAD mRNA to label all interneurons. Interneuron subtypes are identified by specific immunocytochemical markers (e.g., parvalbumin and somatostatin). Stereological methods (optical fractionator) are used to estimate numbers of interneurons. Briefly, an entire structure (dentate gyrus or entorhinal cortex) is sectioned and sampled such that any part within has an equal probability of being sampled. The cumulative volume of sampled sites and total volume of the entire region of interest are calculated and used to extrapolate the number of counted cells to an estimate of the total number of cells per structure. Statistical tests evaluate adequacy of sampling.

Electrophysiology

Horizontal slices are prepared and recordings obtained at ~32 °C. Most recordings are obtained from visualized cells in whole-cell patch-clamp mode; some use sharp intracellular electrodes. Events analyzed include current-clamp responses to orthodromic stimulation, evoked IPSP/Cs, spontaneous and miniature IPSCs. In some experiments laser scanning photo-uncaging of glutamate is used.

Recent Results

Dentate Gyrus

The rat dentate gyrus normally contains approximately 1 million granule cells and 36 000 GABAergic interneurons: 50% in the hilus, 25% in the granule cell layer, and 25% in the molecular layer. Somatostatin-immunoreactive interneurons account for 25% of all GABAergic interneurons in the dentate gyrus, and parvalbumin-positive neurons account for 8%. Proportions of somatostatin- and parvalbumin-positive interneurons are similar in primate dentate gyrus. Like many patients with temporal lobe epilepsy, epileptic kainate-treated rats display obvious loss of Nissl-stained hilar neurons, while granule cells qualitatively appear relatively spared (**Figure 1**). There is significant loss of GAD-positive neurons in epileptic rats, which on average have only 87% of the number of GAD-positive neurons in dentate gyrus as controls. Somatostatin-immunoreactive neurons are highly vulnerable (50–63% of controls); parvalbumin-positive neurons are less so (78% of controls). GABAergic neuron loss is most severe in the temporal pole, which is homologous to the anterior hippocampus in humans – the region that displays the most severe neuron loss and is most typically surgically resected to treat some medically intractable cases. Other laboratories report similar anatomical results in models of temporal lobe epilepsy. Synaptic activation of granule cells evokes prolonged depolarizations and more action potentials in tissue from patients and models of temporal lobe epilepsy than cells in control tissue. Pharmacologically isolated, evoked IPSPs have smaller conductances (<40% of controls), and spontaneous and miniature IPSC frequencies are reduced (<50% of controls). Other laboratories have evaluated models of temporal lobe epilepsy and report similar deficits in granule cell inhibition.

Entorhinal Cortex

The rat medial entorhinal cortex normally contains approximately 460 000 neurons, of which 38 500 are GABAergic. Like many patients with temporal lobe epilepsy, epileptic pilocarpine-treated rats display obvious loss of Nissl-stained neurons in layer III of medial entorhinal cortex (**Figure 1**). Nissl-stained neuron loss is significant but less severe in other layers of medial entorhinal cortex. There is significant loss of GAD-positive neurons (75% of controls) in the medial entorhinal cortex (all layers included) of epileptic rats. Use of sensitive labeling (GAD in situ hybridization) and analysis techniques (optical fractionator method) may account for our detection of interneuron loss in medial entorhinal cortex, in contrast to previous studies. As reported previously by other laboratories, we confirmed that in epileptic tissue, synaptic activation of layer II cells evokes prolonged depolarizations and excessive action potential discharge. In layer II cells of medial entorhinal cortex in epileptic rats, frequencies of spontaneous and miniature IPSCs are reduced to 60% of controls. However, frequency and amplitude of spontaneous EPSCs in layer III GABAergic interneurons are not reduced in epileptic rats, suggesting normal excitatory synaptic input to surviving interneurons. To evaluate recurrent inhibitory circuits in layer II, we focally photostimulated interneurons (by release of caged glutamate) while recording inhibitory synaptic responses in layer II principal cells. IPSCs were evoked five times more frequently in slices from control versus epileptic animals, suggesting loss of interneurons and reduced recurrent inhibition.

Together, these findings suggest that in models of temporal lobe epilepsy, inhibition is reduced in dentate granule cells and layer II cells of medial entorhinal cortex. Previously proposed mechanisms of reduced inhibition include zinc-induced suppression of $GABA_A$-receptor function in dentate gyrus and dormancy of interneurons in dentate gyrus and entorhinal cortex due to loss of excitatory synaptic input. Other possible mechanisms include changes in GABA-receptor subunit expression and GABA uptake. These mechanisms may contribute to hyperexcitability in dentate gyrus and medial entorhinal cortex, but reductions in numbers of interneurons and their inhibitory synaptic input to granule cells and layer II

cells is the most parsimonious explanation consistent with our anatomical and electrophysiological findings. Because layer II EC provides the predominant excitatory input to granule cells, reduced inhibition of the closely linked neurons could lead to synergistic hyperexcitable effects. Furthermore, interneuron loss and reduced inhibition in other nearby regions, including CA1, suggests the loss of inhibitory interneurons might be a widespread and important pathophysiological feature of temporal lobe epilepsy.

Future Goals

There are many different types of inhibition (e.g., presynaptic vs. postsynaptic, $GABA_A$-mediated vs. $GABA_B$-mediated vs. non-GABA-mediated, phasic vs. tonic, feed-forward vs. feed-back) generated by a variety of GABAergic interneuron subtypes. Interneuron subtypes are functionally specialized. For example, highly vulnerable hilar somatostatin interneurons target their axon collaterals to granule cell dendritic shafts and spines in the outer molecular layer, an ideal position for inhibiting excitatory input from entorhinal layer II cells that arrives in the same stratum. Defining deficits in inhibition and interneuron loss more precisely is an important step toward specifically reversing the pathology and testing its clinical relevance. Furthermore, there is much to learn about underlying mechanisms of selective vulnerability or resistance of specific interneuron subtypes. And, it is important to evaluate whether surviving interneurons, including those that display epilepsy-related morphological abnormalities, function differently in epileptic tissue.

If reduced inhibitory synaptic input to granule cells and layer II cells is important for temporal lobe epileptogenesis, then preventing or reversing deficiencies should be therapeutic. In fact, the nervous system appears to compensate, albeit incompletely, through a variety of mechanisms, after hyperexcitability develops. For example, our findings are consistent with reports by the Mody laboratory that in models of temporal lobe epilepsy, amplitudes of miniature IPSCs increase in granule cells because more $GABA_A$ receptors are expressed per synapse. Work by the laboratories of Babb, Mathern, Freund, and others on epileptic tissue from patients suggests surviving interneurons sprout axon collaterals and form new synapses with granule cells. Neurogenesis in the dentate gyrus, especially after epileptogenic injuries, offers the possibility of endogenous sources to replace GABAergic interneurons. Although evidence of GABAergic neurogenesis is limited, recent findings suggest transplantation may be an option. Future work will continue to evaluate and attempt to enhance regenerative and compensatory mechanisms. If we could 'restore' inhibition of dentate granule cells and layer II cells of medial entorhinal cortex, we would better understand the clinical significance of interneuron loss and therapeutic potential of interneuron replacement.

See also: **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; $GABA_A$ Receptor Plasticity in Epileptogenesis; **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; Plasticity and Reorganization of GABA Neurons in Epilepsy; Properties of Interneurons and their Relation to Epilepsy.

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Plasticity and Reorganization of GABA Neurons in Epilepsy

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Introduction

Identifying the changes that occur in GABA neurons in epilepsy, and determining their functional consequences, remain among the most important issues in the epilepsy field. GABA neurons have long been considered important in epilepsy because of their inhibitory actions in many brain regions and their role in controlling neuronal excitability. Thus, it is logical to consider that loss of GABA neurons could contribute to epilepsy. However, it is also clear that changes in the GABA system extend beyond the simple loss of some of these neurons, and encompass an array of changes in remaining GABA neurons. The goal of this chapter is to highlight several types of morphological and neurochemical changes that occur in GABA neurons in the hippocampal formation in temporal lobe epilepsy (TLE) models. These changes emphasize the considerable plasticity of GABA neurons and raise questions regarding the consequences of the changes for the development and control of epilepsy.

Background

Initial suggestions that loss of GABA neurons might be a critical feature in epilepsy were based on alterations in a model of cortical focal epilepsy, in which investigators found a loss of basket and axo-axonic neurons in the cerebral cortex. Interestingly, in recent studies of several genetically-modified mice with an epileptic phenotype, GABA neuron loss has also been demonstrated in the cerebral cortex. Thus in some epileptic animals, loss of GABA neurons certainly occurs and may contribute to seizure development in a relatively clear and predictable manner. However, changes in the hippocampus in models of TLE are more complex.

For many years, the question of whether hippocampal GABA neurons are lost in TLE was vigorously debated, with some groups emphasizing the preservation of GABA neurons and other groups emphasizing the vulnerability of GABA neurons to excitotoxic damage. It now appears that both views are correct, and it is critical to consider both types of changes as the GABA system is analyzed in epilepsy models. Although GABA neuron loss clearly occurs in several models following insults such as status epilepticus, many GABA neurons are preserved. Importantly, these remaining GABA neurons undergo substantial changes, and the altered GABA neurons could be important pieces of the epilepsy puzzle. Since these neurons remain in the hippocampus following the initial insult, their function is critical for maintaining seizure control and their malfunction could contribute to seizure generation. Thus it is important to determine the characteristics of the remaining GABA neurons.

Methods

For morphological studies of GABA neurons in epilepsy, one ideally would like to visualize the cell bodies of the entire population of GABA neurons as well as their axonal arborizations and terminals. Unfortunately, no single method provides this information, and thus multiple methods are often required for a complete picture. Indeed, one's view of GABA neuron changes in epilepsy is likely to depend on the methods that are chosen to visualize these neurons.

Immunohistochemical localization of the two isoforms of the GABA-synthesizing enzyme, glutamate decarboxylase (GAD65 and GAD67), is often used for identifying GABA neurons. While these methods are quite effective for labeling the majority of GABA neurons in some brain regions, such as the cerebral cortex, they do not provide consistent labeling of all GABA neuron cell bodies in the hippocampus with currently available antibodies. For such cell body labeling, in situ hybridization methods for localizing GAD mRNAs are particularly useful. The in situ hybridization methods provide a clear view of GABA neuron cell bodies without occlusion by axon terminals, and the numbers of GABA neurons in all regions and layers of the hippocampus are striking when compared with those identified with other methods (**Figure 1(a)**).

In contrast, immunohistochemical localization of GAD65, the isoform involved primarily in local control of GABA synthesis at synaptic sites, identifies most GABAergic axon terminals but often labels only a limited number of cell bodies. Localization of GAD67, the isoform responsible for maintaining baseline levels of GABA for both neurotransmitter and metabolic functions, reveals many – though not all – GABA neuron cell bodies and proximal dendrites. In addition, GAD67 effectively labels moderate numbers of GABAergic axon terminals, including perisomatic terminals in stratum pyramidale of CA1. Thus, each method has its advantages and provides

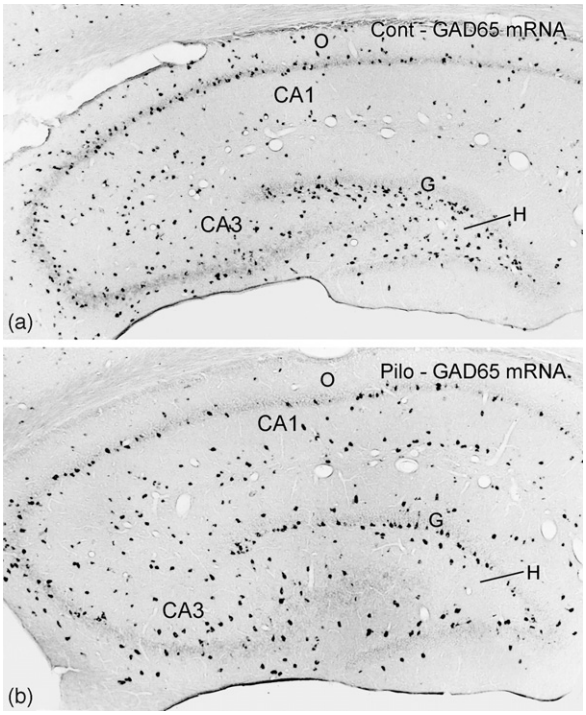


Figure 1 In situ hybridization of GAD65 mRNA in the hippocampus of a control (a) and pilocarpine-treated (b) rat. (a) In a control animal, numerous GAD65 mRNA-labeled cell bodies are evident throughout the hippocampal formation. (b) In a pilocarpine-treated animal during the chronic period, a distinct loss of GAD65 mRNA-labeled neurons is evident in the hilus (H) of the dentate gyrus and stratum oriens (O) of CA1. Many labeled neurons are present along the base of the granule-cell layer (G) despite loss of hilar neurons in the regions immediately below the layer. Numerous strongly-labeled GABA neurons remain in other regions of the hippocampus. Adapted from Houser CR (1999) Neuronal loss and synaptic reorganization in temporal lobe epilepsy. In Delgado-Escueta AV, Wilson WA, Olsen RW, and Porter RJ (eds.). *Jaspers's Basic Mechanisms of the Epilepsies: Advances in Neurology* 3rd ed., vol. 79, with permission from Lippincott Williams and Wilkins.

information about the broad population of GABA neurons in the hippocampal formation.

Subtypes of GABA neurons can be detected by labeling the neuropeptides or calcium-binding proteins that are present in many GABA neurons. These methods have been particularly useful for identifying vulnerable groups of GABA neurons in epilepsy, and have the advantage of focusing attention on specific groups of GABA neurons, although the more limited perspective must also be recognized.

One technically important and interesting aspect of GABA neuron localization in epilepsy is the potential for alterations in protein and peptide expression in many seizure models, either in response to an initial episode of status epilepticus or as a result of altered neuronal activity. Such functional changes contribute to the difficulties in evaluating GABA neuron loss and morphological reorganization with some markers. For example, an increase in GAD expression in either the cell bodies or axon terminals of remaining neurons could conceal GABA neuron loss by producing increased labeling of remaining neurons. Likewise, a decrease in protein expression could suggest loss of a subgroup of interneurons, even though that group remains viable (simply no longer expresses the marker of interest).

Despite some limitations, immunohistochemical and in situ hybridization methods for localizing GABA neurons have provided a wealth of information about these neurons and the changes that occur in epilepsy. It is now important to expand studies of remaining GABA neurons beyond the markers for these neurons, and identify potential changes in other proteins – including those of receptors and ion channels – that could be modified and thus alter the function of these neurons in epilepsy. Immunohistochemical and in situ hybridization studies are ideally suited for identifying interneuron-specific changes that might not be evident with other methods that involve analyses of entire brain regions.

Recent Results

Susceptibility and Resistance of GABA Neurons to Seizure-Induced Damage

Labeling cell bodies of the entire population of GABA neurons clearly demonstrates neuronal loss, and at the same time, emphasizes the large number of GABA neurons that remain in the hippocampal formation in lesion models of TLE (**Figure1(a)** and **1(b)**). GABA neurons in the hilus of the dentate gyrus are considered to be among the most vulnerable GABA neurons in the hippocampal formation (**Figure 1(b)**), and neurons in stratum oriens of CA1 are also frequently decreased in both pilocarpine and kainate models of TLE (**Figure 1(b)**). The loss of these neurons is in sharp contrast to the preservation of many GABA neurons along the base of the granule-cell layer (**Figure 1(b)**) and in the pyramidal-cell layer of CA1 (**Figure 1(b)**). Despite their close proximity, some GABA neurons are susceptible to damage while others appear resistant.

More selective views of GABA neuron loss in epilepsy have come from localization of neuropeptides and calcium-binding proteins that characterize subclasses of GABA neurons. Such labeling has demonstrated that somatostatin and neuropeptide Y (NPY) neurons in the dentate hilus are often severely depleted in human TLE and many animal models (**Figures 2(a)** and **2(b)**). The numbers of somatostatin neurons in stratum oriens of CA1 are also reduced, consistent with the findings from in situ hybridization studies of GAD mRNAs. In contrast, parvalbumin-expressing neurons in the dentate gyrus, many of which are basket cells, are relatively resistant to seizure-induced damage in humans and some animal models, and their labeling may even be enhanced during the chronic period (**Figure 2(c)** and **2(d)**). However, a decrease in

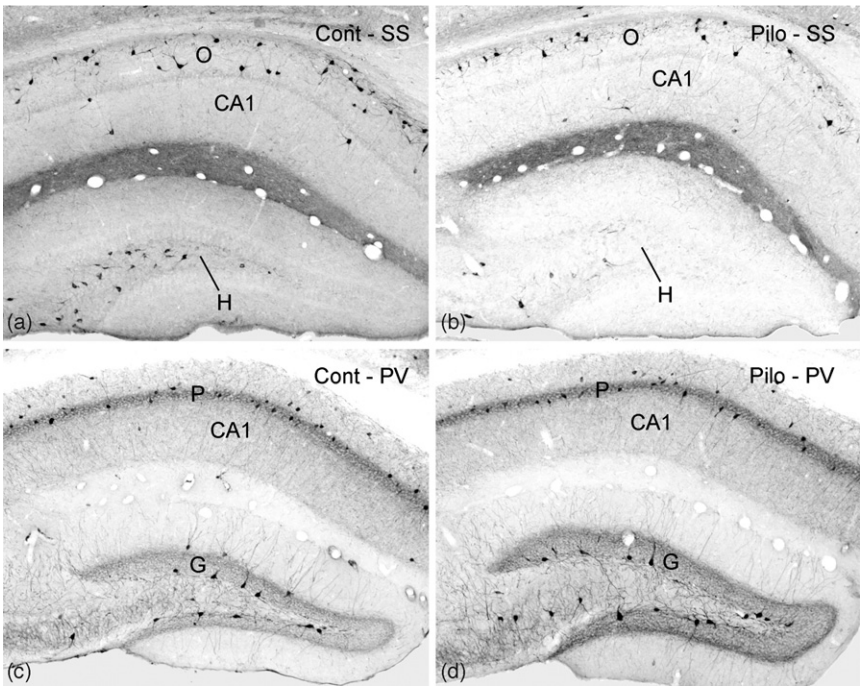


Figure 2 Immunohistochemical localization of somatostatin (SS) and parvalbumin (PV) in control (a,c) and pilocarpine-treated (b,d) mice at two months following status epilepticus. (a,b) In the pilocarpine-treated mouse, SS neurons are severely depleted in the hilus (H) of the dentate gyrus, where they are abundant in the control animal. A moderate loss of SS neurons is also evident in stratum oriens (O) of CA1. (c,d) In a pilocarpine-treated mouse, numerous PV-labeled neurons remain along the base of the granule-cell layer (G) of the dentate gyrus and throughout stratum pyramidale (P) of CA1. The labeling of parvalbumin terminals in the granule-cell and pyramidal-cell layers appears stronger than in the control animal, despite identical processing of the tissue.

parvalbumin-labeled interneurons in the hippocampal formation also has been reported in several models. Differences in the findings could reflect variations in the models, regional or species differences, or (in some instances) decreased parvalbumin immunoreactivity in remaining neurons.

From such findings, a broader perspective on GABA neuron loss in epilepsy is emerging. The data suggest that GABA neurons that innervate dendritic regions are most vulnerable to damage, whereas those that provide perisomatic innervation are often preserved. This view is consistent with loss of hilar somatostatin neurons that innervate the outer molecular layer of the dentate gyrus and preservation of many GABAergic basket cells at the base of the granule-cell layer that innervate the somata of granule cells. In CA1, electrophysiological studies demonstrate a reduction in dendritic but not somatic inhibition in a rat model of TLE, consistent with loss of interneurons that innervate primarily dendritic regions. These patterns have interesting functional implications, for they suggest that GABAergic modulation of the principal cells' response to excitatory input could be deficient while GABAergic control of the final output of the principal cells is preserved. This differential pattern has also led to the interesting suggestion that preservation of strong perisomatic innervation, and possible sprouting of these axon terminals, could promote synchronous firing of the principal cells, particularly when remaining GABA neurons are strongly activated. In this way, some remaining GABA neurons might contribute to seizure activity.

Alterations of Remaining GABA Neurons

Considering the severe loss of hilar somatostatin neurons, a depletion of GABAergic terminals in their target region (the molecular layer of the dentate gyrus) would be expected. However, such decreases are not evident during the chronic period in TLE animal models. Instead, GABAergic fibers and terminals are abundant throughout the molecular layer and have the highest density in the outer part of the layer (as in control animals) (**Figure 3(a)** and **3(b)**). Such findings suggest substantial plasticity of remaining GABA neurons. Changes in these remaining neurons include up-regulation of the GABA-synthesizing enzymes, potential morphological reorganization of GABAergic axons, and altered expression of receptors in remaining GABA neurons.

Up-regulation of GAD mRNAs and proteins

GABA neurons that remain in the hippocampal formation following an episode of status epilepticus show a remarkable up-regulation of the mRNAs for both GAD65 and

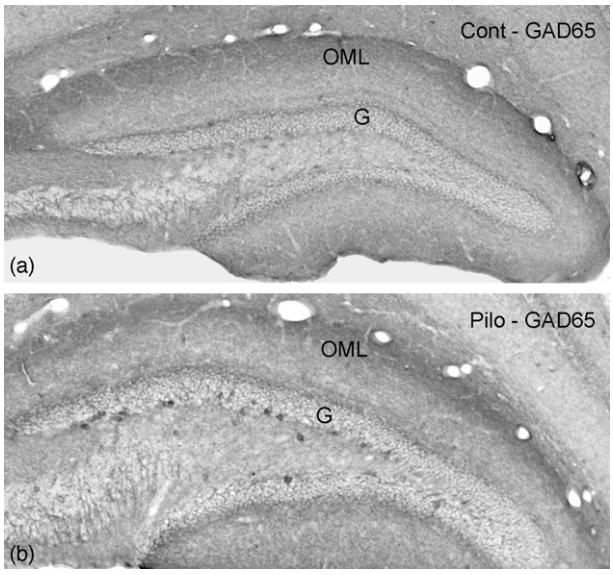


Figure 3 Immunohistochemical localization of GAD65 in the dentate gyrus of a control (a) and pilocarpine-treated (b) mouse during the chronic period. (a) In a control animal, GAD65-labeled axon terminals are most highly concentrated in the outer molecular layer (OML) and in a narrow band along the outer border of the granule-cell layer (G). A few lightly labeled cell bodies are evident at the base of the granule-cell layer. (b) In a pilocarpine-treated mouse, a dense field of GAD65-labeled terminals is present in the OML despite loss of hilar GABA neurons that normally innervate this region. Labeling in the inner and middle molecular layer appears to be increased slightly, and the laminar pattern of GAD65-labeled terminals in the molecular layer is less distinct than in the control mouse. Some GABA neurons along the base of the granule-cell layer (G) are more strongly labeled in the pilocarpine-treated mouse than in the control.

GAD67. This response extends beyond the region of severe GABA neuron loss and is evident in remaining GABA neurons throughout the dentate gyrus and hippocampus. The up-regulation of GAD mRNAs is generally viewed as a compensatory response to increased excitability in the region. The increased GAD mRNA expression presumably leads to increased GAD and GABA levels. These changes could influence GABAergic function as well as affect immunohistochemical labeling within the hippocampus. For example, increased GAD labeling in persisting axon terminals could conceivably mask the loss of GABAergic axons and terminals in regions such as the molecular layer (**Figure 3(b)**). In addition, increased GAD levels could lead to enhanced labeling of some GABAergic cell bodies, including those along the base of the granule-cell layer (**Figure 3(b)**).

Recently, increased labeling of the vesicular GABA transporter (vGAT) has also been demonstrated in GABAergic axon terminals in the hippocampus in a rat model of recurrent seizures, suggesting increased vGAT synthesis and possibly an associated increase in GABA release at some axon terminals.

The up-regulation of such GABA-related mRNAs and proteins suggests a very active GABA system that presumably could control neuronal activity for much of the time. However, with persistently elevated levels of activity, the system might have limited reserves to respond to any further increases in excitability, such as would occur during seizure onset. Another possibility is that the persistently increased activity of the GABA system could lead to additional adaptive changes within the system, including altered levels of GABA receptor subunits, that might then counteract the effects of enhanced presynaptic GABAergic function.

Potential reorganization of GABAergic axons

The preservation of many GABA neurons and an apparent increase in some GABAergic axon and terminal fields during the chronic stage have been described in humans with TLE and several epilepsy models. This has led to the suggestion that GABA neurons may exhibit axonal reorganization, similar to that of mossy fibers in epilepsy. Yet it remains uncertain whether the increased labeling of GABA fibers results from axonal sprouting of remaining GABA neurons or an increase of GAD and GABA in existing terminals. The classical sprouting of mossy fibers from granule cells in TLE has been relatively easy to document because of the aberrant location of the reorganized fibers as well as their high content of zinc, dynorphin, and other unique markers. In contrast, GABAergic axon terminals are normally found in varying densities in nearly all regions of the hippocampus, and sprouting within a local area is thus more difficult to detect.

Conclusive demonstrations of axonal sprouting of GABA neurons may require time course studies with markers for subgroups of GABA neurons that allow more selective analyses. Such studies of remaining somatostatin neurons in the mouse pilocarpine model are in progress. Preliminary findings suggest that axonal sprouting is initiated soon after the degeneration of hilar somatostatin neurons and continues to progress for several months. Such axonal reorganization could account for the difficulties in demonstrating a loss of GABAergic axon terminals in regions such as the outer molecular layer of the dentate gyrus, even when extensive loss of GABAergic innervation to this region has occurred as a result of hilar neuron loss.

If axonal reorganization occurs, the functional consequences will depend on the circuitry that is established. If GABA neurons of the same subtype as those that degenerate were to reinnervate the same regions, similar circuitry could be formed and considerable compensation might occur. Alternatively, the reorganized axon terminals could

originate from GABA neurons in other regions of the hippocampal formation or, possibly, extrahippocampal regions. In such cases, the neurons might not participate in the appropriate circuit and thus could be less effective than the original population of GABA neurons. Although GAD-containing fibers and terminals in the dentate molecular layer are abundant in the pilocarpine-treated animals during the chronic stage, their pattern appears less well-organized than in control animals (**Figure 3(a)** and **3(b)**). These changes are consistent with morphological reorganization of GABAergic innervation, possibly from different populations of interneurons than those that originally innervated the region.

Alterations in remaining GABA neurons that could impair their function

The response of remaining GABA neurons to their afferent input is another important consideration, and decreased responsiveness of these neurons could substantially impair GABAergic function. A decrease in excitatory drive to some GABA neurons, such as basket cells of the dentate gyrus, has been proposed as a mechanism for decreased inhibitory function in some epilepsy models. However, there is currently little direct evidence for a decrease in excitatory input to these GABA neurons. However, other changes in GABA neurons could alter their excitability.

Recently, an interesting change in expression of the δ subunit of the $GABA_A$ receptor was observed in GABA neurons in the mouse pilocarpine model during the chronic period. Expression of the δ subunit was markedly increased in interneurons throughout the dentate gyrus, despite decreased expression in dentate granule cells (**Figure 4(a)** and **4(b)**). This cell-type-specific regulation of the δ subunit in interneurons and principal cells was unexpected, and demonstrates unique plasticity of this $GABA_A$ receptor subunit.

δ Subunit-containing receptors mediate tonic or persistent inhibition that can be distinguished from phasic or synaptically-mediated inhibition, and appear to be quite important in controlling neuronal excitability. These receptors have a high affinity for GABA and are generally located either at the periphery of GABAergic synapses or at extrasynaptic locations where they can respond to ambient levels of GABA and spillover of synaptically-released GABA. Furthermore, activity of δ subunit-containing receptors can be modulated by physiological levels of neurosteroids and low levels of ethanol.

In epileptic mice, increased expression of the δ subunit was particularly evident in interneurons of the dentate

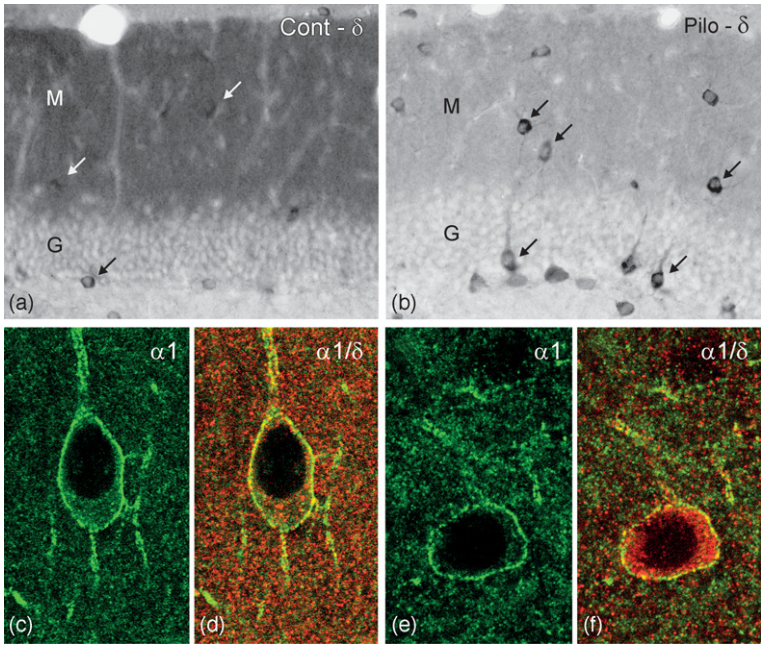


Figure 4 Immunohistochemical localization of the δ and α1 subunits of the $GABA_A$ receptor in the dentate gyrus of control and pilocarpine-treated mice. (a,b) In a pilocarpine-treated mouse during the chronic period, δ subunit labeling of interneurons (arrows) in the molecular (M) and granule-cell (G) layers is substantially increased. The increased labeling of interneurons contrasts with decreased labeling of granule-cell dendrites in the molecular layer and their cell bodies in the granule-cell layer in the pilocarpine-treated animal. (c–f) As revealed by confocal imaging, α1 subunit labeling (green) on the surface of interneurons in the molecular layer is similar in control (c) and pilocarpine-treated (e) mice. In contrast, δ subunit labeling (red) in the cytoplasm is increased in the pilocarpine-treated animal (f) compared with the control (d). Surface labeling of the subunits (yellow) remains prominent in the pilocarpine-treated animal (f).

molecular layer (**Figure 4(a)–4(f)**). Many of these neurons innervate granule-cell dendrites and, through feed-forward inhibition, help regulate the responsiveness of granule cells to excitatory input from the entorhinal cortex. When functioning optimally, input to these interneurons could stimulate their activity, and this activity could in turn help regulate granule-cell responses to entorhinal input. If tonic inhibition of these interneurons increased, as appears to be the implication of enhanced expression of the δ subunit, the interneurons could become less responsive to their normal excitatory input. Inhibition of the granule cells might then be compromised at precisely the times when it is most needed to control granule-cell excitability. Such functional effects remain to be demonstrated, but the possibility that increased inhibition of inhibitory interneurons could contribute to increased seizure susceptibility is intriguing.

Evidence that decreased excitability of GABA neurons can contribute to recurrent seizures has come from recent studies of a transgenic mouse with a loss-of-function mutation of the gene encoding the voltage-gated sodium channel $Na_v1.1$. Voltage-gated sodium (Na_v) channels play critical roles in the initiation and propagation of action potentials, and reduction in these channels was expected to lead to decreased neuronal excitability. Instead, heterozygous mice with decreased $Na_v1.1$ channels showed increased excitability and spontaneous seizures. It was then discovered that the $Na_v1.1$ channels were normally highly expressed on the soma of many hippocampal interneurons. Thus, the mutation produced alterations primarily in interneurons rather than principal cells, and resulted in substantially reduced action potential generation and inability of the interneurons to sustain firing. These findings provide strong support for the suggestion that impaired function of existing GABA neurons can contribute to increased excitability of the hippocampus and spontaneous seizures. Importantly, such alterations could occur in humans with severe myoclonic epilepsy in infancy (SMEI) as a result of the same mutation of the $Na_v1.1$ channel gene.

Additional changes that could alter the excitability of remaining GABA neurons are likely to be found. Such changes could be quite important, for many GABAergic interneurons have extensive axonal plexuses. Thus, changes in excitability of even small numbers of GABA neurons could have widespread influences. These remaining neurons could become targets of pharmacological treatment whereby relatively selective actions on GABA neurons could influence large populations of principal cells.

Future Challenges

Future studies will need to consider both loss and preservation of GABA neurons, and their influences within the reorganized circuitry of each brain region. Current studies have shown that each region of the hippocampal formation, including the dentate gyrus, CA3, CA1, subiculum, and entorhinal cortex, has unique changes in GABA neurons and their receptors. However, with time, common patterns may become evident. For example, GABA neuron loss could be found in virtually all regions, even if the types of vulnerable GABA neurons differ among regions. Following such loss, reorganization of remaining GABA neurons could occur, although the specific types of GABA neurons that undergo reorganization and their targets may differ. Finally, receptors and ion channels that regulate the responses of remaining GABA neurons could be altered in ways that reduce their activity and thus their effectiveness of controlling their target neurons. The functional effects of all of these changes are most likely to be evident when demands on the GABA system are increased. A critical question in all instances is how remaining GABA neurons function within their established circuits in the intact brain. Given the current emphasis on studying 'reduced' or 'simplified' models, it is important to remember that these in vivo functions could differ significantly from the responsiveness of these cells to specified, direct stimulation in vitro.

Identifying additional changes in interneurons that could alter their function is a particularly exciting challenge. Indeed, GABA neurons that remain following the initial insult could be as important as those that are lost, for these neurons maintain control of the system for the majority of time between seizure episodes. Also, it is the function of these neurons that may be deficient at the time of a spontaneous seizure. As alterations in remaining GABA neurons are identified, the challenge is to determine which changes are compensatory and might be enhanced for the prevention and treatment of epilepsy, and which are maladaptive and should be counteracted.

Acknowledgements

Support for my epilepsy research by VA Medical Research Funds and National Institutes of Health Grants NS046524 and NS051311 is gratefully acknowledged. I also thank Drs. Monique Esclapez, Andre Obenaus, Zechun Peng, and Nianhui Zhang for their numerous contributions to our studies of GABA neurons in epilepsy over the years and Christine Huang for superb assistance with the immunohistochemistry studies and figure preparation.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury and Progressive Cell Damage; **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal

Interneuron Types: Implications for Epileptogenesis; GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex; Properties of Interneurons and their Relation to Epilepsy; **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; **Plasticity**: Circuitry Reorganization, Regeneration, and Sprouting.

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Properties of Interneurons and their Relation to Epilepsy

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Introduction

Within the mammalian central nervous system, GABAergic synaptic inhibition is provided by a wide array of local circuit inhibitory interneuron types. Synaptic inhibition contributes to local processes (including information coding and synaptic plasticity) and global processes such as network synchrony and oscillations. Alterations in the intrinsic excitability or synaptic efficacy of inhibitory interneurons may impair their ability to regulate network synchronicity and overall excitability, often resulting in the production of electrographic seizure activity. Indeed, pharmacological agents that reduce inhibition are typically proconvulsant, while agents that selectively enhance inhibitory synaptic tone are widely used as antiepileptic drug therapies.

The excitability of GABAergic interneurons is defined by the unique set of ion conductances that each cell expresses. Potassium conductances are key determinants of cellular excitability, as they are largely responsible for determining the resting potential, input resistance, membrane time constant, and action potential repolarization rate. Therefore understanding the repertoire of potassium conductances expressed by interneurons, and how these conductances contribute to cellular function, will add to our understanding of the mechanisms by which interneurons preserve the optimal level of excitability.

An enormous diversity of potassium (K) channels exists. Over 100 distinct potassium channel subunits have been identified. These subunits can be classified into several groups based on their functional properties, including voltage-gated K channels, Ca^{2+} activated K^+ channels, inward rectifiers, K^+ 'leak' channels, and Na^+ activated K^+ channels. These groups can be further divided into families and subfamilies based upon their molecular similarity. A single neuron expresses many different K^+ channel subunits from each of the families and subfamilies; each of these channels contributes to the excitability of the neuron in a different way. For example, potassium 'leak' channels dominate the resting membrane properties, while voltage-gated channels are activated during synaptic transmission and action potentials.

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Because of the large diversity of K^+ channel subunits and functions, we have focused on how a single K^+ channel subfamily, the Kv3 subfamily, critically contributes to the excitability of interneurons.

Background

The Kv3 subfamily of voltage-gated potassium channels is composed of the subunits Kv3.1–3.4, which assemble as either homomers or heteromers to form tetrameric potassium channels. Of particular interest, in cortex and hippocampus expression of these Kv3 subfamily members is restricted to several inhibitory interneuron subpopulations. Knockout mice have been made of all four Kv3 genes. Of the single mutants, Kv3.2 has the highest propensity to develop spontaneous seizures. Furthermore, seizures are more easily evoked in Kv3.2-deficient mice using electrical stimulation of the thalamus, and Kv3.2-deficient mice injected with the convulsant picrotoxin (a $GABA_A$ receptor antagonist) develop more severe seizures with a shorter latency to onset than wild-type controls. The increased seizure susceptibility of Kv3.2-deficient mice suggests that Kv3.2-containing channels play a critical role in controlling network excitability.

To understand how channels formed from Kv3.2 subunits might regulate network excitability, we need to know which neurons express Kv3.2 and how Kv3.2 channels contribute to the function of these neurons. In the neocortex and hippocampus, Kv3.2 mRNA and protein are predominantly expressed in GABAergic interneurons. More specifically, Kv3.2 expression is limited to subclasses of GABAergic interneurons that are designed to fire at high frequencies. These 'fast-spiking' interneurons mainly consist of two anatomical classes: parvalbumin positive, soma-inhibiting (basket) cells, and somatostatin positive, dendrite-inhibiting cells (**Figure 1(a)**). Soma-inhibiting interneurons form multiple GABAergic synapses on the soma and proximal dendrites of excitatory (pyramidal) cells. Because their synapses are close to the site of action potential initiation, they are able to effectively exert control over action potential generation in pyramidal cells, leading to the hypothesis that they play a significant role in regulating excitation within the cortex. Dendrite-inhibiting neurons exert little control over Na^+-dependent action potential generation. Instead, they modulate Ca^{2+}-dependent spikes and postsynaptic potentials within the dendrites, influencing which signals propagate to the soma. Mice that lack dendrite-inhibiting interneurons have an increased susceptibility to seizures, suggesting that these interneurons have a significant role in determining network excitability. Thus, the two main types of interneurons that express Kv3.2 are involved in controlling pyramidal cell excitability.

As already stated, the ability to fire action potentials in rapid succession is a defining feature of Kv3.2-containing interneurons. Indeed, fast-spiking interneurons in Kv3.2 knockout mice have lower firing rates than fast-spiking interneurons in wild-type mice. The lower firing rates are correlated with broader action potentials and reduced after-hyperpolarizations. The lower firing rates suggest a

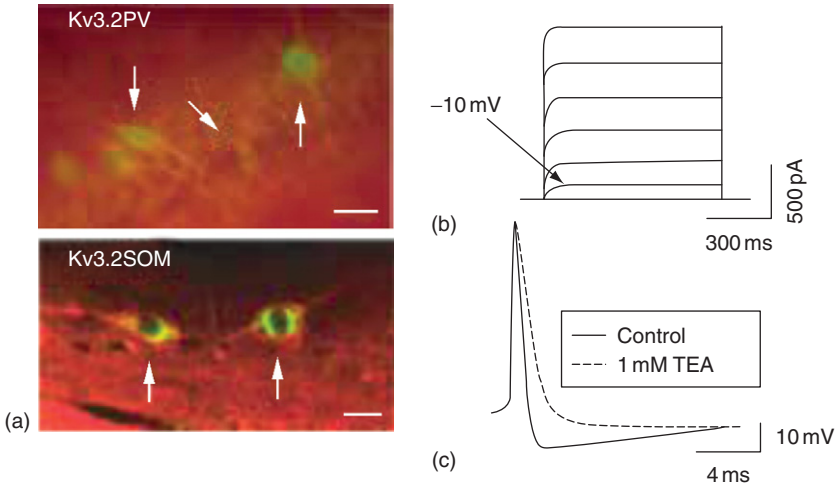


Figure 1 (a) Kv3.2 (red) is coexpressed with the calcium binding protein parvalbumin (green, top) and somatostatin (green, bottom) in hippocampus. While nearly all parvalbumin-positive interneurons express Kv3.2, only about 30% of somatostatin-expression interneurons express Kv3.2. (b) Kv3.2 currents in transfected Chinese hamster ovary cells. Kv3.2 currents are voltage-gated, sustained outward currents that are activated at potentials depolarized to −10 mV. (c) Low concentrations of TEA, sufficient to block Kv3 currents, broaden the action potential duration and decrease the afterhyperpolarization of cortical fast-spiking interneurons. Adapted from Rudy and McBain (2001).

decreased excitability of fast-spiking inhibitory interneurons, leading to reduced cortical inhibitory tone, which may account for the increased susceptibility to seizures observed in Kv3.2 knockout mice.

How do Kv3.2 channels contribute to the fast spiking phenotype of interneurons? Kv3.2-containing channels are optimized to ensure rapid repolarization of the membrane potential after an action potential, speeding Na^+ channel de-inactivation and enhancing the cell's ability to fire action potentials at a high frequency. In heterologous expression systems, Kv3.2 channels activate at −10 mV, well above action potential threshold, and rapidly deactivate upon repolarization of the membrane, suggesting that they are only active during action potentials (**Figure 1(b)**). Moreover, when Kv3.2-expressing HEK293 cells are voltage-clamped to an action potential waveform, current is not activated until the falling phase of the action potential and then quickly deactivates upon membrane repolarization. Thus, Kv3.2 channels do not alter the rising phase of the action potential but contribute a large outward current to the falling phase, a current which serves to more quickly repolarize the membrane potential. Fast repolarization quickly de-inactivates Na^+ channels, enabling them to participate in subsequent action potentials and thus facilitating high action potential firing rates.

The fast repolarization of the membrane potential mediated by Kv3.2-containing channels also occurs in native systems, and contributes to the high firing rate of Kv3-expressing neurons. While no specific Kv3.2 antagonists exists, Kv3 channels in general can be blocked with low concentrations of the nonspecific K^+ channel blockers TEA and 4-AP. Application of these drugs to acute brain slices increases the action potential duration, decreases the after-hyperpolarization, and reduces the firing rate of fast spiking interneurons (**Figure 1(c)**). Furthermore, after pharmacological blockade of Kv3 channels, reinsertion of a Kv3 conductance using a 'dynamic clamp' is sufficient to both reduce action potential duration and increase firing rate, rescuing the fast spiking phenotype. Finally, deep cortical layer parvalbumin positive interneurons in Kv3.2 knockout mice have broad action potentials with reduced after-hyperpolarizations and decreased firing rates, consistent with the view that Kv3 channels regulate inhibition in the cortex by controlling interneuron excitability.

Kv3 channel regulation of interneuron firing rate is almost certainly due to those Kv3 channels located in the soma and proximal axon, near the site of action potential generation. Indeed, Kv3.2, Kv3.3, and Kv3.1b (a splice variant of Kv3.1) are expressed at high levels in the perisomatic compartment. However, Kv3 channel expression is not limited to this compartment. Kv3.2 and Kv3.1b are also present in the axons and axon terminals of cortical interneurons. Furthermore, Kv3.1a is almost exclusively localized to axons and axon terminals. This localization places Kv3 channels in a position to directly influence the release of the neurotransmitter GABA from fast-spiking cells by regulating the shape of the presynaptic action potential within the axon terminal. The shape of the presynaptic action potential determines the time course of the voltage-dependent entry of Ca^{2+} into the presynaptic terminal, modulating the probability of neurotransmitter release. Typically, brief action potentials allow less Ca^{2+} to enter the presynaptic terminal than broad action potentials, reducing the probability of neurotransmitter release. Multiple lines of evidence suggest that Kv3 channels in the axon terminals shorten the duration of calcium entry, thereby reducing the probability of transmitter release. First, in the presence of Kv3 blockers, the size of the inhibitory postsynaptic current (IPSC) is increased and the paired pulse ratio decreased, suggesting an increase in release probability in the absence of Kv3 function. An increase in release probability is also found in Kv3.1/3.2 double knockout mice in comparison to controls. Second, calcium imaging of the presynaptic terminal reveals a larger and longer calcium transient in the presence of Kv3 channel blockers. Finally, TEA applied locally to the terminals affects release probability while somatic application has no effect. Thus, presynaptically localized Kv3 channels are involved in determining the probability of neurotransmitter release.

Kv3.2 plays two apparently antagonistic roles in regulating cortical inhibition. By shortening the time course of action potentials, Kv3.2 increases the frequency at which interneurons can fire action potentials, increasing the amount of inhibition. However, the shortened action potentials also reduce the presynaptic calcium influx, reducing neurotransmitter release probability, and reducing phasic inhibitory input to the postsynaptic cell. Thus, while Kv3.2 knockout mice have less frequent action potentials, they are broader, allowing for more Ca^{2+} influx, enhanced GABA release and increased IPSC amplitudes in the postsynaptic cells. One interpretation of this apparent dichotomy is that in Kv3.2-deficient mice, the reduced firing frequency of interneurons has a larger effect on the overall level of inhibition than the enhanced GABA release. However, this interpretation may be too simplistic and does not take into account the short-term dynamics of neurotransmitter release. When neurotransmitter release probability is increased, there is a concomitant change in the short-term plasticity of the synapse, reducing facilitation or enhancing depression of neurotransmitter release. The increased neurotransmitter release probability of GABAergic interneurons in Kv3.2-deficient mice is accompanied by increased paired pulse depression. The effects of release probability on a long train of stimuli in these mice are unknown; however, it may be that later in the train, the IPSCs are depressed

to the point that they can no longer effectively inhibit network activity. Thus, the effects of altered synaptic transmission may be greater during trains of stimuli than on a single stimulus.

Since Kv3.1 and Kv3.3 largely overlap with Kv3.2 in expression and function, it may be surprising that they do not compensate to maintain network stability in Kv3.2-deficient mice. However, Kv3.2 is unique among the Kv3 subunits in that it contains a protein kinase A (PKA) phosphorylation site on its C-terminus. PKA phosphorylation inhibits current through recombinant Kv3.2 channels. The ability to control channel conductance by phosphorylating or dephosphorylating the channel raises the interesting possibility that PKA phosphorylation of Kv3.2 channels is used to dynamically regulate interneuron, and thus network, excitability. Here we summarize work demonstrating that endogenous PKA inhibits a Kv3.2-like conductance, and that PKA-dependent phosphorylation of Kv3.2-containing channels is regulated by histamine H_2 receptor activation.

Method

To study how Kv3.2-containing channels dynamically-regulate excitability, Marco Atzori and Colleagues performed whole-cell voltage and current clamp recordings from acute hippocampal slices were performed. Since interneurons are located in cell sparse regions of the hippocampus, thin ($\sim$300 μm) sections that were transparent enough to enable the visualization of neurons, facilitating the targeting of inhibitory interneurons during recording. Kv3.2 channels are only expressed in subclasses of interneurons, and even with visualization, determining which cell-types are being recorded can be difficult. To bias recordings toward Kv3.2-containing cells, interneurons targeted for recording were located in the hilus of dentate gyrus, an area rich in both parvalbumin- and somatostatin-positive cells. Furthermore, the cells were filled with a tracer and post-hoc morphological analysis was used to verify that their morphology was consistent with Kv3.2-containing cells.

One of the challenges of this research is to specifically isolating Kv3.2 currents. Typically, a particular channel's currents can be isolated based upon its unique pharmacological and biophysical profile. However, there are no known Kv3.2 channel specific blockers. Two of the most commonly used blockers, 4-AP and TEA, block many classes of potassium channels. Specificity for Kv3 containing channels is increased by using low doses of these drugs (1–2 mM TEA; $<$100 μM 4-AP); however, even at these low doses, all Kv3-containing channels are blocked as well as a small number of other potassium channels. Furthermore, neither the kinetics nor voltage-dependence of Kv3.2 distinguishes it from other Kv3-containing channels. Finally, Kv3.1, 3.2, and 3.3 expressions are largely overlapping in hippocampal interneurons, so the variable expression pattern cannot be used to identify available currents. To overcome the challenges of specifically isolating Kv3.2 currents, the results of pharmacological experiments were verified in Kv3.2 knockout mice.

Recent Results

Similar to neocortical interneurons, in adult mice all hippocampal interneurons that express parvalbumin also express Kv3.2, and approximately one-third of somatostatin-expressing interneurons are Kv3.2 positive. Kv3.2 immunoreactivity is not detectable until P7; then it steadily increases until reaching adult levels by P14. The development of Kv3.2-expression coincides with the expression of parvalbumin and somatostatin and with the ability of these neurons to fire repetitively at high firing rates, providing further evidence that Kv3.2 channel expression contributes to the fast-spiking phenotype.

In the dentate gyrus, Kv3.2-expressing interneurons are located in the subgranular region and within the hilus. Whole-cell voltage clamp recordings of interneurons in this region reveal a TEA-sensitive outward current that is activated at approximately −10 mV. These currents activate rapidly and do not inactivate over the course of a 300-ms test pulse, consistent with Kv3-like current.

While all Kv3-containing channels can be modulated by PKC, only Kv3.2 has a PKA phosphorylation site. Currents through recombinant Kv3.2 are inhibited by PKA phosphorylation. In hilar interneurons, PKA activation also inhibits Kv3-like current. In the presence of the phosphodiesterase inhibitor IBMX, the PKA activators dibutyryl cAMP and 8-Br-cAMP reduce the sustained outward current. This reduction in outward current is occluded by pre-application of TEA, suggesting that the current inhibited by PKA is also TEA-sensitive, consistent with this being a Kv3.2 current. Further evidence that the PKA sensitive outward current is due to Kv3.2 comes from Kv3.2 knockout mice. PKA activation fails to alter the voltage-dependent outward current of hilar interneurons in Kv3.2-deficient mice, suggesting that PKA specifically inhibits Kv3.2 currents.

How is PKA-dependent inhibition of Kv3.2 currents regulated? PKA activity can be modulated by several G-protein coupled receptors, including β-adrenergic, dopamine, serotonin, and histamine H_2 receptors. H_2 receptors

are densely distributed within dentate gyrus, making them a good candidate for modulating Kv3.2 currents via PKA. Indeed, application of histamine or the histamine receptor agonist dimaprit reduced Kv3-like currents (Figure 2(a,b)), and pre-application of TEA or PKA-inhibitors blocks the action of histamine and dimaprit, indicating that activation of H_2 receptors leads to a reduction of Kv3-like currents via PKA phosphorylation. In Kv3.2-deficient neurons, dimaprit has no effect on outward currents, suggesting that H_2 receptor activation specifically inhibited Kv3.2 currents.

H_2 receptor activation can reduce Kv3.2 currents via PKA activation, but is this reduction in Kv3-like currents sufficient to alter the firing rate of interneurons? The maximum firing rates of interneurons were determined by delivering trains of brief, suprathreshold current pulses at various frequencies. The maximum frequency at which the interneuron spikes are able to follow current pulses without failing is reduced by approximately 25–30% in the presence of H_2 agonists or PKA activators (Figure 2(c)). Furthermore, in Kv3.2-deficient mice, H_2 agonists and PKA activators have no effect on the maximal firing frequency (Figure 2(d)). These results suggest that modulation of Kv3.2 currents alters the excitability of interneurons, reducing their ability of fire action potentials at high firing rates.

Kv3.2 modifies firing rate by efficiently and quickly repolarizing the membrane potential during the falling phase of an action potential, which facilitates the de-inactivation of voltage-dependent Na^+ channels, preparing them to participate in subsequent action potentials. Failure to repolarize the membrane potential and de-inactivate Na^+ channels decreases the action potential amplitude. H_2 agonists and PKA activators decreased the amplitude and increased the duration of action potentials, with greater effects occurring late in the train. The changes in action potential shape observed in H_2 agonists and PKA activators are consistent with impaired membrane repolarization and subsequent reduction in available Na^+ channels. The larger changes late in the train are likely due to the cumulative effects of Na^+ channel inactivation. As Na^+ channels fail to de-inactivate before the next depolarizing pulse, fewer Na^+ channels are available for the next action potential, further contributing to changes in action potential shape. PKA activators and H_2 agonists do not affect the shape of action potentials in Kv3.2-deficient neurons, indicating that their effects are specific for Kv3.2 currents.

Although these experiments clearly demonstrate that H_2/PKA dependent modulation of Kv3.2 currents affects the firing properties of individual neurons, these changes must be translated into altered network function for them to be involved in seizure activity. In hippocampal slices, pharmacological removal of excitatory synaptic transmission by bath application of glutamate receptor antagonists reveals spontaneously occurring high-frequency oscillations of the field potential in the principal cell layers. H_2 receptor agonists and PKA activators decrease the incidence of these population events in wild type mice but had no effect in Kv3.2-deficient mice, indicating that PKA-dependent inhibition of Kv3.2 currents is sufficient to reduce the excitability of a network of inhibitory interneurons.

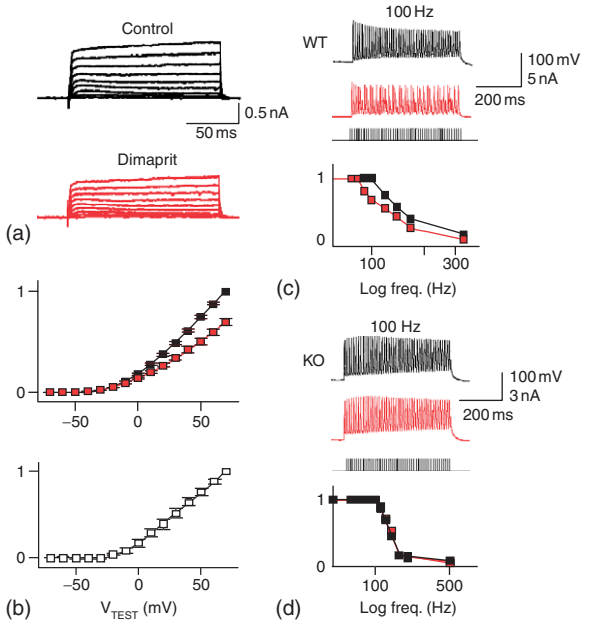


Figure 2 Kv3.2 current is modulated by H_2 receptor activation. (a) Whole cell sustained outward currents in hippocampal interneurons are decreased after application of the selective H_2 receptor agonist dimaprit. Outward currents were elicited by a 300 ms step to from a holding voltage of −70 mV to test voltages between −50 mV and +70 mV in 10 mV increments. (b) Top: Normalized current–voltage relationship measured in control (black) or in the presence of dimaprit (red). Outward current is reduced at positive voltages, consistent with an H_2 receptor dependent decrease in K^+ current. Bottom: The dimaprit sensitive current was isolated by subtraction of the current in dimaprit from control. The dimaprit-sensitive current is an outward current activated at −10 mV, consistent with it being a Kv3.2 current. (c) Dimaprit reduces the maximal firing frequency of wild-type interneurons. A train of stimuli delivered at 100 Hz can be followed for the entire 500 ms test period in control conditions (black); however, after application of dimaprit (red) the train of stimuli cannot be followed faithfully. Bottom: A plot of the action potential probability versus stimulus frequency demonstrates that dimaprit (red) reduces the ability of neuron to follow high frequency trains of stimuli. (d) In Kv3.2 knockout mice, application of dimaprit (red) has no effect on the ability of interneurons to fire high frequency trains of stimuli. Adapted from Atzori *et al*. (2000).

Future Goals

Kv3 channels have similar functional properties and largely overlapping distributions, yet Kv3.2-deficient mice have increased seizure susceptibility whereas other Kv3.x deficient mice do not. Understanding this difference will require dissecting the subtle differences between Kv3 channel function and expression.

One of the few features that distinguish Kv3 channels is their modulation by second messengers. Of the Kv3 channels, Kv3.2 is the only one phosphorylated by PKA activity, suggesting that the regulation of Kv3.2 current by PKA phosphorylation is key to network stability. In neurons, PKA activation can occur via numerous neuromodulatory pathways. Here we have described how the activation of H_2 receptors leads to PKA activation and a reduction in Kv3.2 current. However, PKA formation can be triggered by a variety of monoamine receptors, including adrenergic, serotonin, and dopamine receptors, all of which are present through the cortex and hippocampus. Thus, the PKA-dependent regulation of Kv3.2 receptors may be effected by several neuromodulators. Some of these receptors, such as D1 receptors and beta-adrenergic receptors, increase PKA activity; others, such as D2 receptors, decrease its formation. We hypothesize that activation of different receptor systems may be a mechanism of differentially modulating the system. Further study is necessary to determine which of these neuromodulatory systems act upon Kv3.2.

How can Kv3.2 modulation be important for maintaining network stability? Kv3 current decreases the duration of the action potential waveform, which allows for higher frequency firing, but also decreases the amount of Ca^{2+} influx, reducing the release probability. Modulation of Kv3.2 current by monoamine receptors may dynamically regulate the balance between high firing rates and high neurotransmitter release probability. To understand this action, the link between neuromodulators and neurotransmitter release must be more clearly defined. Importantly, we need to better understand how firing rate and neurotransmitter release probability contribute to overall network inhibition and whether there are situations that favor one over the other.

The study of Kv3.2 has largely focused on cortical structures, and there is some evidence that the lack of Kv3.2 in the cortex may influence the epileptic phenotype. During EEG recordings Kv3.2-deficient mice exhibit abnormal high amplitude sharp potentials that may reflect poor control of corticocortical inputs, indicating an inability of inhibitory interneurons to sufficiently suppress network function. However, Kv3.2 is also prominently expressed in many subcortical structures, particularly thalamic relay neurons, which also may be responsible for an epileptic phenotype. Elucidating the mechanisms underlying the enhanced seizure susceptibility of Kv3.2-deficient mice will require a deeper understanding of Kv3.2 function within individual cell types, and insight into how those cells contribute to network function.

See also: **Glutamate**: Functional Reorganization of Inhibitory Circuits in Epilepsy: mGluR1/5 Signaling Mechanisms and Long-Term Plasticity; **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex; Plasticity and Reorganization of GABA Neurons in Epilepsy.

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ION CHANNELS

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Acquired Ion Channel Dysfunction in Epilepsy

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Introduction

That ion channel dysfunction lies at the root of epilepsy has for decades seemed a self-evident truth. Ion channels, both voltage- and ligand-gated, mediate the excitable behavior of neurons, and since seizures result from neuronal hyperexcitability, surely altered biophysical properties or expression of ion channels must underlie this pathological hyperexcitability. Actual proof of this dogma, however, did not arrive until 1995, with the first publication of a genetic locus found in a human epilepsy syndrome, a mutation in nicotinic acetylcholine receptors underlying autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). Since 1995, a number of other idiopathic human epilepsy syndromes have been conclusively linked to single gene mutations (Table 1). Of note is the fact that all of these genes code for ion channel subunits or presumptive accessory subunits. The alterations of ion channel properties caused by these mutations are diverse, and include loss of expression or function (haploinsufficiency) as well as alterations that influence function of channels assembled from heteromeric subunits (a dominant negative action). The importance of these findings cannot be overstated: they prove that defects in individual ion channel subtypes can produce human epilepsy syndromes, complete with characteristic developmental courses and responses to antiepileptic drugs.

Table 1 Human genetic epilepsy syndromes resulting from ion channelopathy

Syndrome	*Affected genes*
Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE)	*CHRNA4*, *CHRNB2*
Benign familial neonatal convulsions (BFNC)	*KCNQ2*, *KCNQ3*
Generalized epilepsy with febrile seizures plus (GEFS+)	*SCN1B*, *SCN1A*, *GABARG2*
Severe myoclonic epilepsy of infancy (SMEI)	*SCN1A*
Benign familial neonatal–infantile convulsions (BFNIC)	*SCN1A*
Idiopathic generalized epilepsy and episodic ataxia	*CACNB4*
Autosomal dominant partial epilepsy with auditory features (ADPEAF)	*LGI1*

Does ion channel dysfunction similarly underlie epilepsy arising from acquired causes, such as head injury, brain tumors, or cerebral hemorrhage as it does in genetic epilepsy? (Temporal lobe epilepsy (TLE), the most common cause of epilepsy in adults, is widely assumed to also be an 'acquired epilepsy.') It is reasonable to hypothesize that ion channel dysfunction forms the final common pathway resulting from a variety of acute insults to the CNS. However, unlike the case for some genetic human epilepsy syndromes, proof of this concept is still lacking. Reasons for our slow progress in testing this hypothesis include the relative inaccessibility of human brain tissue from patients with epilepsy – unless they come to epilepsy surgery – with which to study ion channel function (compared with the relative ease of finding pathology in a patient's genomic code). Also problematic is the lack of control data from unaffected patients. Thus, animal modeling is the principal means of studying the causes of acquired epilepsy.

Background

The animal models in wide use present a range of advantages and disadvantages in their similarity to human

epilepsies. Models that replicate typical CNS insults would seem the best candidates to study the biological processes underlying the induction of the epileptic state, or epileptogenesis. However, experiments thus far with rodent models of traumatic brain injury, cerebral hemorrhage, hypoxic–ischemic encephalopathy, and febrile seizures have produced animals with relatively mild epileptic phenotypes – with either subtle partial or subclinical seizures, an epilepsy course that may remit over time, or even no evidence of spontaneously recurring seizures. Thus, the most widely used models are the poststatus epilepticus (SE) models involving induction of SE by pilocarpine or kainate. These models produce a robust epileptic phenotype in rodents, with spontaneously recurring seizures that span (or exceed) the Racine scale (a behavioral measure of seizure type/severity), and which do not remit over time. Like human acquired epilepsy, there is a variable 'latent period' between the insult and the development of spontaneous seizures, although the duration of this period is dependent on model methodology and in some cases may be just a few days; also like human TLE, these post-SE models produce hippocampal pathology reminiscent of medial temporal sclerosis. A significant drawback to these models is their lack of a naturalistic insult: humans are rarely exposed to chemical convulsants, and it is unclear how status epilepticus is related to traumatic brain insult (or to TLE). It has been argued that post-SE models are relevant because some human cases of adult TLE are associated with a history of febrile SE as a child, but this finding is the exception rather than the rule.

An additional rationale for the use of post-SE models of epilepsy pertains to the doctrine of 'seizures beget seizures.' This idea, that the natural course of untreated epilepsy shows an accelerating seizure frequency, dates to observations of Gowers in the nineteenth century and suggests that successive seizures alter brain excitability in such a way as to make further seizures more likely. If true, one mechanism by which this progression might occur is through seizure-dependent modification of ion channel properties that results in increased neuronal excitability. Although 'seizures beget seizures' is an oft-quoted dictum, there is little clinical proof supporting it. While retrospective clinical observations do show that the interval between seizures tends to decrease for the first several seizures after the onset of epilepsy (but before treatment), a definitive prospective test of this hypothesis is impossible for ethical reasons. In animal models of acquired epilepsy, such as kindling, kainate, and fluid percussion injury, there is clear evidence that seizure frequency and intensity increase after the initial insult, eventually achieving a plateau level. Whether the accelerating seizure rate seen is seizure-dependent, or instead reflects an underlying progressive process of epileptogenesis set in motion by the initial insult, is unclear. This question can be experimentally tested by pharmacologically blocking seizures during the early period after the insult, when an accelerating seizure frequency would be expected. Then, after the antiepileptic treatment is discontinued, it can be determined whether seizures begin at their usual low, accelerating rate, or whether they immediately begin at their higher plateau frequency. An interesting example of this test comes from a study in the WAG/Rij rat model of generalized epilepsy in which blockade of seizures during the period when they make their spontaneous developmental appearance significantly decreases their age-dependent frequency after the antiepileptic drug is discontinued. This finding suggests that for one model of epilepsy with a genetic basis, spontaneous seizures appear with an accelerating time course that is independent of underlying developmental processes. Conclusive data for acquired epilepsy models on this question are thus far lacking.

Thus, there are multiple ways in which acquired alteration of ion channel function can contribute to epileptogenesis – either as a fundamental cause of hyperexcitability or as a mediator of the process in which spontaneous seizures progressively worsen the course of epilepsy. It is also possible that some ion channel alterations retard the development of epilepsy. When interpreting studies of ion channel property changes in epilepsy models, it is important to consider whether the changes seen reflect a seizure-dependent process occurring after the onset of epilepsy, or whether they are instead set into motion by the initial insult and precede the onset of seizures. A further question is whether the changes seen are likely proconvulsive versus homeostatic. That is, do these changes demonstrably increase the excitability of neurons, or do they diminish intrinsic excitability in an attempt at maintaining brain normality? With these thoughts in mind, we can consider recent evidence for acquired ion channel changes in various animal models of epilepsy.

Methods

The results described later derive from a survey of the literature published in the last 12 years using animal models of acquired epilepsy to study altered ion channel properties with electrophysiological, biochemical, and molecular biological techniques.

Results – Specific Ion Channel Changes in Epilepsy

Na^+ Channels

Na^+ channels represent an obvious target of seizure-induced plasticity. Gain of function mutations in the α subunit of Nav1.1 channels or the accessory β1 subunit

have been implicated in the inherited human epilepsy syndrome GEFS+; these mutations generally cause an increase in I_{Na}, the current mediated by Na^+ channels, either through slowed or incomplete voltage-dependent inactivation. In contrast, a significant loss of I_{Na} is seen in the progressive human epilepsy syndrome SMEI. This counterintuitive result may be explained by a selective loss of Na^+ channels in cortical interneurons, rather than in principal neurons, leading to diminished inhibition.

Acquired changes in I_{Na} in dentate granule cells (DGCs) have been seen after pilocarpine-induced SE, with voltage-clamp recordings showing a hyperpolarizing shift in I_{Na} activation coupled with a depolarizing shift in fast inactivation, producing a net increase in the 'window current,' or current activated at physiological potentials. Measurement of mRNA from microdissected dentate gyrus extracts, at dates postpilocarpine that were comparable to the physiological experiments, shows downregulation of Nav1.2 and 1.6 transcripts, along with the β1 accessory subunit. There does not appear to be any change in DGC I_{Na} current density corresponding to the decreased transcription of Nav subunits, but the loss of β1 transcription is possibly consistent with the depolarizing shift in I_{Na} inactivation.

An intriguing related question is whether ion channel plasticity in epilepsy affects the response to antiepileptic drugs (AEDs), most of which have been hypothesized to act through direct modulation of ion channels, especially Na^+ channels. Examination of tissue from both epileptic rats and humans undergoing temporal lobectomy has found that epilepsy diminished the inhibitory actions of carbamazepine (CBZ) on I_{Na}. Specifically, the reduction in rate of recovery from inactivation that CBZ induces under normal conditions is nearly absent in DGCs from epileptic tissue. The effect of CBZ in shifting I_{Na} voltage dependence in a hyperpolarizing direction, however, is maintained under epileptic conditions. These results suggest that changes to Na^+ channels in epilepsy render them less susceptible to AED actions, possibly contributing to medical intractability. However, it is unclear which of the actions of CBZ on I_{Na} are most important in reducing neuronal excitability. Another issue that limits interpretation of some in vitro studies is the relatively high concentrations of drug used to study biophysical actions, often an order of magnitude higher than clinically relevant concentrations seen in human cerebrospinal fluid. Nonetheless, studies of AED action in epileptic animals raise the important question of whether 'epileptic ion channels' might themselves be a different pharmacologic target than the native or heterologously expressed channels usually studied under normal conditions.

Ca^{2+} Channels

Upregulation of Ca^{2+} channel activity has been shown in experimental acquired epilepsy in a number of studies. This work began with the observation that during the chronic phase of epilepsy induced by pilocarpine, the proportion of 'bursting' (i.e., firing of multiple action potentials from a single short depolarizing current injection) CA1 hippocampal pyramidal neurons increased from <5% to nearly 50% of cells. Low-threshold T-type currents have been implicated on physiological and pharmacological grounds, with an increase in T-current density. When activated, this augmented T-current could mediate a depolarizing potential that promotes burst AP firing. In accord with prior work that had shown localization of low-threshold Ca^{2+} currents to the apical dendrites of pyramidal neurons, focal application of the T-channel blocker Ni^{2+} to the dendrites abolishes bursting, while application to the soma does not. It is interesting that this condition represents an acquired channelopathy confined to the apical dendrites, one of several examples to be discussed later. There has been no evidence thus far as to the mechanism underlying the upregulation of T current. Because the AED ethosuximide blocks T-channel activity, this work would suggest that ethosuximide, already used against absence epilepsy, might also be effective in temporal lobe epilepsy; however, there is no clinical evidence presently supporting this idea.

K^+ Channels

Bertil Hille commented in his classic textbook that K^+ channels were like the 'stops on an organ,' able to fine-tune neuronal excitability by their diverse biophysical properties. Somewhat surprisingly then, K^+ channel dysfunction has been underrepresented in genetic epilepsy, with only one syndrome (BFNC) clearly due to K^+ channel mutation. In experimental acquired epilepsy models, however, a number of changes in K^+ channel activity have been identified, some of which appear to be proconvulsive, while others appear to represent a homeostatic change.

An important milestone in the study of ion channel properties was the recognition that many ion channel species in pyramidal neurons are distributed in highly nonuniform somatodendritic patterns. The A-type (or transient) K^+ current was the first example, found to exist in pyramidal neuron dendrites in a density gradient pattern, with the apical dendrites containing a sevenfold higher density than the soma. The high dendritic density of rapidly activating Kv4.2 channels (the main channel type underlying the A-current in pyramidal neurons) reduces the amplitude of excitatory postsynaptic potentials, activity-evoked intracellular Ca^{2+} transients, and backpropagating dendritic action potentials. The dendritic location of Kv4.2 channels, while directly influencing integration of dendritic synaptic potentials, also hampers electrophysiological study. However, measurements of Kv4.2 channel expression in chronic epilepsy (i.e., tissue from

postpilocarpine rats) have shown that Kv4.2 channel number is diminished by about one-third, as quantified by both Western blotting and mRNA expression. The electrophysiological consequence of the loss of A-current is increased AP backpropagation in the dendrites, potentially allowing increased opening of other voltage-gated channels such as Ca^{2+} channels. An interesting additional finding is that the Kv4.2 channels that remain in this tissue are more phosphorylated at a site that is recognized by the extracellular stimulus-related kinase (ERK). Phosphorylation by ERK of Kv4.2 shifts the voltage-dependent activation of these channels to more depolarized levels, diminishing their activity. Thus, the Kv4.2 channel dysfunction seen in this model of chronic epilepsy is twofold: a loss of channel number, and downregulation of the channels that remained.

Whether or not changes in Kv4.2 function are a significant contributor to the epileptic phenotype remains to be seen. Despite its significant role in dendritic physiology, a knockout of Kv4.2 by itself does not appear to cause obvious seizures in the transgenic animals. One possible explanation for this observation is that there is upregulation of other Kv channels (i.e., non-Kv4.2) that can mediate transient A-type currents in Kv4.2 knockout mice; these currents, putatively mediated by the Kv1 family and localized to the cell soma or axon initial segment, may counteract the loss of dendritic Kv4.2 channels. Also, because Kv4.2 expression has been measured only in the chronic phase of epilepsy, it is unclear whether the loss of A-currents is a cause or a consequence of ongoing seizures. Indeed, one study has found that Kv4.2 mRNA expression was reduced by pentylenetetrazole-induced seizures alone, in the absence of established epilepsy.

Another K^+ channel modulated by seizures is the Kv2.1 channel that produces a delayed rectifier current. Unlike Kv4.2, Kv2.1 is localized to the soma and proximal apical and basal dendrites, and it appears to depress excitability during periods of repetitive action potential firing. Shortly following kainate-induced SE, several changes in the localization and biophysical properties of Kv2.1 channels occur. For example, their distribution on the somatic membrane transforms from clusters of ion channels to a more diffuse pattern, without a loss of overall density. Further, the voltage-dependent activation of I_K (the delayed rectifier current mediated in part by Kv2.1) shifts in a hyperpolarizing direction, potentially increasing I_K activation. These changes in Kv2.1 properties are mediated by glutamatergic stimulation followed by calcineurin-dependent dephosphorylation of a number of residues on Kv2.1 itself. These changes reflect a putative signaling pathway by which seizure activity can produce acute changes in ion channel properties. Unlike the changes seen in Kv4.2 with epilepsy, it would be predicted that upregulation of Kv2.1 activation would act in a homeostatic, anticonvulsant fashion. (It is not clear what the effect of declustered Kv2.1 channels would be on excitability.) Also, the changes in Kv2.1 were induced by SE, but were not assessed in animals after the development of epilepsy; thus, it was not determined if they persisted beyond the acute SE-related phase in the kainate model. Indeed, the declustering of channels is observed to gradually revert to near-control levels several hours following stimulation. However, these studies provided solid mechanistic evidence for the links between seizures and ion channel modifications, and lay the groundwork for investigation of these mechanisms in chronic epilepsy.

HCN Channels

Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels seem unlikely candidates for a pathogenic role in epilepsy. Originally, identified in the sinoatrial node as a key regulator of heart rate, they have since been found widely in brain, with the HCN1 subtype distributed primarily to neocortex and hippocampus, while the HCN2 subtype is found in subcortical regions such as thalamus. Recent evidence has implicated dysfunction of these channels in both genetic and acquired epilepsy. Genetic loss of function of HCN channels clearly causes epilepsy; knockout of the HCN2 channel produces a generalized epilepsy phenotype in rodents, while inbred rat strains with epilepsy demonstrate altered function of both HCN2 and HCN1 channels. It is unclear whether knockout of the HCN1 channel also produces an epileptic phenotype, although anecdotal evidence suggests that HCN1 knockout mice lack spontaneous seizures but show more severe seizures provoked by kindling or pilocarpine. To date, no human genetic epilepsy pedigrees have been associated with HCN channel mutation.

There is increasing evidence that acquired experimental epilepsy is associated with altered HCN channel function and expression. One of the first studies to make this association found an increase in I_h, the current mediated by HCN channels, in hippocampal pyramidal neuron somatic recordings after hyperthermia-provoked seizures. However, like Kv4.2 and T channels, HCN channels are predominantly localized to the apical dendrites of pyramidal neurons, so it was of particular interest that subsequent studies, measuring dendritic I_h, found that HCN channels show an acute loss of expression within the first week post-SE. This loss of expression is maintained in chronic epilepsy, and is associated with a hyperpolarizing shift in voltage-dependent gating that further downregulates I_h. (Since HCN channels are activated with hyperpolarization, a hyperpolarizing shift in activation reduces the amount of I_h present at resting potential.) Interestingly, when recurrent seizures post-SE are blocked with phenobarbital administration, the altered HCN channel gating reverts to normal, while the loss of HCN channel expression persists. This result

suggests that there are separate mechanisms of HCN channel downregulation in epilepsy, some clearly dependent on ongoing seizures, and not integral to epileptogenesis per se. Similar caveats may apply to other observed changes in ion channel function.

Changes in HCN channel function in epilepsy, at least for pyramidal neurons, represent another example of ion channelopathy confined to the dendrites, similar to that seen for A-type K^+ channels. The mechanisms underlying the loss of dendritic HCN and A-channel expression are unclear; although there is evidence that HCN1 channel transcription is chronically reduced following hyperthermia-provoked seizures, the rapid loss of dendritic HCN channels (with 24-h post-SE) suggests additional, probably posttranslational mechanisms. In pyramidal neurons, HCN channel downregulation produces neuronal hyperexcitability, although this is not as straightforward to demonstrate as it is for Na^+ and K^+ channels – given the sometimes conflicting influences of I_h on neuronal input resistance, time constant, and resting membrane potential.

$GABA_A$ Receptors

Blocking inhibition mediated by $GABA_A$ receptors ($GABA_A$Rs) reliably provokes seizures in in vitro and in vivo models of epilepsy. Thus, it would be expected that alterations of $GABA_A$Rs are well represented in genetic and acquired epilepsy. In fact, there are several reports of human pedigrees of generalized epilepsy associated with loss of function of various $GABA_A$R subunits. Good evidence also exists for acquired alteration of $GABA_A$Rs in experimental models. Because $GABA_A$Rs are composed of five subunits in varying combinations, numerous possibilities exist for altered $GABA_A$R function through differential up- or downregulation of its component subunits. A careful study of this phenomenon in the pilocarpine model has found that α1 and β1 subunit expression in dentate granule cells is decreased in the latent period prior to the onset of seizures, with increases in δ and ε subunits. These changes are maintained in the chronic phase of epilepsy and appear to contribute to increased Zn^{2+} sensitivity of $GABA_A$Rs. Because Zn^{2+} diminishes $GABA_A$R function, this change promotes hyperexcitability.

Contrasting findings with regard to the δ $GABA_A$R subunit have been seen using the pilocarpine model in mice. Immunohistochemistry in DGCs has found a diffuse loss of δ subunit labeling, beginning in the latent period postpilocarpine, and continuing into the chronic phase. Interestingly, δ subunit expression is increased in dentate gyrus interneurons. It is unclear how to explain the contrasting findings of these two studies; differing methods to measure $GABA_A$R subunit expression may contribute to these differences. However, the recent correlation of δ subunit-containing $GABA_A$Rs and extrasynaptic, tonic inhibitory currents suggests that these channels may mediate a component of GABAergic inhibition that is not subject to the phasic rundown shown by non-δ-containing receptors. Thus, the loss of δ subunits may constitute an additional mechanism by which selective changes in subunit expression could influence neuronal excitability through attenuation of a steady-state inhibitory current.

Downregulation of GABAergic function has also been seen in a model of perinatal hypoxia. While this model in itself does not produce epilepsy, it does produce persistent neuronal hyperexcitability, and this study is instructive for the mechanisms found to underlie altered inhibition. Acutely following a hypoxic episode in immature rats, evoked IPSCs in CA1 hippocampal pyramidal neurons are decreased. This effect is dependent both on Ca^{2+} influx through Ca^{2+}-permeable AMPA receptors, and activation of the serine-threonine phosphatase calcineurin. Correspondingly, calcineurin activity is increased postseizure and the phosphorylation of $GABA_A$R β2 and β3 subunits is also decreased (although it is not clear that these subunits are responsible for the decrease in IPSCs observed). This work points to signaling pathways, such as calcineurin, that might be acutely activated following status epilepticus. Identification of such mechanisms underlying the induction of ion channelopathy after a neural insult is a significant advance, for it opens the possibility of future therapeutic advances that might target these pathways. For example, phosphorylation signaling is an active area of clinical drug development, with numerous inhibitors of kinases and phosphatases either in preclinical testing, or in a few cases, already in clinical use (e.g., FK-506, an inhibitor of calcineurin used for immunosuppression in organ transplant). These agents are useful tools to test whether interruption of the signaling that underlies acquired channelopathy might prevent the ion channel alterations, and perhaps influence epileptogenesis as well.

Future Directions

The studies described here clearly demonstrate that experimental acquired epilepsy is associated with alterations in both voltage-gated and ligand-gated ion channel expression, including their biophysical properties. It is tempting to conclude that, as with genetic epilepsies, these changes in ion channel properties underlie the basis of neuronal hyperexcitability that produces seizures. But the situation with acquired epilepsy is not nearly so straightforward as with genetic epilepsy, and there are numerous challenges to be surmounted before we understand how *acquired* channelopathy contributes to epilepsy. While the genetic epilepsy literature shows that alteration of the properties of a single species of ion

channel is sufficient to cause a human epilepsy syndrome, the sheer number of ion channel changes observed in acquired epilepsy makes it unlikely that any one of them is causative on its own. The post-SE models used in experimental epilepsy induce a diffuse insult to the CNS that triggers widespread changes in neuronal function and viability. Associated with these changes in cell properties are multiple changes in ion channel function – some of which may contribute to epileptogenesis, some which may cause hyperexcitability but which may not influence epileptogenesis, and some of which may be homeostatic in nature, opposing the induced hyperexcitability.

To better understand how acquired channelopathy contributes to the induction and maintenance of the epileptic state, we will need to study ion channel changes at a more comprehensive level of detail than has often applied. One issue is that the course of epileptogenesis in the different experimental models is still to be detailed and 'validated'; thus, an assumption that a given ion channel change is occurring during the latent phase (and may be contributing to epileptogenesis) may be unwarranted. Measuring the onset of spontaneous seizures, using chronic video-EEG recordings, is a time-consuming but important step to determine the true extent of the latent period and to identify ion channel changes that are relevant.

Another question is whether ion channel changes observed in chronic epilepsy are independent of ongoing seizures. This point can be tested with periods of AED treatment after epilepsy is established. A change in ion channel properties that occurs before the onset of seizures and persists after seizure suppression is a possible contributor to epileptogenesis, while those that revert to normal after AED treatment are likely to be a result of the epileptic activity – although they may be involved in the maintenance of the epileptic state by promoting hyperexcitability. Another issue that often remains unproven in experimental epilepsy is whether alteration of a given ion channel actually causes hyperexcitability. While it might be assumed that a downregulation of K^+ channel activity or an upregulation of Na^+ activity is necessarily proconvulsive, counterintuitive examples abound – for example, the loss of I_{Na} in most cases of SMEI, and a recent description of a gain of function in a Ca^{2+}-activated K^+ channel in a genetic epilepsy pedigree.

Finally, evidence for a causative role of ion channelopathy in epileptogenesis will be strengthened by demonstration that reversing the channelopathy modifies the course of epileptogenesis. A recent exciting example of this approach was illustrated using gene therapy to increase expression of downregulated $GABA_AR$ subunits, delaying the onset of spontaneous seizures post-SE. Continuing advances in understanding the molecular mechanisms underlying acquired channelopathy offer the promise that similar benefits might result from pharmacological blockade of signaling intermediaries, such as calcineurin. Such clinical applicability, of course, remains the ultimate goal of understanding the process of epileptogenesis – the hope of intervening to prevent epilepsy after an acute insult to the CNS.

See also: **Epileptogenesis**: Surrogate Markers for Epileptogenesis; **Ion Channels**: Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Post-Translational Modifications of Ion Channels in Epilepsy; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Post-Traumatic Epilepsy**: Posttraumatic Models.

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Channelopathies in Epilepsy

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Introduction

Genetic factors are thought to account for approximately 70% of the risk of developing epilepsy. Although the genes responsible for the vast majority of this risk are not known, an increasing number of mutations in single genes have been identified in rare families. These families generally exhibit autosomal dominant transmission with high penetrance. Most of the genes identified in these families encode ion channels. This finding provides an unparalleled insight into possible mechanisms of sporadic epilepsy, which has – in many cases – very similar clinical features as monogenic epilepsy. The role of these 'channelopathy' genes furthermore suggests that inherited variation in ion channel genes may account for much of the heritability of common forms of epilepsy, although this possibility is yet to be proved.

This overview of the 'epileptic channelopathies' briefly considers how mutations can affect ion channel biogenesis and function, describes some of the syndromes that have been identified in association with such mutations (see Figure 1, Table 1), and then considers each of the main ion channels affected. We have, where possible, attempted to 'explain' the clinical phenotype on the basis of what is known of the normal function of the ion channels and of the mechanisms of the mutations.

Background

How do Ion Channel Mutations Affect Brain Function?

In principle, mutations can affect almost every step of gene expression – from transcription through ion channel function, including translation, assembly, trafficking from the endoplasmatic reticulum to the normal location in the neuronal membrane, gating, and permeation. However, mutations in promoter sequences have not yet been identified (although this fact could simply reflect our poor knowledge of promoter sequences). Mutations have been identified in introns and exons, and can consist of single nucleotide changes, or deletions or insertions. Many mutations lead to frameshifts or splice site alterations that generally result in aberrant sequences terminating in premature stop codons. Missense mutations in coding sequences can substantially alter channel properties if they are located in strategic positions (such as voltage-sensing or pore-forming parts of the channel, or regions necessary for correct folding of the peptide, or interaction with accessory subunits or other proteins).

Most attempts to understand the consequences of missense gene mutations have relied on heterologous expression in nonneuronal systems, although mouse knock-in models are providing invaluable insights. Intuitively, seizures might be expected to result from gain-of-function mutations of Na^+ and Ca^{2+} channels or loss-of-function mutations of Cl^- channels such as associated with $GABA_A$ receptors (which mostly mediate inhibitory neurotransmission), or some types of K^+ channels (which set the resting potential and terminate action potentials). Some channelopathies indeed conform to this assumption, but there are some notable exceptions, as are discussed below. Furthermore, this simple view of ion channels cannot account for the differences in phenotypes associated with distinct mutations affecting any one class of ion channels.

The link from ion channel mutation to clinical manifestation can be complicated for a variety of reasons.

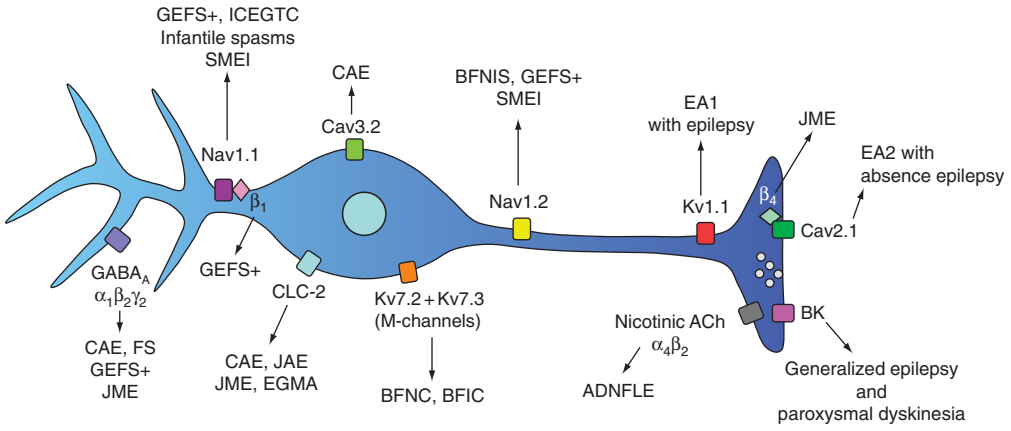


Figure 1 Summary of ion channels whose mutations cause epilepsy in some families.

Table 1 Overview of ion channels and modulating molecules whose mutations are associated with epilepsy

Channel type	*Gene*	*Protein*	*Clinical phenotype*
Voltage-gated			
Na^+ channels	SCN1A	α subunit of Nav1.1	• GEFS+ • ICEGTC • Infantile spasms • SMEI
	SCN2A	α subunit of Nav1.2	• BFNIS • GEFS+ • SMEI
	SCN1B	β_1 subunit	• GEFS+
K^+ channels	KCNQ2 KCNQ3	M-type K^+ channel subunits	• BFNC with or without myokymia • BFIC
	KCNA1	α Subunit of Kv1.1	• EA1 with epilepsy[a]
	KCNMA1	α Subunit BK channels	• Generalized epilepsy and paroxysmal dyskinesia[a]
Ca^{2+} channels	CACNA1A	α_{1A} Subunit of Cav2.1 (P/Q-type)	• EA2 with absence epilepsy[a]
	CACNB4	β_4 Subunit	• JME
Cl^- channels	CLCN2	CLC-2	• CAE • EGMA • JAE • JME
Ligand-gated			
Nicotinic ACh receptors	CHRNA2	α_2 Subunit	• ADNFLE
	CHRNA4	α_4 Subunit	
	CHRNB2	β_2 Subunit	
$GABA_A$ receptors	GABRA1	α_1 Subunit	• CAE • JME
	GABRG2	γ_2 Subunit	• CAE • FS • GEFS+
Molecules with influence			
on Ca^{2+} channels	EFHC1	EFHC1	• JME
on K^+ channels and/or AMPA receptors	LGI1	LGI1	• Autosomal dominant temporal lobe epilepsy with auditory features

[a]Mixed clinical phenotypes with epilepsies and nonepileptic paroxysmal neurological disorders.

Ion channels often display precise developmental and regional, cellular and subcellular expression patterns, and can undergo differential splicing and post-translational modifications. These factors crucially affect how mutations change the properties of neurons and neuronal networks. For instance, it has been argued that loss-of-function mutations of one Na^+ channel may cause epilepsy because it plays an especially important role in inhibitory interneurons. Among other factors that can distort the relationship between the molecular consequence of the mutation and its associated phenotype is that different splice variants of a given ion channel can alter its properties, and that the same channel can have different roles depending on the accessory subunits with which it assembles. Mutations can also have unexpected effects on the expression of other channels with similar or different properties.

Clinical Phenotypes in the Epileptic Channelopathies

Many of the epileptic channelopathies that have been identified exhibit clinical features indistinguishable from common sporadic idiopathic generalized epilepsies. The latter include childhood absence epilepsy (CAE), juvenile absence epilepsy (JAE), juvenile myoclonic epilepsy (JME), and epilepsy with grand mal seizures on awakening (EGMA). Only a very small proportion of patients with these syndromic diagnoses have been shown to have mutations in the known epilepsy genes.

Among other syndromes that have been identified in patients with ion channel mutations are various types of benign neonatal or infantile seizures, which tend to resolve spontaneously. When there is demonstrated familial clustering, these syndromes have been named benign familial neonatal convulsions (BFNC), benign familial infantile seizures (BFIS), and the intermediate condition benign familial neonatal–infantile seizures (BFNIS). Again, making a diagnosis of benign neonatal or infantile convulsions does not predict that a mutation in one of the candidate ion channels associated with the familial disorders will be identified. Some channelopathies are associated with a very pleomorphic presentation, suggesting a strong influence of modifying genes or environmental factors. For example, patients with generalized

epilepsy with febrile seizures plus (GEFS+) carrying the same mutation exhibit highly variable forms of generalized epilepsy with respect to seizure type and severity, but with the common factor that they tend also to exhibit febrile seizures in childhood persisting beyond the age of 6 years. At the more severe end of this spectrum is severe myoclonic epilepsy of infancy (SMEI, Dravet syndrome), which is characterized by refractory and fever-sensitive generalized clonic, tonic–clonic or unilateral seizures that usually start in the first year of life, tend to worsen, and are frequently accompanied by cognitive deterioration. Clinically very similar, except for the absence of myoclonic seizures, is intractable childhood epilepsy with frequent generalized tonic–clonic seizures (ICEGTC). A diagnosis of SMEI carries a high probability that the affected child carries a mutation in the Na^+ channel gene SCN1A.

Finally, the rare syndrome of nocturnal frontal lobe epilepsy presents with focal motor seizures, often arising from sleep. If inherited in an autosomal dominant manner (ADNFLE), this disorder should prompt screening for a mutation in one of the nicotinic receptor subunit genes.

Methods

Gene Identification

Many genes underlying monogenic epilepsy have been identified by linkage mapping in large kindreds that exhibit Mendelian inheritance, followed by sequencing of genes in the interval thus defined. Among obstacles frequently encountered are difficulties in ascertainment, phenotypic variability, and the occurrence of sporadic epilepsy in the background population. Almost all epileptic channelopathies thus elucidated are transmitted in an autosomal dominant manner. It is not clear whether many recessively transmitted channelopathies remain to be discovered.

Although mutations in ion channel genes have, for a long time, been considered strong candidates to cause epilepsy, there are several hundred genes in this family, most of which have not been implicated in epilepsy. Nevertheless, the discovery of mutations in some ion channel genes has prompted sequencing of closely related genes in families or individuals with epilepsy, with some success.

Functional Expression

Some mutations are predicted to disrupt ion channel synthesis, for instance because they lead to premature truncation of the peptide. However, many other mutations alter one or a few amino acids, and in this situation heterologous expression combined with voltage clamp electrophysiology can shed light on the consequences of the sequence change for ion channel function. Briefly, this method consists of expressing the mutant ion channel gene, together with necessary auxiliary proteins, in cells grown in vitro. Among cells that have been used are oocytes of the Xenopus frog, cell lines such as human embryonal kidney (HEK) cells, and primary cultures of rodent neurons (although these tend to be more difficult to transfect). The ion channel kinetics can be studied by recording the current passing across the membrane in response to imposed voltage steps or agonist applications. Taken together, information about total current density and the behavior of individual channels provide a reasonably complete description of channel properties, which can be compared with properties of wild-type channels. Thus, it is generally possible to infer whether the channel mutation is likely to give rise to a loss or gain of function. Coexpression of the mutant channel with wild-type channels can also shed light on how mutations are likely to interact with the unaffected allele in heterozygous individuals.

Animal Models

Since ion channels may have very different roles in different neurons, and normally exhibit highly diverse patters of expression in the brain, it is not always possible to extrapolate from altered ion channel function to altered circuit function. Mouse models have been generated for several mutations, either by knocking out the orthologous ion channel gene (for nonsense or loss-of-function mutations) or by knocking in a missense mutation. Such animals can be studied with video and EEG for spontaneous seizure pattern, and with provocation methods (for instance with chemoconvulsant administration to look for altered seizure threshold). They can also be sacrificed for acute brain slice electrophysiology, which can shed light on changes in synaptic properties and neuronal excitability. Although these are very powerful methods, species differences can hinder a clear explanation for why some mutations predispose to seizure occurrence.

Results

Voltage-Gated Ion Channels

Sodium channels

Voltage-gated Na^+ (Nav) channels are composed of a pore-forming α-subunit, a large trans-membrane protein with four repeated domains, and accessory β-subunits (β_{1-4}), which influence the channel's cell surface expression and its biophysical properties. Five of the nine mammalian α-subunit genes are expressed in the brain (SCN1A-3A, SCN5A, SCN8A), with differential regional and subcellular expression patterns.

SCN1A mutations

A large number of inherited and de novo heterozygous mutations in SCN1A (chromosomal location 2q24) have been identified in GEFS+, SMEI, and ICEGTC.

Mutations associated with GEFS+ tend to be missense, whereas those associated with SMEI also include nonsense or frameshift mutations.

Functional expression studies of GEFS+ mutant channels have not yielded an unambiguous indication whether the disease results from gain- or loss-of-function. Some mutations appear to slow Na^+ channel inactivation, or alter its voltage dependence, resulting in an increase in Na^+ current. Other effects of GEFS+ mutations, such as depolarization of the activation threshold, slowed recovery from inactivation, and decreased current density, point instead to a reduction in Na^+ current. Although distinct mutations have different effects, some of the inconsistencies may also reflect the limitations of heterologous expression systems in which these channel properties are analyzed, which do not fully capture the behavior of channels in their native environment.

The fact that most mutations identified in association with SMEI result in disruption of one allele of SCN1A argues strongly for loss of Na^+ channel function in this disorder. Although several of the missense mutations also identified in this disorder have been shown to affect a broad range of biophysical properties, haploinsufficiency is the commonest finding in SMEI. This feature is perhaps unexpected, because loss of Na^+ currents would be expected to cause neuronal hypoexcitability. A possible resolution of this paradox, suggested by a recent analysis of a mouse SCN1A knock-out mutant, is that inhibitory interneurons, unlike principal cells, fail to tolerate a reduction in gene dosage. Thus, the product of the wild-type allele, and of genes coding for related Nav channels, is not sufficient to allow efficient spiking, resulting in a net increase in neuronal network excitability.

SCN2A mutations

Missense and nonsense mutations of SCN2A (chromosomal location 2q23-q24) have been associated with BFNIS, GEFS+, and a SMEI-like phenotype. Again, it has proved difficult to establish a consistent pattern of biophysical consequences for the different mutations identified.

SCN1B mutations

The β_1 subunit of Nav channels consists of a single membrane-spanning protein with a large N-terminal extracellular domain. β-Subunits increase the functional expression of α-subunits when coexpressed. They also accelerate the inactivation of sodium currents by interactions between their immunoglobulin-like fold and α-subunits. Only a few mutations in the SCN1B gene (chromosomal location 19q13) coding for β_1-subunits have been reported in GEFS+. Since β_1-subunits normally help to traffic Na^+ channels to the membrane, and also modulate inactivation kinetics, it is again difficult to establish with certainty whether epilepsy results from a net increase or decrease in Nav function.

Potassium channels

K^+ channels are a very large and heterogeneous group of ion channels, only a few of which have been implicated in monogenic epilepsy.

KCNA1 mutations

KCNA1 encodes the α-subunit of Kv1.1 channels. One α-subunit consists of 6 transmembrane segments with a voltage-sensor (S4) and the pore-loop between S5 and S6, and is homologous to each of the four repeated domains of Na^+ channels. Four α-subunits (which can be different members of the Kv1 family) assemble together with four β-subunits to constitute a complete channel, and the identities of the subunits determine its biophysical properties. Kv1.1-containing channels are mainly expressed in the axons of neurons throughout the nervous system, where they contribute to membrane potential stabilization and repolarization after action potentials. They are also thought to curtail the presynaptic depolarization that drives Ca^{2+} channel opening and neurotransmitter release.

Heterozygous missense KCNA1 mutations (chromosome location 12p13.32) cause autosomal dominant Episodic Ataxia type 1 (EA1), which can be accompanied by partial or generalized seizures. The mutations cause loss-of-function, although some have also been shown to interfere with inactivation. It remains unclear why only some of the mutations are associated with epilepsy.

KCNQ mutations

Voltage-dependent Kv7.1-Kv7.5 channel α-subunits are encoded by KCNQ1–5. Kv7.2 and Kv7.3 co-assemble to form channels that underlie the so-called 'M' current, which is modulated by muscarinic acetylcholine receptors, and which contributes to the neuronal input resistance at resting membrane potential. Heterozygous mutations in KCNQ2 or KCNQ3 (chromosomal locations 20q13.3 and 8q24) occur in BFNC, and generally decrease current density in heterologous expression. Since the affected individuals are heterozygous, only relatively small degrees of loss of function are sufficient to give rise to this syndrome. It remains unclear why BFNC usually remits spontaneously after a few weeks of age.

KCNMA1 mutations

KCNMA1 (chromosomal location 10q22) encodes the BK (or SLO1) channel. BK is a voltage- and Ca^{2+}-activated K^+ channel with a large ('big,' hence BK) conductance. Although BK shares features in common with other voltage-dependent channels, it has two special features:

each α-subunit consists of seven instead of six transmembrane segments, and the cytoplasmic-terminal contains a large Ca^{2+}-binding tail domain. BK channels are widely expressed in the brain and present on cell bodies, dendrites, and presynaptic terminals, where they contribute to the repolarizing phase of action potentials and the early part of the afterhyperpolarization.

A heterozygous missense mutation in KCNMA1 was found to cause generalized epilepsy and paroxysmal dyskinesia. Surprisingly, the mutation has a gain-of-function effect, increasing the Ca^{2+} sensitivity of the channels, which would be expected to decrease firing and neurotransmitter release. Among possible explanations for the neurological phenotype is that faster repolarization could minimize Na^+ channel inactivation, or facilitate the opening of I_h channels, which have a pacemaker function in some neurons. Either or both of these effects could theoretically allow neurons to fire at higher frequencies.

Calcium channels

Ca^{2+} channels have the same overall structure as Na^+ channels, with one pore-forming α_1-subunit containing four domains, each consisting of six transmembrane segments that resemble voltage-gated K^+ channel subunits. Ca^{2+} channels, however, tend to have slower kinetics, and the outer pore selectivity filter makes them permeable to Ca^{2+} ions. Accessory β-, $\alpha_2\delta$-, and (in some cases) γ-subunits influence their biophysical properties and surface expression. Nine different α_1-subunits occur, and are grouped into three classes (Cav1–3) with distinct biophysical and pharmacological properties, and different regional, cellular and subcellular expression patterns.

CACNA1A mutations

CACNA1A encodes the α_{1A}-subunit of Cav2.1 channels (also known as P/Q-type channels). Apart from the cerebellum (where they are abundantly expressed in the cell bodies and dendrites of Purkinje and granule cells), Cav2.1 channels are predominantly expressed on presynaptic terminals throughout the nervous system, where (together with Cav2.2 and, to a lesser extent, Cav2.3) they mediate the Ca^{2+} influx that triggers neurotransmitter release. Heterozygous mutations in CACNA1A (chromosomal location 19p13.2–p13.1) are associated with familial hemiplegic migraine, spino-cerebellar ataxia type 6, and episodic ataxia type 2 (EA2). Some patients with EA2 also suffer from absence seizures. Functional expression studies reveal loss-of-function effects of the mutations associated with EA2 and absence epilepsy.

CACNB4 mutation

To date, only one premature stop codon mutation in CACNB4 (chromosomal location 2q22–23, encoding the cytoplasmic β_4-subunit) has been identified in association with JME. This mutation, too, is expected to have a loss-of-function effect.

How does a reduction in Ca^{2+} current cause absence epilepsy? Interestingly, homozygous mutations in CACNA1A and CACNB4 have been identified in several inbred mouse strains, which have in common frequent spike-wave seizures and gait ataxia. Some indirect evidence obtained in these mice suggests that the balance of excitatory and inhibitory neurotransmission within the thalamocortical loop may be disturbed, leading to absence seizures. Compensatory changes in expression of Cav2.2 (N-type) and Cav3.x (T-type) channels may also contribute to seizure initiation. The involvement of Cav3.2 in absence epilepsy is, furthermore, supported by the finding that CACNA1H mutations occasionally occur in association with CAE. Some of these mutations have been shown to confer gain-of-function effects, in line with the prominent role of Cav3 channels in intrinsic oscillations of thalamic neurons. However, since the mutations do not cosegregate with CAE within families with affected individuals, CACNA1H may actually be a susceptibility locus that interacts with other genetic or environmental factors to determine the risk of developing this form of epilepsy.

Chloride channels

CLC channels are unrelated to other voltage-gated channels, and have a dimeric structure. Five of the nine members of the CLC family are expressed in the CNS. Although CLC-2 encoded by CLCN2 is widely present in the brain, the roles of this channel in neuronal function are poorly understood. The channels are modulated by membrane potential, internal Cl^- concentration, and pH, and mediate an inwardly rectifying current.

CLCN2 mutations

Three heterozygous CLCN2 (chromosomal location 3q26) mutations have been identified in association with different forms of idiopathic generalized epilepsy, including CAE, JAE, JME, and EGMA. Although two mutations resulted in nonfunctional channels, the third missense mutation has been reported to have a gain-of-function effect (although this report has been disputed). A possible explanation for epilepsy in association with loss of CLC-2 function is that the neuronal capacity to regulate internal chloride is impaired, thereby interfering with $GABA_A$ receptor-mediated inhibition.

Ligand-Gated Ion Channels

Ligand-gated ion channels (or ionotropic receptors) generally open when a specific neurotransmitter binds to an extracellular domain of the receptor/channel complex.

The channels implicated in epilepsy all belong to the nicotinic family, which are pentameric; each subunit has four transmembrane segments (termed M1-M4) with M2 contributing to the ion-conducting pore.

Nicotinic acetylcholine receptors

Neuronal nicotinic acetylcholine receptors (nAChRs) are either homo- or heteropentameric. The heteropentameric channels are composed of α_{2-6} and β_{2-4} subunits with an $(\alpha_x)_2(\beta_y)_3$ stoichiometry. A common subtype in the brain is $\alpha_4\beta_2$, with predominant expression in the thalamus and cortex. These channels have a high affinity for acetylcholine and nicotine, and are permeable to Na^+ and (to a lesser extent) Ca^{2+} ions. Because of predominant presynaptic expression, neuronal nAChRs are thought to modulate neurotransmitter release, but how they affect neuronal networks remains poorly understood.

CHRNA2, CHRNA4, and CHRNB2 mutations

Heterozygous mutations in the CHRNA2, CHRNA4, and CHRNB2 genes have been identified in about 10% of patients suffering from nocturnal frontal lobe epilepsy (NFLE), most commonly when this is transmitted in an autosomal dominant fashion (ADNFLE). Seven mostly missense mutations have been reported in CHRNA4 (chromosomal location 20q13) encoding the α_4-subunit and CHRNB2 (chromosomal location 1q21) coding for the β_2-subunit, whereas only one mutation has been reported so far for CHRNA2 (chromosomal location 8p12.3–8q12.3) which codes for the α_2-subunit. Overall, these mutations mostly affect the transmembrane segment M2 which contributes to the ion pore. However, the functional consequences of the different mutations are highly diverse, with different effects on Ca^{2+} dependence and Ca^{2+} permeability, and also opposite effects on the apparent acetylcholine affinity or desensitization kinetics. It is therefore difficult to identify a single common biophysical feature that could point to a net loss- or gain-of-function. The situation is further complicated by the various possible stoichiometries that can potentially exist if the affected subunit is substituted by wild type or other subunits. Mouse knock-in models have been generated for several ADNFLE mutations. Although homozygous mutant mice exhibit spontaneous and/or nicotine-evoked seizures and, in some cases, dystonic posturing, the neural circuits mediating these phenomena are not clear. Thus, a mechanistic explanation for epilepsy in association with nicotinic receptor mutations is still elusive. Why these nicotinic receptor-associated seizures selectively originate from the frontal lobe, and why they tend to occur during light sleep, remain to be explained.

$GABA_A$ receptors

$GABA_A$ receptors are heteropentameric and share many structural features with nicotinic receptors, although they are selectively permeable to Cl^- and, to a lesser extent, HCO_3^-. There are 16 different subunits (α_{1-6}, β_{1-3}, γ_{1-3}, δ, ε, π, and θ), with channels composed of $\alpha_1\beta_2\gamma_2$ being the most abundant in the brain. $GABA_A$ receptors open upon GABA-binding and permit chloride ions to flow across the cell membrane. This channel opening results in shunting of membrane currents as well as in a membrane potential change, the polarity of which depends on the intracellular Cl^- concentration; the intracellular Cl^- concentration generally decreases with development, making most GABAergic synapses hyperpolarizing in adult neurons. The receptor kinetics, as well as their sensitivity to drugs and endogenous modulators such as zinc and neurosteroids, depend on the subunit composition of the channel. $GABA_A$ receptors are widely expressed in the CNS, with predominant postsynaptic localization on cell bodies and dendrites where they mediate phasic inhibition. Extrasynaptic $GABA_A$ receptors have also been identified, which mediate tonic inhibition of neuronal activity.

GABRG2 and GABRA1 mutations

Heterozygous mutations in GABRG2 (chromosomal location 5q32–34) coding for the γ_2 subunit, have been shown to cause GEFS+, CAE, and febrile seizures. The missense mutations all lead to loss-of-function, although through different molecular mechanisms. Heterozygous mutations in GABRA1 (chromosomal location 5q34) encoding the α_1 subunit have also been found in association with autosomal dominant JME and a sporadic form of CAE. These also result in loss-of-function.

A reduction in functional $GABA_A$ receptors leads to impaired neuronal inhibition, favoring the occurrence of seizures. A recent experimental study has suggested that temperature-dependent impairment in membrane trafficking of the γ_2 subunit may explain, at least in part, why febrile seizures occur.

Molecules Modulating Ion Channels

Only two of the genes identified as causing relatively 'pure' epilepsy do not encode ion channels.

EFHC1

The EFHC1 gene encodes a protein also called EFHC1, which contains an EF-hand motif which is able to bind Ca^{2+} ions. Missense mutations in the EFHC1 gene have been identified in JME. Interestingly, EFHC1 is co-localized with Cav2.3 (R-type) Ca^{2+} channels, which contribute to the generation of neuronal afterdepolarization and bursting, and to Ca^{2+} influx in dendrites and presynaptic terminals. Furthermore, wild-type EFHC1 has been shown to increase Cav2.3 currents and to enhance apoptosis in vitro, while the mutations prevent these effects. A possible explanation linking EFHC1

mutations and epilepsy is that reduced apoptosis interferes with the normal elimination of neurons during development, thereby leading to hyperexcitable circuits. This proposal remains speculative.

LGI1

Leucine-rich glioma inactivated gene 1 (LGI1, chromosomal location 10q24) is a secreted non-ion channel protein that is mutated in autosomal dominant lateral temporal lobe epilepsy (ADLTLE, also know as autosomal dominant partial epilepsy with auditory features, ADPEAF). This unusual syndrome differs from other forms of temporal lobe epilepsy in that seizures can be precipitated by auditory stimuli or accompanied by auditory hallucinations. Two cellular mechanisms that could possibly relate mutant LGI1 to seizure generation have been proposed, both involving ion channel modulation. First, wild-type LGI-1 has been suggested to modulate the fast inactivation of K^+ channels composed of Kv1.1, Kv1.4, and Kvβ1 subunits. ADLTLE-associated mutant LGI-1, in contrast, appears to be incapable of slowing rapid inactivation, resulting in K^+ channels that inactivate more rapidly. This feature could, in turn, result in excessive presynaptic Ca^{2+} influx and neurotransmitter release, favoring seizure generation. This hypothesis is problematic because fast inactivation takes place on the cytoplasmic aspect of Kv channels, and yet LGI-1 is mainly extracellular. Second, wild-type LGI1 has been reported to increase the surface expression of excitatory AMPA-type glutamate receptors, via an interaction with the transmembrane adhesion protein ADAM22. As mutant LGI1 appears not to be able to bind ADAM22, this mutation could result in a decrease in excitatory AMPA currents. How this effect could result in epilepsy remains unclear, let alone why the seizures have auditory features.

Conclusion and Directions for Future Research

The list of ion channels mutated in epilepsy will, no doubt, continue to grow. Two main challenges for future research stand out. First, although 'explaining' epilepsy is reasonably straightforward in a few cases (for instance for the $GABA_A$ receptor mutations), it remains difficult to relate the molecular lesion to the syndrome for many other ion channels. Progress will require a detailed understanding of brain physiology, and benefit from mouse models of monogenic disease. Second, the monogenic epilepsies remain extremely rare, but idiopathic epilepsy is very common and shows strong heritability. The ion channel genes are obvious candidate susceptibility factors, and some progress has been made in identifying CACNA1H as one quantitative trait locus. To what extent subtle genetic variants in other ion channel genes, inherited in combination, account for the bulk of idiopathic epilepsy, remains to be determined.

See also: **Genetics**: Clinical Genetics of Epilepsy; Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Sodium Channel Mutations that Cause GEFS+.

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Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies

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Introduction

Epileptic seizures are the result of excessive discharges arising in a population of neurons with altered excitable properties. These alterations are relevant to epileptic discharge when they involve a critical mass of hyperexcitable cells in highly synchronized activities. Most epileptic seizures are due to discharges generated in the cerebral cortex (neocortex) and hippocampal structures (also referred to as the paleocortex), although subcortical structures may also be involved. Here, the neocortical cell properties relevant to epileptic discharges are discussed.

Investigations in experimental animals have demonstrated that a number of changes in cell excitability can lead to the generation of epileptic discharges. An understanding of these changes, and in particular the pathways through which expression of gene mutations give rise to these changes, will help us understand the bases for many human epilepsies. Investigations in experimental preparations focusing on neurophysiological–biomolecular analysis of genetically determined structure–function alterations in ligand and voltage-gated channels have further advanced our understanding of the cell mechanisms responsible for excessive excitability.

Background

Excitable Properties of Nerve Cell Membrane

The lipid bilayer of the cytoplasmic membrane of nerve cells is largely impermeable to ions. Neuron excitability depends on Ion movements across the cell membrane through selective gates, schematically represented in **Figure 1(a)** The sodium (Na^+)–potassium(K^+) pump transports Na^+ from the intracellular to the extracellular compartment and K^+ in the opposite direction, in a ratio of 3:2. As Na^+ and K^+ are both monovalent, the result of Na^+–K^+ pump activity is membrane polarization, with the intracellular compartment becoming more negative. Under resting conditions, the difference between extra- and intracellular potentials (resting membrane potential) can be measured by means of a suitable electrode; its value is about −70 mV (**Figure 1(b)**, green lines). The energy required for transmembrane Na^+–K^+ ion transport is provided by adenosine triphosphate (ATP) molecules, hence the term Na^+–K^+ ATP pump.

Ion movements through the voltage-sensitive and the ligand-activated channels are simply due to electrostatic forces and the different ion concentrations between the extra- and intracellular compartments (electrochemical gradient), and therefore do not require an energy supply. Ionic movements through these gates (which are selectively permeable to specific ions) generate transient positive (depolarizing) or negative (hyperpolarizing) changes in membrane potential that determine cell excitability. When a channel opens, the type of ion to which it is selectively permeable moves passively on the basis of its electrochemical gradient until membrane potential attains the specific equilibrium potential for that ion. As the intracellular/extracellular Na^+ concentration gradient can be actively reconstituted by the Na^+–K^+ ATP pump, it can be said that ionic pumps create and maintain the resting membrane potential that is the prerequisite for excitation, whereas the ionic currents flowing through the channels are the main determinants of an excitable state.

The examples in **Figure 1(a)** refer to the Cl^- channel associated with the γ-aminobutyric acid (GABA) subtype A receptors (blue) and the voltage-activated Na^+ channel (red).

As a result of the extraordinary advances that have been made in molecular biology, we can now correlate the function and structure of ion channels, which are hetero-oligomeric membrane proteins typically consisting of 2–6 subunits, each subunit containing transmembrane segments that are assembled in a variable number of domains. The identification of the molecular structure of the various subunits and their corresponding coding genes has revealed a surprising multiplicity of distinct subunits whose assembly can lead to a great variety of channel subtypes with different properties. One experimental approach that has greatly contributed towards defining channel structure–function relationships is to express the channel constituents into elementary preparations – such as *Xenopus* oocytes, cell lines, or cultured neurons. This combined neurophysiological and biomolecular approach has made it possible to characterize the genetically determined alterations in intrinsic excitable properties that are responsible for human epilepsies.

Epileptogenic Alterations in Excitable Neuronal Properties

The role of ion channels in epileptogenesis has been recognized since the 1960s, when it was shown that

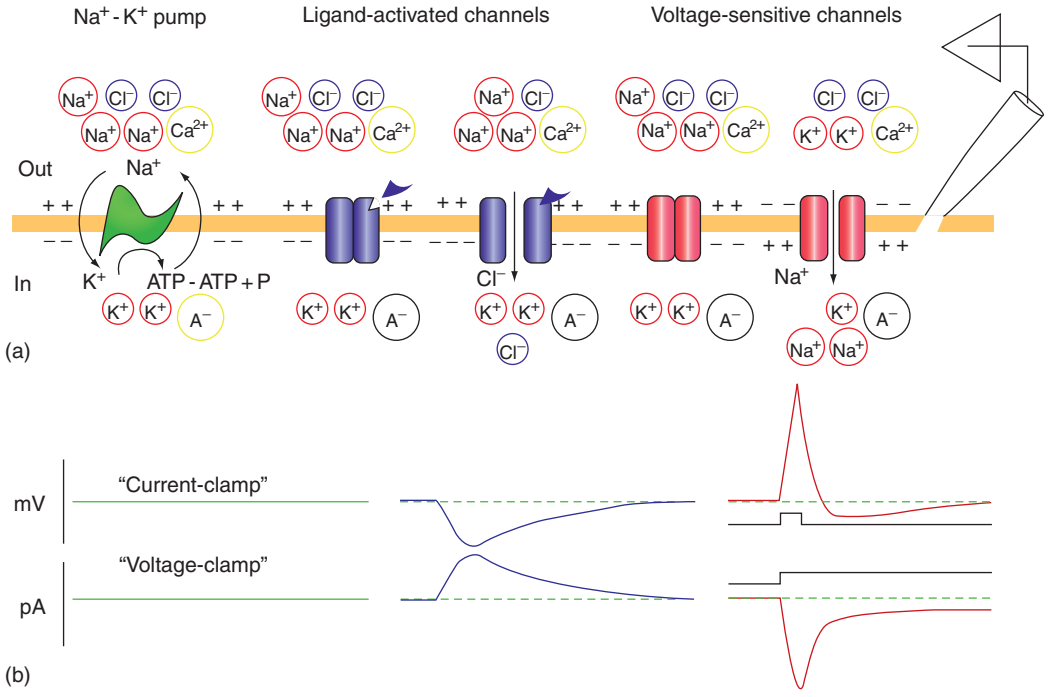


Figure 1 (a) Schematic representation of the cell membrane with Ionic pumps, ligand and voltage gated channels. The membrane is orange, and *out* and *in*, respectively, designate the extracellular and intracellular compartments. The main types of membrane gates are ion pumps (green), ligand-gated channels (blue) and voltage-gated channels (red). Ion movements across the membrane gates determine membrane potential and its changes relevant to neuron excitability. GABA receptors are key components of the inhibitory synapse between a presynaptic terminal of an inhibitory interneuron and a postsynaptic neuron. The inhibitory GABA neurotransmitter released from the presynaptic terminal binds postsynaptic receptors, which form a pore portion that is selectively permeable to either Cl^- (the $GABA_A$ subtype receptor) or K^+ (the $GABA_B$ subtype receptor). In the example shown in (a), the GABA-receptor interaction leads to a conformational change in the pore portion of the $GABA_A$ receptor that allows Cl^- to flow through it. At resting or positive-to-resting membrane potential, the Cl^- electrochemical gradient drives Cl^- from the extracellular to the intracellular compartment, thus leading to a negative-going change in membrane potential (b, upper blue trace) that represents the $GABA_A$ component of the inhibitory post-synaptic potential (IPSP). If the membrane potential is kept constant by means of voltage clamping, the time-course of the Cl^- current can be shown (b, lower blue trace). As Cl^- equilibrium potential is only slightly more negative than the resting potential, the ionic current has a limited amplitude. The Na+ permeable voltage-gated channel is shown in red. Depolarizing current pulses (b, black traces) open the channel and allow Na^+ to enter; as more Na^+ enters the intracellular compartment, the more the membrane depolarizes, thus putting a larger number of channels in the open conformation. This incremental depolarization (recorded by the microelectrode-b, upper red trace) creates the rising arm of the action potential. Once again, voltage clamping makes it possible to analyze the kinetics of the inward Na^+ current (b, lower red trace), which depends on its highly effective electrochemical driving force, its very positive equilibrium potential (around +60 mV), and the kinetics of Na^+ channel activation–inactivation. After opening, the large majority of Na^+ channels take on an inactivated conformation that does not allow any further ion flow. Reproduced from Avanzini G and Franceschetti S (2003) Cellular biology of epileptogenesis. *Lancet Neurology* 2: 33–42, with permission from Lancet Neurology.

neurons within experimentally induced epileptic foci have altered excitable properties that depend on transmembrane ion currents. Studies of various epilepsy models have shown that all of the neurons belonging to an epileptic aggregate consistently discharge in the form of protracted action potential bursts arising from a depolarized plateau (**Figure 2**). This 'paradigm' of an elementary epileptic discharge was initially observed in penicillin cortical foci and christened the paroxysmal depolarization shift (PDS) by Matsumoto and Ajmone Marsan; the PDS was later reported in different animal models and in human epileptogenic tissue, and therefore is considered reliable hallmark of an active epileptogenic process (see Further Reading section).

Similar discharges can be obtained using a number of experimental manipulations that enhance depolarizing ion currents (mainly carried by Na^+ and Ca^{2+} ions) or reduce hyperpolarizing ion currents (mainly carried by Cl^- and K^+ ions). However, it is only recently that we had direct evidence of the relevance of ion channel dysfunctions to naturally occurring epilepsies. One important step in this direction was made as a result of a series of experiments on the Drosophila Shaker mutant (carried out by the Jans and their colleagues), the results of which indicated that a mutation of a gene coding for a potassium channel caused the hyperexcitability that is responsible for the abnormal behavior of the Shaker mutant. The subsequent development of the analysis of genetically

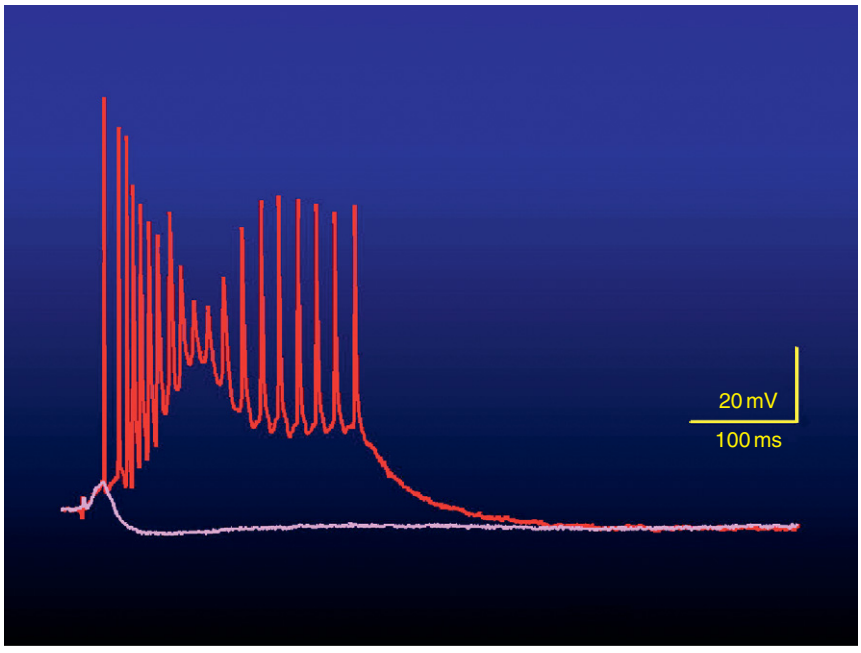


Figure 2 Stimulus-evoked paroxysmal depolarization shift (PDS) recorded from a pyramidal neuron in a bicucullin bathed hippocampal slice. Reproduced from Avanzini G and Franceschetti S (2004) Mechanisms of epileptogenesis. Shorvon S, Perucca E, Fish D, and Dodson E (eds.) *The Treatment of Epilepsy*, 2nd edn., pp. 74–83, with permission from Wiley – Blackwell.

determined channel alterations was greatly facilitated by the availability of techniques allowing gene mutations to be expressed in simple systems, which made it possible to characterize their functional consequences. The main types of experimental models used for such studies are summarized in **Figure 3**. Both in vitro and in vivo models are powerful means of assessing the functional consequences of the gene mutations that have been found to be associated with human epilepsies.

Methods

In vitro Models

Xenopus oocytes

The oocytes of the African clawed frog *Xenopus laevis* have been widely used as an expression system ever since it was first shown that messenger RNA (mRNA) injected into the cytoplasm can be translated into functional proteins and that receptors and channels could also be functionally expressed. Oocytes have a number of advantages: they can be easily removed without the need for animal sacrifice, they are large (a diameter of approximately 1–1.2 mm) and quite resistant to experimental manipulations, and they can be kept alive in vitro for a few weeks. They also have only a few endogenous currents, are easily impaled by microelectrodes, allow long and stable recordings, and make it possible to control the ratio of differently expressed subunits by injecting fixed amounts of RNA. Unfortunately, their large volume does not allow fast clamping of the membrane potential, a drawback that can be overcome by making patch–clamp recordings of macroscopic currents from a relatively large patch of oocyte membrane (macropatch), an approach that allows a good signal-to-noise ratio and fast voltage clamping. Alternatively, the cut-open oocyte voltage-clamp technique allows both fast voltage clamping and control of the composition of the intracellular medium. The most serious disadvantage of the oocyte system is its cell background; the expressed channels may interact with endogenous proteins and undergo posttranslational modifications that may be different from those occurring in mammalian cells. It is therefore not surprising that the ion channels expressed in *Xenopus* oocytes often have properties different from those studied in mammalian cell lines.

Mammalian cell lines

Heterologous expression in mammalian cell lines transfected with cDNA makes it possible to study the properties of the channel of interest while preserving a mammalian cell background. Two cell lines have become standard for ion channel studies: Chinese hamster ovary (CHO) cells and human embryonic kidney (HEK) cells, both of which have an epithelial-like morphology and very small endogenous currents, and are easy to maintain and transfect. They are also easy to use in electrophysiological experiments (typically, whole-cell patch–clamp recordings), thus allowing the relatively rapid screening of channel mutations, fast and accurate clamping of membrane voltage, and good control of the composition of the

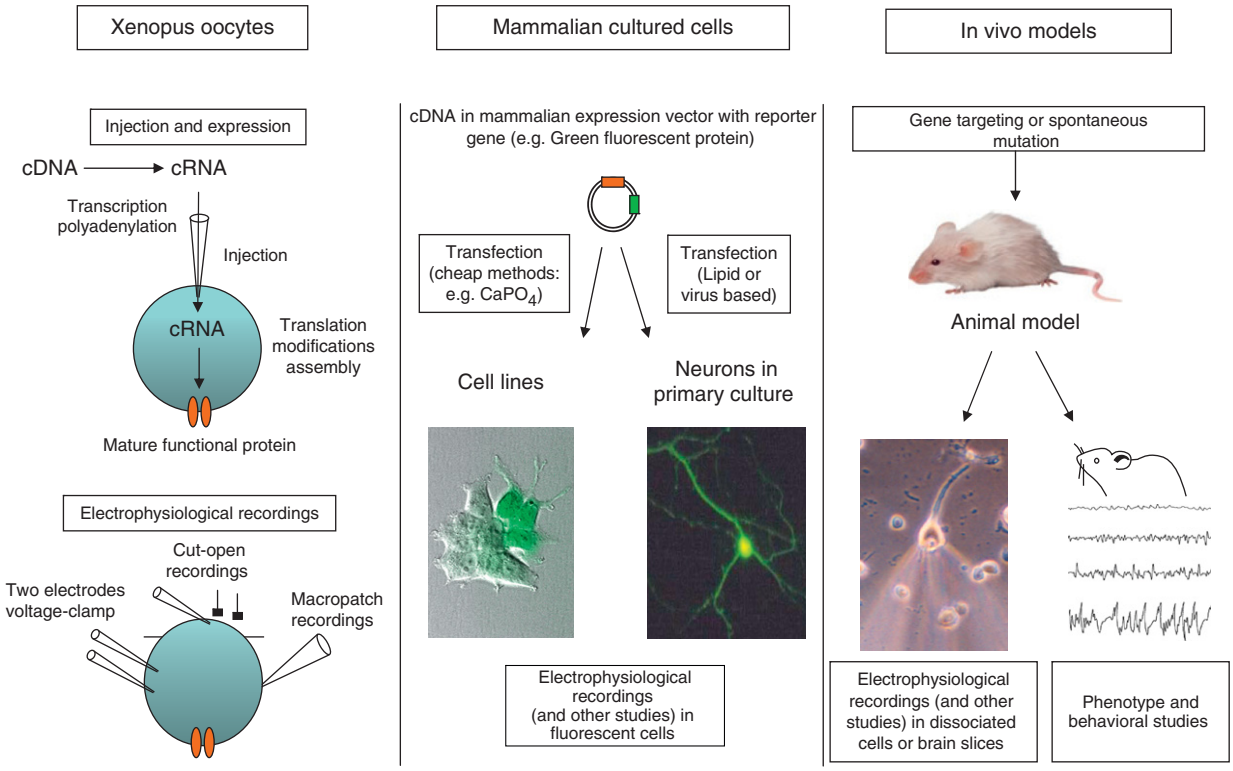


Figure 3 Experimental models for channelopathies. The most popular in vitro models are *Xenopus* oocytes and mammalian cultured cells. In vivo models include gene targeted animals and animal with spontaneous mutations. Detailed descriptions are to be found in the text. Reproduced from Avanzini G, Franceschetti S, and Mantegazza M (2007) Epileptogenic channelopathies: Experimental models of human pathologies. *Epilepsia* (supplement 2): 51–64, with permission from Blackwell publishing.

intracellular medium. In particular, HEK cells (especially the derived tsA-201 cell line) have been used because they have a human cell background (and express high density of the channel of interest when transfected). However, there are striking differences in channel properties when these channels are expressed in different mammalian cell lines (or even different subclones), possibly because the various cell lines express a variety of neuronal genes, including many accessory subunits of ion channels that may differently modulate exogenous channels and thus produce cell line-specific properties. Moreover, different cell lines can also have specific membrane lipid compositions, which may be surprisingly important when setting the functional properties of ion channels. Furthermore, cell lines do not completely reproduce the neuronal genetic background, cell variety, or subcellular specialization.

Primary cultured neurons

Transfected neurons in primary cultures provide an expression system that makes it possible to study exogenous channels while preserving neuronal properties, background, and variety. Neurons isolated from various brain areas can now be efficiently transfected and, in neocortical and hippocampal cultures, it is also easy to identify and make selective recordings of excitatory neurons (with typical pyramidal shapes) or inhibitory interneurons (bipolar, and positive for glutamic acid decarboxylase). Primary cultured neurons should therefore be the system of choice for reproducing differential neuronal cell backgrounds and studying the biophysical properties of wild-type and mutant subunits. However, these cells do have some disadvantages. First, neurons express a plethora of endogenous channels that need to be blocked in order to study exogenously transfected channels in isolation; therefore, the cultured neurons must be transfected with engineered exogenous channels that are resistant to specific blockers. Second, cultured central nervous system neurons develop long processes that make it difficult to space clamp membrane voltage; this problem can be overcome by making macropatch recordings as discussed earlier. Last, electrophysiological experiments with transfected cultured neurons are more difficult than those using cell lines.

In vivo Models

There are a large number of intact animal models in which to explore the consequences of genetic mutation on neuronal excitability. These models include animal strains presenting with spontaneous seizures, in which genetic studies have revealed specific ion channel

dysfunctions, and gene-targeted animals generated by means of genetic manipulation. The first group includes recessive mouse mutants in which Ca^{2+} mutations have been identified (e.g., tottering, lethargic, stargazer, and ducky mice). These animals exhibit spontaneous seizures similar to those of human epileptic syndromes with simple 'absence' seizures (both behavioral and EEG patterns); however, unlike human absence epilepsy (which often remits spontaneously and occurs in neurologically healthy subjects), the seizures in these animal models persist in adult and old rodents, and are associated with other neurological symptoms. Regarding the second set of 'targeted' models, there are currently relatively few that replicate human mutations. The phenotypes of some gene-targeted models are discussed below in comparison with the phenotypes of the corresponding human disorders.

Results

Functional Evaluations of Epileptogenic Channel Gene Mutations

Mutations of genes coding for nicotinic acetylcholine receptors (nAChRs)

Mutation of the nicotinic acetylcholine receptor was one of the first recognized epileptogenic channel mutations, associated with autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE). The phenotype is characterized by clusters of motor seizures during light sleep, and complex behavioral symptoms including gasping, vocalizations, and thrashing hyperkinetic activity. In different families, ADNFLE is associated with mutations of the CHRNA4 and CHRNB2 genes, which respectively encode the α4 and β2 subunits of nAChRs, a heteropentameric ligand-gated ion channel that is permeable to cations with little selectivity. The α4 subunit is expressed in most brain areas and generally associated with the β2 subunit to form an α4–β2 pentameric complex. Studies of mutated channel subunits expressed in *Xenopus* oocytes and cultured cell lines, as a means of evaluating the functional consequences of the genetically determined receptor changes, have given rise to complex and sometimes contradictory data. Evidence for increased ACh affinity and an increased and/or delayed rate of channel desensitization, followed by a prolonged desensitization period, has been reported to account for epileptogenesis. Moreover, CHRNA4 mutations cause nAChR loss of function, whereas CHRNB2 mutations cause a gain of function; the final net effect depends on the pre- or postsynaptic location of the receptor. More work is needed to understand the pathophysiology of ADNFLE. Since the α4 and β2 subunits are expressed ubiquitously in the central nervous system, it remains unclear why the epileptic focus is in the frontal lobe.

K^+ channel mutations

The heterotetramer products of the KCNQ genes form M-channels that regulate the threshold of neuronal electrical excitability. M-currents activate at close-to-resting-membrane potentials, counteract membrane depolarizations, and are sensitive to muscarinic (M) acetylcholine receptor agonists. By expressing KCNQ2 and KCNQ3 genes in *Xenopus* oocytes, investigators demonstrated that M-currents require the coexpression of both subunits. Mutations in the KCNQ2 or KCNQ3 gene are associated with the human epileptic syndrome, benign familial neonatal convulsions (BFNCs). The clinical picture of BFNCs is characterized by clonic seizures (rarely tonic) that are lateralized and sometimes change side, and associated with apneic spells lasting 1–3 min. The most frequent age of onset is the third day of life, with frequent recurrences until the seventh day, and subsequent isolated occurrences. Spontaneous remission occurs in the ensuing weeks or months. Work in the in vitro system provided a logical explanation of BFNC's genetic basis, insofar as mutations in genes coding for either subunit lead to a similar loss of function, and consequently decreased the efficiency of the hyperpolarizing voltage-dependent currents carried by the K^+ ions flowing through the channels.

Na^+ channel mutations

Mutations involving the alpha (SCN1A) or beta (SCN1B) Na^+ channel subunits are associated with simple febrile seizures (FS), generalized epilepsy with febrile seizures plus ($GEFS^+$), and severe myoclonic epilepsy of infancy (SMEI). FS occur during infancy and childhood, and usually subside around the sixth year of age. The $GEFS^+$ phenotypes include febrile seizures ceasing by the age of six years or extending beyond it, afebrile tonic–clonic, myoclonic, absence or atonic seizures, and myoclonic–astatic epilepsy. The SMEI phenotype has a typical natural history starting with tonic, clonic, or tonic–clonic seizures that are often prolonged and associated with fever, followed by febrile myoclonic, tonic–clonic, absence, and simple and complex partial seizures later in life. There is early-onset photosensitivity. The prognosis is poor in terms of cognitive function and seizure control. Interestingly, the $GEFS^+$ phenotype was only recognized after an Na^+ mutation was identified in a large New Zealand family. Although clinically well characterized since the 1990s, SMEI was never considered a genetically determined epileptic encephalopathy until it was found to be associated with a de novo SCN1A mutation.

The functional consequences of SCN1B mutations in *Xenopus* oocytes consist of a longer-lasting Na^+ current flow, consistent with an enhanced cell excitability. SCN1A mutations enhance the fraction (2–5%) of non-inactivating Na^+ currents, which normally account for about 1% of the maximal Na^+ current peak amplitude; this enhancement reduces the depolarization threshold required for neuronal firing, again consistent with

neuronal hyperexcitability. Characterization of the functional consequences of the SCN1A mutations associated with SMEI has been more difficult, since these mutations lead to a truncated protein that would completely inactivate the channel (thus making it impossible to test dysfunction by expressing it in an in vitro system). However, a suitable alternative was provided by the in vivo NaV1.1 knockout mouse, obtained by deleting the last exon of SCN1A. This animal presents with severe seizures early in life and has a very high, early postnatal mortality rate. Recordings from dissociated hippocampal neurons taken from heterozygous SCN1A knockout mice clearly show that the mutated subunit is selectively expressed by inhibitory interneurons, thus leading to a disinhibition of the principal pyramidal neurons that could account for the epileptic symptoms.

Ca^{2+} channel mutations

It has been found that mutations in genes coding for high-voltage-activated (HVA) Ca^{2+} channels are associated with absence epilepsies in rodent models. These mutations lead to reduced P/Q current density in neurons and, in principle, imply reduced cell excitability because P/Q-type Ca^{2+} channels play a dominant role in synaptic transmission. On the basis of what is known concerning the physiology of the thalamocortical circuitry responsible for absence epilepsy with spike-wave (SW) discharges (Figure 4), it would be expected that the channels responsible for the low threshold T-type Ca^{2+} currents would be primarily involved in spike wave generation, as the integrity of the T-current in the thalamic nuclei (in the thalamic reticular nucleus) is a prerequisite for SW expression in rodents. Investigators have described an association between genetic variations in the T channel gene Cav3.2 and childhood absence epilepsy. However, attempts to create an absence model by targeting the genes coding for Ca^{2+} channels have so far been unsuccessful, so the role of Ca^{2+} in epileptogenesis has still to be clarified. In this regard, it is worth remembering the complexity of Ca^{2+} cell functions, which include signal transduction, cell differentiation/proliferation, and synaptogenesis, as this complexity greatly complicates the interpretation of the results.

Gene mutations affecting GABA receptors or Cl^- channels

Rare GEFS+ phenotypes can associate with mutations affecting the γ2 subunits of the GABA receptor (GABRG2, 5q31-33). Various mutations of GABA receptor subunits have been reported in families with absence seizure with or without febrile seizures (γ2 subunit and idiopathic myoclonic epilepsy (α1 subunit)). These mutations lead to various defects in channel function that are generally consistent with a decreased effect of exogenous GABA, including a decrease in the amplitude of GABA-induced currents, altered channel gating, and probably reduced surface expression of functional receptors. A mutation affecting the CLCN2 gene (3q27-28), coding a voltage-gated Cl^- channel that is possibly involved in physiological GABA-dependent inhibition, has also been reported in families with various types of idiopathic generalized epilepsies with absence and convulsive seizures. The mutated channels show complete loss of

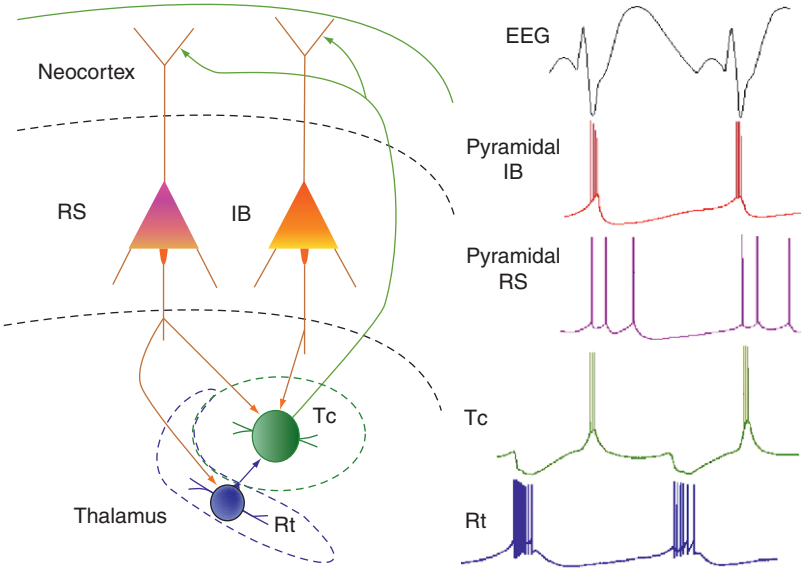


Figure 4 On the left the thalamocortical circuits responsible for absence seizures are schematically depicted. They include regular spiking (RS) and intrinsically bursting (IB) neurons of neocortex and thalamic reticular (Rt) and thalamocortical (Tc) neurons. In the right part of the figure spike-wave associated cell discharges are of the different neuronal populations are reported. Reproduced from Avanzini G and Franceschetti S (2003) Cellular biology of epileptogenesis. *Lancet Neurology* 2: 33–42, with permission from Lancet Neurology.

function or altered voltage-dependent gating, possibly causing impaired Cl^- homeostasis or membrane depolarization, and favoring hyperexcitability.

Conclusions and Directions for Future Research

The combined neurophysiological and biomolecular approach to the analysis of genetically determined alterations in ligand- and voltage-gated channel has opened up a new and exciting scenario in the neurobiology of epilepsy. The findings, as reviewed earlier, provide direct evidence that channel dysfunctions due to single gene mutations may account for epileptogenesis. However, it must not be forgotten that alterations in individual excitable properties have to be considered within the context of the neural circuitry involved in epileptogenesis. Although altered neuronal excitability is a primary prerequisite of epileptogenesis, epileptic discharges do not come from the abnormal activities of individual neurons but require the synchronous activation of large populations of hyperexcitable cells. Moreover, during a seizure, discharges tend to propagate through normal or pathological fiber connections, and so the role of neuronal circuitry in the generation and spread of epileptic discharges should never be overlooked.

Despite this caveat, the demonstration that the structurally altered ligand- and voltage-gated channels found in some human epilepsies can lead to potentially epileptogenic changes in ionic currents is a major breakthrough for our understanding of epileptogenesis. The implications of genetically determined channel dysfunctions in other paroxysmal muscular and cardiac disorders have been increasingly acknowledged since the 1991 publication of the seminal paper by Ptaceck *et al.* on hyperkalemic periodic paralysis. The recognition that some epilepsies may fall within the category of 'channelopathies' has proved to be highly advantageous for epilepsy research, which can now benefit from the results obtained in other excitable tissues.

Many new pieces of information have been collected so far but even more are expected to be gained in the near future. We have learned that, depending on their subunit composition, a variety of channel subtypes can be identified whose functional properties are still to be carefully characterized. The first objective for the ongoing research is to systematically correlate the subunit composition of different subforms of ionic channels with their function. The second important objective is to map the distribution of the subtypes across the different brain areas, and their differential expression in different cell types. This information could lead to predict the effect of a given channel mutation in different neural systems and ultimately to link the epileptic phenotype with the respective genotype.

Most of what we know about epileptogenic channelopathies comes from investigations of relatively rare forms of monogenic epilepsies. The main challenge for the future is to understand the role of channel dysfunctions in the pathogenesis of more common types of idiopathic, probably polygenic, epilepsies and of symptomatic epilepsies – due to exogenously determined channel alterations ('acquired channelopathies').

From the practical point of view, a better understanding of the role of ligand- and voltage-gated channels in epileptogenesis can provide sound criteria for the ongoing revision of the classification of epilepsy, and for the molecular/pharmacological development of selective and targeted antiepileptic drugs.

Acknowledgement

This work was supported in part by a grant from the EU Sixth Framework Thematic Priority Life Sciences, Genomics and Biotechnology for Health, contract number LSH-CT-2006-037315 (EPICURE).

See also: **Genetics**: Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; Ion Channel Mutations in Familial Eplepsy; Proepileptic Effects of BK Channel Gene Mutations; Sodium Channel Mutations that Cause GEFS+.

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Ion Channel Mutations in Familial Epilepsy

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Abbreviations

GEFS+	generalized epilepsy with febrile seizures plus
SMEI	severe myoclonic epilepsy of infancy
BFNIC	benign familial neonatal/infantile convulsions
EBN	epilepsy benign neonatal
BFNS	benign familial neonatal seizures
JME	juvenile myoclonic epilepsy
JAE	juvenile absence epilepsy
EGMA	epilepsy with grand mal on awakening
ADNFLE	autosomal dominant nocturnal frontal lobe
ADLTE	autosomal dominant lateral temporal lobe epilepsy

Introduction – Neuronal Ion Channels: From Molecules to Complexity

Ion channels are macromolecular protein complexes mediating flux of physiological ions through cellular membranes. With hundreds of ion channel genes identified in the human genome, there is scope for enormous functional diversity. Splicing and heteromeric assembly contribute further to this diversity – a feature that presumably confers a selective advantage by optimizing signal processing in the various cell types throughout the body. Functional diversity of neurons is highly dependent on the repertoire of ion channels expressed, and a given neuron may express dozens of different ion channel genes. Further diversity is provided by the regulation of the subcellular distribution of these channels within the neuron. In these ways, the computational power of networks is amplified by the creation of specialized neurons with unique integration and firing properties. The three major cell types are the excitatory and inhibitory neurons and glia, with each cell type encompassing numerous subclasses.

Figure 1 summarizes expression and localization of ion channel classes in these cells. Underlying this elegance and complexity are highly concerted and intricate molecular regulation processes that govern ion channel spatial and temporal expression, assembly, trafficking, modification, and degradation.

Neuronal communication is effected by synapses, located primarily on the dendritic and somatic membranes of a neuron. At most chemical synapses, information flow is unidirectional and involves the conversion of an electrical signal to a chemical signal in the presynaptic neuron, and then to an electrical signal in the postsynaptic neuron. A neuron integrates signaling from dendritic and somatic inputs (up to 200 000) to create an all-or-none output in the form of an action potential in the axon. Action potentials generated by the coordinated gating of sodium and potassium channels enable a means of long-range communication within and between neurons. At the presynaptic terminal, Ca^{2+} channels are responsible for 'translating' action potentials into the release of transmitter, which then acts postsynaptically on a range of excitatory or inhibitory ion channels. Thus, ion channels play a central role not only in determining the functional properties of a single neuron, but also in the fundamentals of signaling between neurons.

Such signaling permits neurons to operate within 'networks,' separable functional units within the brain that serve its myriad of functions. Networks possess

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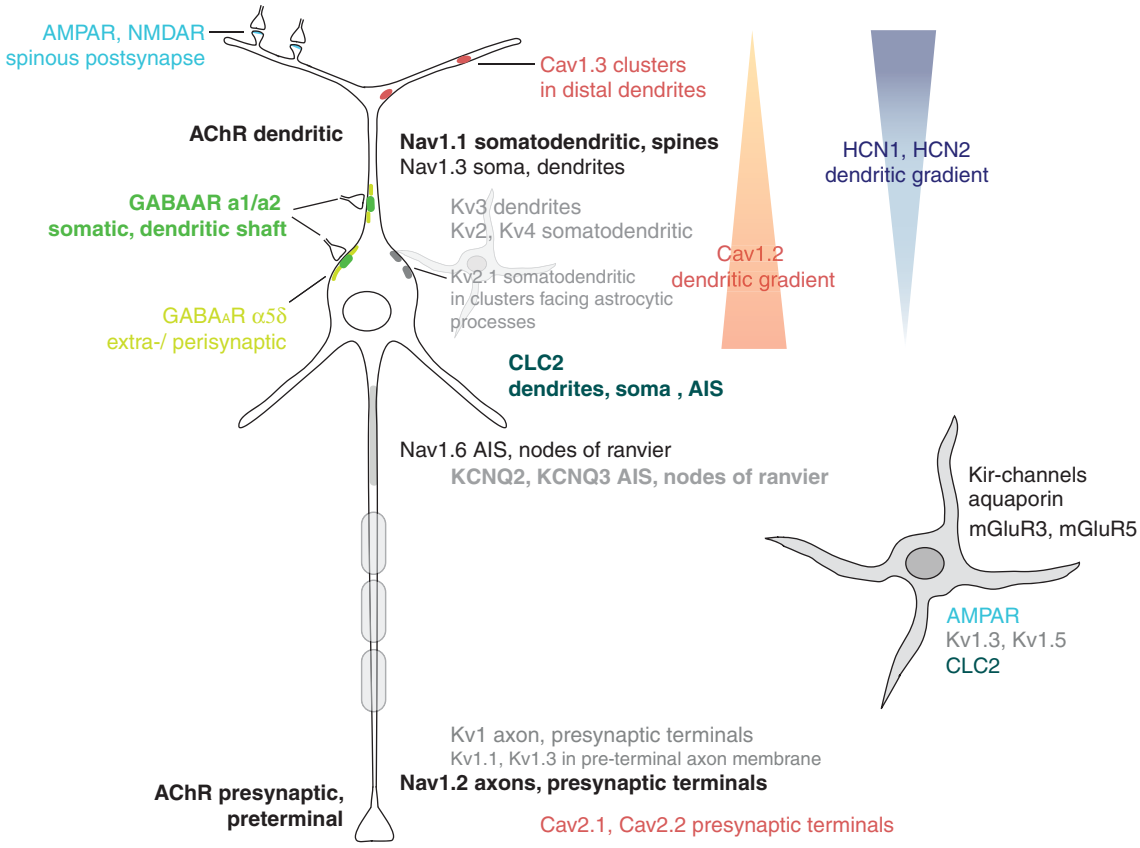


Figure 1 Schematic depiction of a typical excitatory neuron highlighting heterogeneous distribution of key ion channels. Channel types were selected because they are implicated in epilepsy (bold font); they are functionally critical for neurons and illustrate the complexity of ion channel distribution within a single neuron. The list of channels is by no means exhaustive (e.g., glycine receptors, 5-HT_3 receptors, kainate receptors, gap junctions, and others have been omitted).

Based on the mechanism of channel gating, ion channels can be classified: voltage gated, ligand gated, and others. Epilepsies associated with channel mutations are shown later in brackets.

Voltage-gated channels

Na^+ channels (Nav) are responsible for action potential generation and propagation and passive signal conductance involved in synaptic transmission [GEFS+, SMEI, BFNIC]. Four subtypes are found in the brain, three of which reside in the axon initial segment (AIS), nodes of Ranvier, and presynaptic terminals of the mature brain; Nav 1.1 and 1.3 also localize to soma and dendrites.

K^+ channels roughly divide into two functional subgroups: Dendritic **Kv**-channels regulate dendritic excitability, backpropagation of action potentials, and also the amplitude and waveform of excitatory postsynaptic responses. Axonal Kv subtypes limit action potential (AP) duration and membrane depolarization. Kv-subunits form heteromeric channels, which vary in gating, expression, and localization, and a simplified distribution is shown in **Figure 1**. Glial Kv1.3 and Kv1.5 function as delayed rectifying channels. KCNQ channels [EBN, BFNS] are slower delayed rectifying channels. They are found in AIS and nodes of Ranvier where they set subthreshold membrane potential, regulate excitability, and are themselves regulated by G protein-coupled pathways.

Ca^{2+} channels (CaV) mediate synaptic transmission in presynaptic terminals and are responsible for dendritic Ca^{2+} signaling [JME].

Hyperpolarization-activated channels (HCN) mediate a mixed cation current and regulate neuronal excitability and rhythmicity of AP firing. They show a gradient of distribution with a maximum at the distal dendrites declining toward the soma.

Voltage-gated Cl^- channels (CLC) are localized to spines, dendrites, soma, and AIS and are responsible for chloride homeostasis, thereby possibly modulating $GABA_A$R-mediated inhibition [JAE, EGMA].

Ligand-gated channels

Also known as ionotropic receptors, this group of channels open in response to specific ligand molecules binding to the extracellular domain of the receptor protein.

AMPA (α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid) and *NMDA* (*N*-methyl-D-aspartic acid) receptors are gated by glutamate and mediate the bulk of excitatory neurotransmission in the brain. They mostly reside in postsynaptic densities in dendritic spines.

Nicotinic acetylcholine receptors (*nAChRs*) are cation-permeable and are found both pre and postsynaptically [ADNFLE]. In the CNS, ACh has a mostly neuromodulatory role.

Excitation is balanced by anion-permeable γ-aminobutyric acid-gated $GABA_A$ receptors (*$GABA_A$Rs*) [GEFS+]. $GABA_A$Rs are located in inhibitory synapses found at the soma and at proximal dendritic shafts. Synaptic $GABA_A$Rs are important for phasic inhibition, whereas extra and perisynaptic $GABA_A$Rs containing α5 or δ subunits mediate tonic inhibition.

G protein-coupled receptors (*mGluR3* and *mGluR5*) are the predominant metabotropic glutamate receptor subtypes expressed by glial cells. Activation of these receptors inhibits cAMP accumulation and leads to an increase in intracellular Ca^{2+}, respectively.

redundancy and plasticity, permitting dynamic and adaptive behaviors. Once again, ion channels are the molecular determinants of these emergent properties.

The delicate balance between the excitatory and inhibitory events within these networks is maintained by the exquisite control exerted by the function of ion channels. Although networks are inherently robust to variations in physiological input, subtle changes in the molecular constituents of networks may dramatically alter their properties and give rise to pathological instability – as seen in neurological disorders such as epilepsy.

Background – Familial Epilepsy: From Molecules to Malady

Subtle changes in ion channel function are a hallmark of familial epilepsy syndromes. These familial epilepsies are largely confined to the idiopathic syndromes that cover a spectrum of severity from relatively benign syndromes such as febrile seizures or childhood absence epilepsy to severe myoclonic epilepsy of infancy (SMEI, Dravet's syndrome). The genetic architecture also covers a spectrum, from 'complex monogenic' to polygenic inheritance. The majority of mutations studied to date fall into the former category, although several studies have identified risk factor mutations associated with polygenic diseases.

In sum, these studies are beginning to provide insight into the genetics of epilepsy – but they do not bridge the knowledge gap from molecular deficit to behavioral seizure. Establishing a chain of understanding from molecule to malady requires an understanding of the impact of a mutation at each of the levels of brain organization discussed earlier. Obtaining such insight requires analysis of the in vitro consequences of a mutation on ion channel kinetics, permeation, and pharmacology to give a 'molecular phenotype,' as well as elucidation of the in vivo regulation of channel expression, biogenesis, assembly, trafficking, and turnover, to establish a 'neuronal phenotype.' At the next level of neuronal organization, it is important to understand how affected neurons alter the function of networks in which they reside and, therefore, it is necessary to investigate synaptic properties and network behavior to establish the 'network phenotype.' Because epilepsy is often viewed as a disease of brain networks, fundamental knowledge of the epileptic process should emerge by the successful investigation and integration of these three levels of organization.

Rather than attempt to review all studies that have been done to date in pursuit of these goals, we focus below on selected examples that illustrate studies lending themselves to this stepwise integrative approach.

Methods

Molecular Biological Methods

Interactions at the level of individual molecules are frequently studied using biochemical assays like pull-down experiments, western blotting, or mass spectrometry. Expression patterns and expression levels can be assessed at the RNA level with quantitative polymerase chain reaction (qPCR) and in situ hybridization, or at the protein level using immunohistochemical approaches.

Heterologous Expression Systems

Heterologous expression experiments, that is, artificial expression of genes from a different species, have been used widely to study ion channel function in a well-defined, controllable environment. Amphibian oocytes, human embryonic kidney cells (HEK), and different immortalized cell lines, for the most part, do not natively express neuronal ion channels and can be transfected with cDNA encoding the ion channel genes under investigation. Therefore, they can be used to describe the function of specific combinations of ion channel subunits, regulatory proteins, or channel mutants in the absence of contaminating currents. Sharp electrode or patch clamp techniques are used to measure the currents produced by the expressed channels. Patch clamp allows measurement of not only whole cell currents but, in the cell-attached or excised-patch mode, enables the direct observation of single channel function in real time.

In addition to functional assessment, heterologous systems inform our understanding of the cell biological consequences of ion channel mutations. For example, the impact of mutations that alter interactions with intracellular matrix proteins can be visualized using fluorescent labeling and quantified using digital imaging methods such as confocal microscopy and fluorescence energy transfer (FRET).

Glial and neuronal *Kir-channels* are inwardly rectifying K^+ channels. They contribute to K^+ reuptake and spatial K^+ buffering in the extracellular space.

Neurons also express G protein-activated inwardly rectifying channels (*GIRKs*). These play a powerful inhibitory role downstream for the action of several neurotransmitters.

Others

Aquaporins function as 'water channels' in many cell types and tissues in which fluid transport is crucial. Both extracellular K^+ concentration and osmolarity have been shown to dramatically modulate neuronal excitability.

Neuronal in vitro Studies: Imaging and Electrophysiology

Neurons in cultures or brain slices are accessible to a multitude of different functional and morphological approaches. Patch clamp electrophysiology has been instrumental in understanding functional changes in single neurons – for example, using input–output curves, high-frequency stimulation, or pharmacological manipulation. Functional imaging techniques directly monitor the flux of specific ions, most commonly Na^+ and Ca^{2+}, in identified cells or small networks, and voltage-sensitive imaging can directly monitor membrane potential changes with subcellular resolution.

The advantage of fixed tissue is the good preservation of neuronal anatomy, and the high resolution preserved for microscopy. Such tissue can be used for immunohistochemistry and other morphological techniques (e.g., Golgi staining, TUNEL staining, etc.) as well as electron microscopy.

Genetically Modified Viruses

In the last few years, recombinant viruses have become popular tools in neuroscience. They are used for expression of tagged proteins and mutants, biosensors (e.g., Ca^{2+} indicators), enzymes (e.g., cre-recombinase), light-activated ion channels (channelrhodopsins), and other molecules that not only report function but can also perturb the system under investigation in a controlled manner. Advantages of viral expression systems are a quick turnaround time, and spatially (by virtue of where the virus is injected) and temporally targeted expression of the gene of interest, with the added potential of targeting different cell populations using cell type-specific promoters.

In vivo Approaches

Recently, several knock-in mice of human epilepsy mutations have been generated. These models specifically recapitulate the human genetic lesion and frequently share many of the core pathological features. They are, therefore, extremely useful in studying the systems level biology of epilepsy; for example, pharmacoresponses or pharmacodynamics (of antiepileptic drugs as well as proepileptic drugs), neuronal network function, and animal behavior. Similarly, epileptic rat strains, such as WAG/Rij or GAERS, provide insight into polygenic inheritance of epilepsy.

Other epilepsy models generate recurrent seizures in response to electrical stimulation (e.g., amygdala or corneal kindling) or pharmacological activation (e.g., kainic acid, PTZ).

Imaging techniques in rodent models include electrocorticographic (EEG, or ECoG) monitoring, MRI, voltage-sensitive dye imaging, and 2D and 3D in vivo multiphoton imaging. These approaches, together with single and multiunit recordings and in vivo patch recordings, allow for examination of neuronal network activity in an intact brain, in some cases even in freely behaving animals.

Behavioral tests are employed to study the impact of a particular ion channel mutation on seizures, cognition, and motor or psychosocial skills such as learning and memory, coordination, and anxiety.

Results

Molecular Phenotype

Mutations acutely altering ion channel function

Neuronal excitability is critically dependent on electrochemical gradients that drive the passage of ions across the membrane. Therefore, it seems very satisfying and logical to assume that epilepsy arises from malfunction of a mutated ion channel, leading to increased excitatory or decreased inhibitory currents.

It is, however, extremely difficult to infer network hyperexcitability from the mere presence of mutated receptors. For example, autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is associated with mutations in neuronal nicotinic acetylcholine receptors (nAChR). nAChRs are pentameric ligand-gated ion channels composed of different combinations of α and β subunits. In total, six mutations in the most abundant subtype, $\alpha4\beta2$, have been identified. Several pharmacological and biophysical properties of the receptors are influenced by the mutations; interestingly, changes in ACh sensitivity, ACh kinetics, and Ca^{2+}-dependency were common to all ADNFLE mutations, whereas use-dependent potentiation, peak ACh current response, or Ca^{2+} permeability were altered in some but not all mutations. Most investigators believe that these changes represent a gain of function in the mutant receptors, but the pathophysiological process that leads to seizures is unclear.

Mutations in modulatory subunits

The flow of ions through an ion channel can be modulated by several different factors. An example is the voltage-gated sodium channels, which comprise a pore-forming α subunit and a choice among four modulatory β subunits. These β subunits are not necessary for channel function and do not form functional channels on their own. However, in combination with the channel forming α subunit, they modulate a range of channel properties that include ion channel gating, intracellular localization of the ion channel complex, membrane expression, and protein–matrix adhesion and cell–cell adhesion.

Epilepsy-causing mutations occur in both the α and β subunits, demonstrating the critical role of the modulatory β subunit in normal and disease states. Given the functional scope for β subunit modulation, there is

enormous potential for mutations to influence channel and neuronal function in epilepsy. The C121W mutation in the sodium channel β1 subunit (SCN1β) is an excellent example to illustrate this point.

SCN1β comprises an intracellular C-terminus, one transmembrane helix, and a large extracellular N-terminal domain, the structure of which closely resembles an immunoglobulin (Ig) domain; Ig-like folds are common structural protein subdomains involved in cell–cell recognition and adhesion. The single C121W amino acid mutation disrupts a disulfide bond in this Ig-like region. The functional consequences of this mutation have been tested both in *Xenopus* oocytes and mammalian cell lines. The results differed remarkably between these model systems: after coexpression with sodium channel α subunits in oocytes, wild-type SCN1β increased sodium channel inactivation fivefold, but mutant SCN1β(C121W) had no effect on inactivation. In mammalian cell lines, in contrast, α subunit current inactivation was not faster than in oocytes; furthermore, the effects of SCN1β coexpression were less marked. In comparison to wild-type SCN1β, mutant SCN1β(C121W) caused a subtle positive shift in voltage dependency and a decreased use-dependence. Interestingly, a study in insect cells demonstrated that SCN1β(C121W) did not promote cell-to-cell adhesion – in contrast to wild-type SCN1β, suggesting a disruption of homophilic β subunit interactions in the mutant.

Clearly, the single point mutation SCN1b(C121W) influences many of the functions ascribed to accessory subunits, suggesting that combined effects of subtle changes in channel function that modify the cell's biology may cause an epileptic phenotype. In this context, it is interesting that the spectrum of clinical presentations associated with the SCN1B–C121W mutation is exceptionally broad, ranging from febrile seizures to SMEI and temporal lobe epilepsy (TLE). This diversity of phenotypes might reflect mutations in different domains of SCN1b, leading to perturbation of one or more of its multiple functions.

Mutations influencing ion channel biogenesis and trafficking

$GABA_A$ receptors are the main mediators of fast inhibition in the central nervous system. They are pentameric ligand-gated chloride channels consisting of two α- and two β-subunits in combination with either a γ-, δ-, θ-, ϕ-, or ε-subunit; the most common stoichiometry is α1β2γ2. Different $GABA_A$ receptor subtypes are located in postsynaptic compartments versus peri- or extrasynaptic membranes, where they mediate phasic and tonic inhibition, respectively.

Several $GABA_A$ receptor mutations associated with epilepsy have been discovered. One intensively studied mutation in the GABARγ2 subunit leads to an arginine to glutamine amino acid exchange at position 43 in the mature protein, and causes childhood absence epilepsy and febrile seizures in patients. The mutation is localized in the N-terminal extracellular domain of the γ2 subunit, most probably at the interface between γ2 and β subunits.

The functional effects of the R43Q mutation on GABA receptor kinetics are still a matter of some debate. There is, however, accumulating evidence suggesting a deficiency in channel assembly and cell surface expression or localization of the channels containing the mutated γ2 subunit. Experiments in several expression systems indicate a failure in subunit interaction between γ2 and α1/β2, leading to intracellular retention of mutant channel complexes. In neurons, α1β2 channels of unknown stoichiometry still reach the cell membrane and, it is speculated, induce an imbalance between phasic (synaptic) and tonic (extrasynaptic) inhibition.

In conclusion, inhibition of channel assembly and perturbed intracellular trafficking, in combination with changes in receptor kinetics, is likely to be responsible for the general cortical hyperexcitability and the epileptic phenotype observed in patients with this mutation.

Mutations influencing protein–protein interaction

Ion channels are embedded in a meshwork of proteins, which coordinate their intracellular localization, trafficking, and use-dependent recycling, mediate attachment to the cytoskeleton, and modulate their function. Several mutations in 'non-ion channel genes' have been identified in epileptic patients; for the purpose of this review, we will focus on *LGI1*, which was identified as the causative gene for ADLTE.

The LGI1 protein is characterized by leucine-rich repeats in its N-terminal half, indicating a function in protein–protein interaction. Recent publications have revealed that LGI1 is tightly associated with Kv1.1 potassium channel complexes. One effect of native LGI1 is to counteract Kvβ1 by selectively removing rapid inactivation from the A-type Kv channel. This function was completely abolished by the ADLTE mutation, probably resulting in frequency-dependent AP broadening.

Analysis in heterologous expression system reveals alterations in the molecular and biochemical properties caused by ion channel mutations, but such membrane-level analysis usually cannot fully explain the pathophysiological properties of cellular networks that express the channel. Therefore, unanswered questions remain. Which, if any, of the changes observed are important for epilepsy? Are there other changes in function that cannot be recapitulated or observed in the expression system? Why are seizures episodic? Does expression during brain development cause chronic changes that cannot be predicted from analysis of the acute deficit alone? Answers to these questions will require capturing the nature of the deficit in the natural environment of the channel – that is, the neuron – where a more complete repertoire of modulatory,

regulatory, and anatomical influences can exert their effects. Methodologically, this type of analysis can be achieved by transfer of the mutated gene to a neuronal cell culture, by creation of transgenic or gene-targeted mouse models, or by viral gene transfer.

Neuronal Phenotype

Ultimately, an epilepsy-causing mutation will alter the way in which a neuron functions, to result in abnormal electrical discharges characteristic of the disease. An appreciation of the consequences of a mutation on neuron behavior is critical to our understanding of disease genesis and progression. Furthermore, neurons are broadly divided into excitatory and inhibitory types, and the balance between inhibition and excitation is important for normal brain function. Any influence that disrupts this fine balance can lead to an epileptic state as a result of 'overexcitation' or 'underinhibition' (more commonly referred to as 'disinhibition'). Excitatory and inhibitory neurons may independently or collectively be affected by an ion channel mutation, and therefore different cell types may differentially participate in disease genesis.

One important property of neurons is that they fire action potentials in response to synaptic input. Each class of neuron has a unique transfer function that governs action potential output for a given input. That transfer function is generally determined by the morphology of the dendritic tree, the distribution of ligand-gated and voltage-gated conductances, properties of the axon initial segment, size of the soma, and (for certain neurons) the properties of dendritic specializations called spines. Passive properties, such as the membrane capacitance and leak conductances, also influence firing. Epilepsy-associated mutations that alter one or more of these parameters can alter neuronal input–output function to cause network instability and seizures.

Sodium channels and firing properties in epilepsy

Voltage-gated sodium channels are a well-studied and essential components responsible for action potential generation and propagation. Whole-cell voltage clamp studies in clonal cell lines (e.g., HEK293) of mutated sodium channels typically identify a range of changes in channel properties, including alterations in inactivation kinetics, channel availability, and voltage sensitivity of gating. It may be impossible to predict which of these changes result in an alteration of a neuron's firing pattern to cause epilepsy. Collectively, this problem is compounded because some changes cause increases in channel excitability while others cause decreases in channel excitability.

Numerical/computer modeling has been used to predict the consequences of such mutations on excitability, and for some mutations has encapsulated the range of biophysical changes associated with a mutation. For example, for a given input current, a model neuron containing the measured properties of mutated sodium channels would fire at a much higher rate than wild type. However, such modeling could not have predicted the mechanism of action of Dravet's syndrome-associated mutations in SCN1A. In a mouse model harboring a human disease-causing mutation (a truncation mutation), investigators showed a specific reduction in firing only in inhibitory interneurons; excitatory cell firing was rescued because of cell-type specific expression of Scn1a. This example highlights the potential strength of modeling, but also reveals a limitation that can only be overcome by a combination of genetic, cell biological, and electrophysiological analyses in a mouse model.

The axon initial segment (AIS) is a specialized region of the axon, adjacent to the soma, and is the site where action potentials are often initiated in response to somatic and dendritic input. Nav1.6 channels are enriched at the AIS, and epilepsy-causing mutations in Nav1.6 may result in an increased propensity for the AIS to trigger an action potential (i.e., for an input that would otherwise encode a lower chance of firing). In this case, it was important to understand not only the electrophysiological properties altered by a given mutation, but also the cellular localization of the mutant channel itself. It is possible that trafficking deficits caused by a mutation may not alter action potential initiation as assessed in a clonal cell (such as HEK293 cell) that lacks either the specialized trafficking mechanisms or the specialized geometry of a neuron; nonetheless, such a mutation could alter enrichment at the AIS and thereby impact firing.

Early ideas of the function of the dendritic tree emphasized the concept that the dendrite was a largely passive structure that relayed synaptic inputs to the soma and axon. More recent work showed that dendrites contain voltage-gated sodium, potassium, and Ca^{2+} channels, so that action potentials can either arise in, or 'backpropagate' into, the dendrite. The importance of this neuronal structure cannot be underestimated, and the role of dendritic function (or dysfunction) in epilepsy is likely to grow as our understanding of the basic biology increases. A number of sodium channels shown to be associated with epilepsy (SCN1A, SCN2A) localize to the dendritic tree, where they are likely to cause changes in synaptic integration and in action potential backpropagation. There is scant knowledge on the dendritic consequences of familial channel mutations.

Network Phenotype

The preceding sections have outlined the importance of understanding the molecular and neuronal consequence of familial ion channel mutations. However, neurons do not function in isolation; they participate in local and remote brain networks whose dysfunction is a hallmark of epilepsy.

Thus, understanding network behavior is a major challenge facing basic and clinical epilepsy research.

Networks and 'local' destabilization in idiopathic generalized epilepsy

Anatomically and functionally distinct regions of the brain express unique repertoires of ion channels and receptors; thus, genetic mutations may only directly impact particular brain regions. Nonetheless, these affected regions can participate in broad networks that include unaffected regions of the brain. The new network states created by the incorporation of affected regions could then create the unstable network behavior seen during epilepsy. An example of this scenario is provided by recent work in our Melbourne laboratory. We have shown that a mutation in the $GABA_A\gamma2$ gene appears to cause a specific synaptic deficit in the cortex, yet gives rise to absence-like seizures that involve thalamocortical networks. Patch clamp studies of neurons showed reduced amplitude of miniature inhibitory synaptic currents in mice heterozygous for the $GABA_A\gamma2$(Q43) allele when compared with wild-type controls. This abnormality could result in a reduction in inhibitory drive in local cortical microcircuits, which feed back to thalamic nuclei. In this example, it is possible that the thalamus – which itself would function normally if it were isolated or if it were connected to a normal cortex – would be driven into a pathological state because of the aberrant function of a component of the network in which it participates. In this way, local functional deficits can be amplified to create global pathological states through network mechanisms. In short, aberrant spike trains in a neuron may arise via a non-cell autonomous process.

Development and wiring

A potentially neglected aspect of the pathology of inherited epilepsies is the impact of ion channel mutations on brain development. Because ion channels play a central role in normal brain development, it is possible that familial mutations alter the developmental trajectory of the brain such that wiring of networks is subtly but importantly altered. One class of channels mutated in epilepsy, the $GABA_A$Rs, are particularly well positioned to impact development and network connections, since these receptors are critical in the regulation of neuronal proliferation, migration, and synaptic refinement. This regulation is due largely to $GABA_A$R-mediated depolarization, leading to Ca^{2+} influx, which occurs in the developing brain. Ligand-gated channels that themselves have significant Ca^{2+} fluxes, such as $\alpha7$ nicotinic, 5-HT3A, and NMDA receptors, are also likely to trigger signal transduction cascades with development consequences.

Other genetic mechanisms may impact network function. For example, dendritic arborization in the seizure-prone WAG/Rij rat is significantly different from that of nonepileptic controls, supporting the idea that morphological changes may underlie seizure susceptibility. Differences in dendritic branching may be developmentally altered by genetic variation unique to the WAG/Rij. In this context, it is becoming increasingly necessary to clarify whether a genetic ion channel mutation causes epilepsy because (a) it changes fundamental properties of migration, network wiring, or neuronal morphology in the developing brain; (b) it changes the acute, moment to moment function of ion channels; or (c) both developmental and acute effects combine to generate an epileptic phenotype. Separating these possibilities may be impossible in humans, but with recent advances in genetic and drug-inducible mouse models, answers to these questions should be within reach.

Future Challenges

Neural network level dysfunction is a predominant manifestation of seizures in both humans and animal models and it is not surprising that epilepsy is thought of as a 'neuronal network disease.' Genetic epilepsies have provided a wealth of information about the molecular and cellular changes that may underpin disease genesis but have not significantly impacted our understanding of pathogenic mechanism at the network level. This disconnect likely arises from the technical difficulty in translating single molecular or cellular changes to network dysfunction. Pathological changes at the level of a single neuron can be extremely subtle yet cause major effects on the networks in which they reside. Understanding the fundamentals of this 'amplification' of subtle molecular changes is crucial to our understanding of epileptogenesis. Long-term potentiation or depression may be mechanisms that are pathologically activated to amplify subtle molecular changes and impact network function, and thereby enhance seizure susceptibility. The challenge is to develop methods and approaches that can establish a chain of causality from the molecular defect to the network and behavioral consequence. Therefore, in vivo approaches, coupled with realistic computational modeling, will be necessary to bridge the various pathogenic mechanisms at work in familial epilepsies, and should provide an understanding of epileptogenesis from 'molecule to malady.'

See also: **Genetics**: Clinical Genetics of Epilepsy; Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; **Neurotransmitter Function**: Synaptic Activities and Transmitter Function: Receptor Trafficking; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy; **Transporters**: GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability.

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Post-Translational Modification of Ion Channels in Epilepsy

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Introduction

Temporal lobe epilepsy (TLE) may develop following a remote episode of status epilepticus (SE). There are several rodent epilepsy models that closely mimic TLE. The chemoconvulsants, kainate and pilocarpine models, have distinct mechanisms of action, but both agents induce SE within hours of injection followed by the development of chronic limbic motor epilepsy with hippocampal sclerosis. During the 'early phase' following drug injection, rats exhibit limbic motor seizures that can be observed both behaviorally and electrographically; the seizures then worsen and progress to SE. In most animal studies, an anticonvulsant medication, such as diazepam or pentobarbital, is used to decrease the duration of SE and to reduce the associated mortality. The 'late phase' (several months following SE) in these models is characterized by extensive loss of CA3 pyramidal cells, mossy fiber sprouting, and the development of spontaneous seizures involving limbic structures. These models are commonly used to evaluate the acute and late consequences of SE. During the acute phase, convulsant-induced seizures and SE are associated with multiple complex changes in the brain. Some of these changes include activation or repression of signal transduction pathways in the CNS. Such an effect on signaling pathways could influence numerous effector molecules throughout the nervous system that may have broad functional consequences. In the following sections, we focus on one candidate mechanism contributing to the alterations seen following convulsant-induced seizures and SE: aberrant regulation of Kv4.2 channels, mediated through the extracellular signal-regulated kinases 1 and 2 (ERK1/2) of the mitogen-activated protein kinase (MAPK) superfamily. We propose that through their coupling to ion channel regulation, kinase pathways have the potential to rapidly modulate neuronal excitability and contribute to seizures and SE.

Background

ERK1/2 Pathway Regulation of Excitability in Hippocampal Dendrites

The MAPK pathway consists of a superfamily of kinases that include ERK 1/2, c-Jun N-terminal kinases, p38 isoforms, and ERK5. MAPKs have been implicated in physiological processes involving the CNS. Specifically, the ERK pathway is involved in the regulation of neuronal excitability, learning, and memory. Cyclic AMP-dependent protein kinase A (PKA) and protein kinase C (PKC) are upstream regulators of the ERK pathway in neurons. Indeed, PKA and PKC activation leads to increased excitability in hippocampal CA1 pyramidal cell dendrites, through decreasing the amplitude of the rapidly-activating, A-type K^+ current (I_A) and increasing the amplitude of back-propagating action potentials (b-APs). ERK inhibition blocks this effect of PKA and PKC activation on I_A and the b-APs in dendrites. In addition, ERK inhibition shifts the voltage-dependence of activation of I_A toward more hyperpolarizing potentials. The implication of these findings is that ERK activation leads to a depolarizing shift in the voltage dependence of I_A, and

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thereby increases excitability in the dendrites. These changes are thought to be due to ERK pathway activation coupling to phosphorylation of Kv4.2 channel subunits, which are the major α-subunits of the channels underlying I_A in CA1 pyramidal cell dendrites.

Kv4.2 Channels Underlie I_A in Hippocampal Dendrites

Kv4.2 is a voltage-dependent potassium channel α-subunit that is localized to the somatodendritic regions of hippocampal neurons and contributes to I_A in the dendrites. Because of the role of I_A in reducing action potential initiation and back-propagation in the dendrites, this channel plays a major role in the regulation of postsynaptic excitability. Kv4.2 knockout mice show a complete loss of I_A in the hippocampal CA1 dendrites and have an increase in b-APs. Furthermore, the Kv4.2 knockout mice show a decreased threshold for the induction of long-term potentiation. Modulation of Kv4.2 channels can occur through multiple kinase regulatory sites, which alter channel kinetics, expression, or trafficking.

Activation of the ERK Pathway in Epilepsy

The contribution of protein kinase cascades to synaptic plasticity – particularly to LTP, learning and memory – has received significant attention. Recent studies have begun to examine the role of protein phosphorylation in neuropathology, including epilepsy. In particular, the ERK pathway has been implicated in a number of pathological processes involving the central nervous system. Modulation of ERK activation has been demonstrated following seizures and SE in rodent models in vivo and in hyperexcitability models in vitro. The ERK pathway regulates a broad range of target molecules through proline-directed serine/threonine phosphorylation. Identification of the effector molecules for the ERK pathway, under both physiologic and pathologic conditions in the nervous system, is an area of active investigation. In previous studies, we identified the potassium channel subunit Kv4.2 as a phosphorylation target of the ERK1/2 pathway. Following phosphopeptide mapping, we generated antibodies that specifically recognize Kv4.2 proteins in rat brain extracts when phosphorylated at the ERK sites. As noted above, Kv4.2 channels are a major contributor to I_A in the dendrites of hippocampal CA1 neurons, and the ERK1/2 pathway regulates I_A. Thus, our working hypothesis is that the ERK effect on I_A is via direct phosphorylation of Kv4.2 channel subunits.

Kv4.2 Alterations in Epilepsy

Potassium channelopathies on a genetic basis are associated with an epilepsy phenotype. Recently, a mutation in the Kv4.2 gene leading to a truncation of the carboxyl-terminus has been described in TLE. In addition, defects in the gene encoding an auxiliary subunit for Kv4.x channels, Kvβ2, have been described in human developmental epilepsy. These studies suggest that defects in the primary, pore-forming Kv4.2 channel subunit or an auxiliary subunit for Kv4.x channels result in increased excitability and seizures in humans. Furthermore, alterations in I_A have also been reported in human epilepsy. Thus, there may be acquired or activity-dependent alterations in Kv4.2 channels, and potentially other ion channels, that contribute to seizures and epilepsy. It has recently been shown in a heterotopia model of cortical malformations and epilepsy that a down-regulation of Kv4.2 may contribute to seizures in this model. Studies in adult rodent epilepsy models have demonstrated activity-dependent downregulation of Kv4.2 channels with associated increased dendritic excitability. These studies also demonstrated an increase in phosphorylation of Kv4.2 by ERK, which would further downregulate I_A. Following pentylenetetrazole- and kainate-induced seizures and electroconvulsive shock, there is a reduction in Kv4.2 mRNA levels in hippocampus. In the kainate model, animals that did not exhibit seizures did not have altered Kv4.2 expression.

Even though it is clear that Kv4.2 can modulate hyperexcitability and play a role in epilepsy, it is unclear whether the increase in ERK activation seen in the early phase following chemoconvulsant stimulation directly couples to Kv4.2, and whether the expression of Kv4.2 proteins is altered during this early phase of epileptogenesis. Studies in our lab have focused on this possibility.

Methods

Video-EEG analysis was used to monitor behavioral and electrographic seizure activity, and to correlate seizures and SE with ERK activation and Kv4.2 phosphorylation following convulsant stimulation (using kainate injection intraperitoneally in adult rats). At various time points following the onset of seizures and SE, the animals were sacrificed and the hippocampus rapidly subdissected. Some of the animals were allowed to survive until they developed epilepsy (3–4 months after kainate-induced SE). Video-EEG monitoring was used to confirm that these animals had developed chronic, spontaneously recurring seizures. Homogenates, total membranes, and synaptosomes were prepared from the hippocampal tissue. The samples were then used for Western blotting with antibodies that detect levels of total protein (phosphorylated and unphosphorylated) or that selectively recognize only the phosphorylated form of the protein. Commercially available antibodies were used to recognize total ERK1/2 (the dually phosphorylated activated form of ERK1/2) and total Kv4.2. For the studies

evaluating levels of ERK-phosphorylated Kv4.2 (phospho-Kv4.2), we developed antisera based on the results of phosphopeptide mapping studies.

Recent Results

An early poststimulation change in Kv4.2 channel phosphorylation and localization could produce dramatic alterations in hippocampal excitability and contribute to continuous seizure activity during SE. In the first series of studies, we examined whether early seizures and SE were associated with ERK activation and coupling to Kv4.2 phosphorylation in hippocampus. In parallel, we evaluated Kv4.2 localization and phosphorylation in the synaptosomal subcellular compartment. We found an increase in ERK activation and phosphorylation of Kv4.2 in hippocampal homogenates during kainate-induced SE compared to sham controls (**Figure 1(a)**). There was no change in total Kv4.2 levels in whole cell membrane extracts from hippocampus in either group. Next we examined ERK phosphorylation of Kv4.2 channels in hippocampal synaptosomal extracts from rats following SE. We found that after SE there is an increase in the levels of ERK phosphorylated Kv4.2 and a decrease in total Kv4.2 in hippocampal synaptosomes isolated from animals with SE compared with sham controls (**Figure 1(b)**). We predict that ERK phosphorylation of Kv4.2 channels and a decrease in the number of Kv4.2 channels postsynaptically will lead to a decrease in I_A in this region. The net result of such an alteration would be a significant increase in excitability in the hippocampal dendrites, which could contribute to alterations in neuronal network behavior (and potentially seizures) in this region.

Through these experiments we have been able to demonstrate that rapid alterations in the ERK pathway are associated with convulsant stimulation and that Kv4.2 is one candidate effector. In addition, there are associated changes in the synaptic localization of Kv4.2 channels during SE, but the mechanism underlying this effect is unknown. We have provided a figure to illustrate the effects of SE on the localization of total and phosphorylated Kv4.2 channels (**Figure 2**). We also have examined whether there are long-term alterations in Kv4.2 following kainate-induced SE. In rats with kainate-induced epilepsy, there was a downregulation of total Kv4.2 protein levels and increased ERK phosphorylated Kv4.2 (**Figure 3**), as also seen in the pilocarpine epilepsy model. These findings suggest that an *acquired* (activity-dependent) alteration in Kv4.2 channels, in part as a result of kinase-mediated events, occurs in SE and epilepsy.

Future Goals

The findings from our and other laboratories indicate that posttranslational mechanisms play an important role in

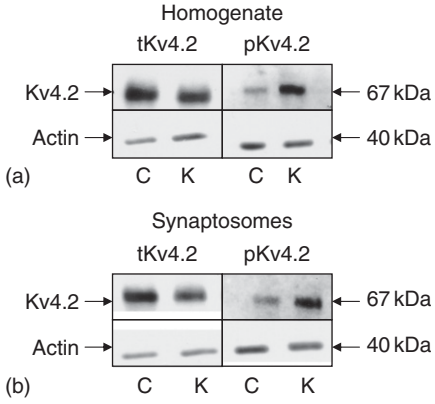


Figure 1 Status epilepticus (SE) is associated with an increase in ERK-phosphorylated Kv4.2 and a decrease in Kv4.2 protein in hippocampal synaptosomes during SE. Following 1 h of SE hippocampi were dissected and total cell (a) and crude synaptosomal (b) extracts were prepared. Western blotting was performed for ERK phosphorylated Kv4.2, total Kv4.2 and actin levels. There was an increase in phosphorylated Kv4.2 but no change in the total Kv4.2 levels using homogenates from SEK compared with control (C) (upper panel a). In crude synaptosomes there was increased ERK-phosphorylated Kv4.2 and a decrease in total Kv4.2 levels in SE (upper panel b). The actin levels were unchanged in both conditions (a & b lower panels).

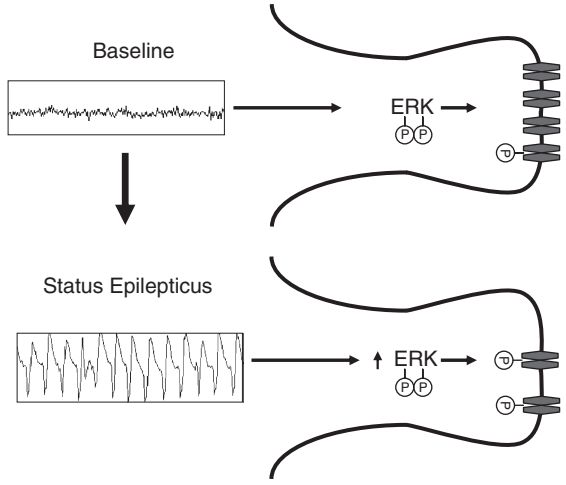


Figure 2 Model of dendritic spine with alterations in ERK activation and Kv4.2 in hippocampus during SE. Under baseline conditions in sham animals there were basal levels of ERK activation and ERK phosphorylation of Kv4.2, which was seen in total membranes and in the synaptosomal regions (top panel). After prolonged SE there was increased ERK activation in hippocampus. In addition, there was increased ERK phosphorylated Kv4.2 in hippocampus (upper panel). There was also a decrease in the level of Kv4.2 following SE, which was seen only in synaptosomes. In contrast, Kv4.2 levels in whole-cell extracts were stable, suggesting altered Kv4.2 trafficking following SE.

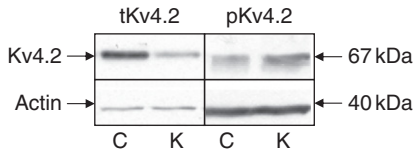


Figure 3 Hippocampal levels of ERK-phosphorylated Kv4.2 are increased and total Kv4.2 levels are decreased in epileptic animals. Following kainate-induced SE, rats developed epilepsy. Sham controls were assessed in parallel. The hippocampus from the epileptic animals (K) and sham controls (C) was removed and membrane extracts were processed for western blotting. Increased ERK-phosphorylated Kv4.2 (right upper panel) and a downregulation of total levels of Kv4.2 channels was found in hippocampal membranes (left upper panel) from epileptic animals. The actin levels were unchanged in both conditions (lower panels).

epilepsy. A number of studies have indicated that post-translational regulation of ion channels plays an important role in channel function and trafficking. In future studies, we plan to further characterize the localization changes in total and ERK-phosphorylated Kv4.2 during SE, and to evaluate whether signal transduction mechanisms play a role in the alterations in Kv4.2 trafficking. It is known that several other kinases are involved in the regulation of Kv4.2 channels in hippocampus. One possibility is that several kinase pathways converge at the point of Kv4.2 regulation, to modulate excitability in neurons during SE and possibly in epilepsy.

SE is associated with significant mortality and morbidity nationwide. Our studies are focused on understanding the cellular mechanisms that are involved in the development of prolonged, continuous seizure activity. We propose that activity-dependent alterations in ion channel regulation contribute to increased network excitability and possibly the potentiation of SE. Our studies are focused on understanding the mechanisms underlying the regulation of a particular ion channel, Kv4.2, during SE. Kv4.2 channels are critical regulators of postsynaptic excitability in the hippocampus. Thus, we predict that alteration in the phosphorylation and total levels of Kv4.2 channels during SE play a significant role in neuronal hyperexcitability and seizures. Our hope is that the findings from these studies will provide novel insights into the mechanisms involved in the regulation of Kv4.2 under physiologic and pathological conditions (such as SE), and that these studies will provide insights into the development of new interventions for the treatment of SE. Possible loci for therapeutic interventions would be at the level of Kv4.2 or the upstream kinase signaling pathways that are modulated during seizures and SE, and are involved in the regulation of Kv4.2. Some of the mechanisms that are involved in the modulation of Kv4.2 during SE may apply to other ion channels that are altered during seizures. Furthermore, these studies should provide the foundation for future investigations evaluating whether the regulatory mechanisms that are key during the early phase of SE persist in later stages of epileptogenesis and in chronic epilepsy.

See also: **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; Channelopathies in Epilepsy; **Neurotransmitter Function**: Synaptic Activities and Transmitter Function: Receptor Trafficking; **Transporters**: GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability.

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Proepileptic Effects of BK Channel Gene Mutations

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Introduction

What are BK Channels?

BK channels are large-conductance, voltage, and calcium-activated potassium channels. A cartoon showing essential features of this protein is presented in **Figure 1**(**a**). The pore-forming α subunit belongs to the voltage-dependent potassium channel family. Thus, they have a typical set of six transmembrane domains (S1–S6) with charged S4 domains that moves across the electric field upon membrane depolarization and imparts energy to channel opening. Unique to BK channels, they have charged residues in S2 and S3 domains that also contribute to voltage sensing, and an additional S0 transmembrane domain. They also have intrinsic calcium binding sites in their carboxyl terminal tail that impart low affinity calcium activation. They have a potassium selectivity sequence that allows high conductance while maintaining selectivity for potassium. BK channels also assemble with a family of two-transmembrane accessory β subunits (β1–β4), through interactions in the N-terminus-S3 domain.

There are several additional features unique to BK channels that have important implications for how they regulate membrane excitability. As implied by the name, BK (Big K^+ conductance, also called MaxiK) channels have an especially large single channel conductance of $\sim$250 pS. This is in the order of 10–20 times larger than most other potassium channels (for example, Kv1.1 channel is $\sim$12 pS). The large conductance means that the opening of relatively few BK channels can have a significant impact on membrane voltage. The relevance of this is seen in vascular smooth muscle where BK channels have an established role in control of vascular tone. Smooth muscle continually gauges global calcium concentration via spontaneous calcium-induced activation of ryanodine receptors in the sarcoplasmic reticulum. As global calcium rises, the probability of ryanodine receptor opening rises to cause a transient and local release of calcium (so called 'calcium spark') that activates BK channels in the apposed plasma membrane. A single calcium spark has been estimated to activate a cluster of only $\sim$15 BK channels, that in turn hyperpolarize the membrane by greater than 20 mV. This hyperpolarization then deactivates voltage-dependent calcium channels and relaxes smooth muscle.

BK channels have the specialized ability to regulate calcium influx that is coincident with membrane depolarization. This is a unique property among potassium channels, and its biophysical basis is shown in **Figure 1**. BK channels are activated by both voltage and calcium, independently (**Figure 1**(**b**)). At a fixed voltage, increasing calcium raises channel open probability. At a fixed calcium concentration, increasing voltage also increases channel opening. However, physiological calcium rise or depolarization alone is insufficient to raise BK channels open probability to significant levels. This property is demonstrated in **Figure 1**(**c**). In nominal (nanomolar) calcium, BK channels open in a voltage-dependent manner but well outside the physiological voltage range of any cell type ($> +100$ mV). As calcium is increased to micromolar concentrations, the conductance–voltage relationship becomes shifted to physiological ranges. Fairly high calcium concentrations (e.g., 7 μM, as shown in **Figure 1**(**c**)) nevertheless still require membrane depolarization to activate significant numbers of channels. Thus, BK channels require the concerted activation by depolarization and relatively high calcium to promote significant channel openings. As a consequence, they are frequently physically and functionally colocalized with a calcium source, such as voltage-dependent calcium channels, NMDA receptors, or ryanodine receptors. By repolarizing membranes in response to coincident calcium influx and depolarization, BK channels serve as a negative feedback regulator of voltage-dependent calcium channels.

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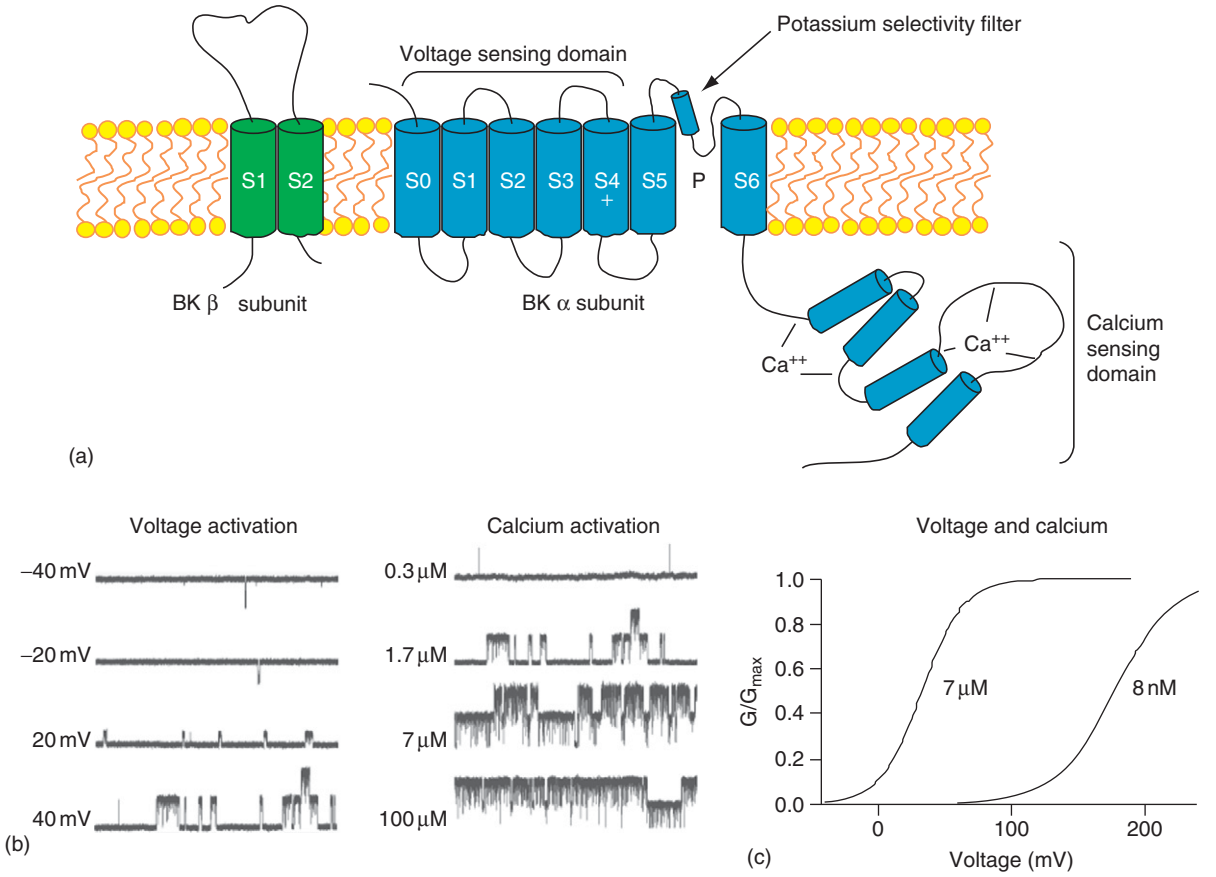


Figure 1 (a) Cartoon showing essential features of BK channels. (b) Example single channel recording of BK channels (N is 2 channels in the patch) activated by calcium or voltage. Left panel shows record traces at constant 1.7 μM internal calcium and using step potentials from −40 mV to +40 mV to activate the channel. Right panel is the same patch recorded at constant +40 mV holding potential and perfusing different buffered internal calcium. (c) BK channel steady-state normalized conductance–voltage curves for indicated calcium concentrations. High calcium shifts conductance–voltage curves to physiological voltage ranges. Conductance–voltage curves are derived from boltzman fits of mean data obtained from macroscopic currents. Currents were from mouse slo channels expressed in transfected HEK293 cells using the inside-out patch clamp configuration using high potassium concentrations (140 mM potassium outside and inside the patch).

Despite expression from a single gene, BK channels represent a diverse group of channels. This is partly mediated by a family of tissue-specific accessory subunits (β1–β4) that alter biophysical properties and pharmacology of BK channels. BK channels can be broadly classified as inactivating and non-inactivating BK channels. The β2 subunit and most splice products of the β3 subunit underlie inactivating BK channels. The non-inactivating channels are further categorized into 'type I' and 'type II' channels, based on bilayer recordings from synaptosome membrane preparations. Type I BK channels are relatively more calcium sensitive, have faster gating kinetics, and are sensitive to scorpion venoms iberiotoxin and charybdotoxin. Type II BK channels have slow gating kinetics, are less sensitive to calcium, and are insensitive to scorpion venoms. Association with the β4 subunit converts fast-gated, type I BK channels to the slow gated, iberiotoxin resistant type II BK channels. Finally, the β1 subunit slows gating and enhances BK channel activation but is predominantly expressed in smooth muscle.

BK Channel Impact on Epilepsy

Given the complexity of the nervous system, the impact of BK channels on epilepsy cannot be inferred solely from cellular neurophysiology. Before a BK channel knockout mouse was available, studies using specific blockers of BK channels indicated that reducing BK channel activity was not pro-epileptic. Injection of A-type and delayed rectifier potassium channels blockers into the brains of mice caused convulsions and seizures, whereas BK channel blockade did not. Interestingly, blocking BK channels does cause motor impairment. Consistent with the pharmacology, subsequent studies of BK channels gene knockout mice also do not demonstrate evidence of seizure activity, but do show defects in motor coordination and motor strength.

In contrast to effects of BK channel antagonists, gain-of-function of BK channel mutations have been shown to promote epilepsy. In humans, a mutation was identified

in one of the calcium binding domains of the BK channel (D434G) that is associated with epilepsy and paroxysmal movement disorders in family members that carry the allele. The epilepsy type associated with this mutation is absence, but generalized tonic-clonic seizures are occasionally seen in some individuals. Analysis of the mutation in heterologous expression systems (*Xenopus oocytes* and transfected cells) indicates that the mutation is a gain-of-function for BK channels. Activation is faster and channel steady-state openings are shifted to more negative membrane potentials. Similarly, knockout of the BK channel β4 subunit, which causes a gain-of-function of BK channels, also causes epilepsy in mice. In this case, perhaps because the β4 subunit has highest expression in the hippocampus, the seizures appear to be temporal lobe in origin, secondarily generalized, and of a non-convulsive type seizure. In both cases, the effects of the β4 knockout or D434G mutation are, perhaps paradoxically, to speed BK channel activation to rates that theoretically would allow a greater contribution of BK channels to repolarization of action potentials.

Background

BK Channel Regulation of Membrane Voltage

Because BK channels require relatively high calcium in combination with depolarization to be activated, they generally do not contribute to resting membrane potentials in neurons. Rather, BK channels are activated during the repolarization phase of an action potential, when calcium influx is greatest. They contribute to a transient outward current that sharpens the action potential and, in many neurons, also produce a transient afterhyperpolarization (fast AHP or fAHP). The fast decay of the AHP (<1 millisecond) is thought to occur because BK channels are also voltage sensitive and therefore deactivate quickly following repolarization. Although BK channels are not thought to directly regulate action potential firing frequency, they nevertheless have an important role in shaping action potential width, which impacts the relative voltage-dependent calcium channel activation and calcium influx into the cell. As will be discussed later, BK channel effects on action potential width and calcium influx may indirectly affect the recruitment of other potassium currents that control firing frequency.

It is worthwhile mentioning two other important calcium-activated potassium currents that contribute to AHPs in neurons. They have a more sustained activation and greater impact on directly regulating action potential firing rates. The decays of medium and slow AHPs (mAHP and sAHP, respectively) occur on the order tens of milliseconds to seconds, respectively. Although several channels affect the mAHP, the predominant calcium-activated channel is the small conductance, SK channel. Unlike BK channels, these channels are voltage-insensitive but have greater calcium sensitivity (submicromolar calcium). They are blocked by the bee venom, apamin. The channels that mediate the sAHP in peripheral neurons (such as enteric neurons) are the related family member, intermediate conductance (IK) calcium-activated potassium channels. However, in most central neurons, the channel underlying the sAHP is unknown.

How Do Bk Channels Increase Excitability?

Before there were indications that BK channels may have pro-epileptic effects, the precedence for a potassium channel that increases excitability was provided by the Kv3 voltage-gated potassium channels. These channels are expressed in a variety of 'fast-spiking' neurons, including inhibitory interneurons that fire action potentials at high rates with little adaptation. The Kv3 channels are high voltage activated, fast-gated potassium channels tailored for the fast-spiking rates. Their ability to quickly repolarize action potentials effectively removes sodium channel inactivation. Thus, during a spike train, action potentials can be faithfully driven at rates of 100 Hz or greater. Knockout of Kv3.2 reduces firing frequency and causes action potential failure during high current injections. As a consequence of reduced excitability in inhibitory neurons, the Kv3.2 KO mice are susceptible to seizures.

A survey of BK channel function in mammalian neurons indicates that it is also BK channels' effect on action potential firing frequency where we see effects that increase excitability. A conceptual scheme for BK channel's effect on action potential firing rates is shown in **Figure 2**. There are some common themes that emerge between different studies. One concept is that BK channel sharpening of action potentials may preclude activation of other, slower-gated potassium channels that would otherwise reduce excitability. This effect is seen in anterior pituitary somatroph neurons that require long plateau-bursts of action potentials to raise calcium to sufficient levels to evoke growth hormone release. The mechanism for plateau bursts is partly mediated by BK channel's effects on action potentials. BK channels activation in these cells reduces the height of the action potential, but is not sufficient for complete repolarization of the action potential, thereby generating the long-plateau bursts. Block of BK channels allow a larger depolarization and activation of a high-voltage activated, delayed rectifier potassium channel that sharply repolarizes the action potentials and converts plateau bursts to spikes.

In hippocampus CA1 pyramidal neurons, action potential trains progress with an early burst of spikes that is followed by spike-frequency adaptation. The early, high frequency action potential firing is promoted by the sharp repolarization and fAHP conferred by BK channels. Block of BK channels broadens action potentials and allows

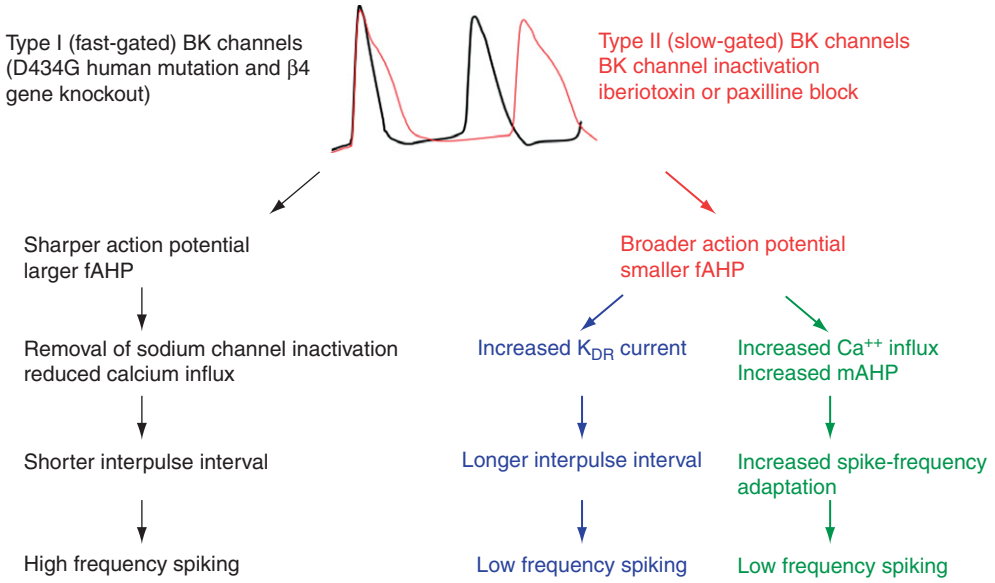


Figure 2 Scheme for BK channel effects on firing frequency. Consequences of fast-gated BK channels (black) encompassing Type I BK channels, the pro-epileptic D434G human mutation or the β4 gene knockout in mice are shown on the left. Fast-gated BK channels are effective at removing sodium channel inactivation and precluding activation of other potassium currents to enhance repetitive firing. Consequences of slow-gated (red) type II BK channels, BK channel inactivation or pharmacological block are shown on right. Slow-gated BK channels or their absence results in action potential broadening. The consequences are the recruitment of other potassium conductances such as the delayed rectifier in CA1 cells (blue scheme), or enhanced calcium influx and SK channel activation in dentate gyrus granule cells (green scheme) to reduce firing frequencies. ''Scheme for BK channels effects. ... ''has an acknowledgment that I emailed to Margaret Crick but for some reason was not forwarded to you. Please append to Figure 2 legend the following acknowledgement'' [Action potential traces (black trace is absence of iberiotoxin, red trace is with iberiotoxin block) were adapted from Gu N, Vervaeke K and Storm JF, *Journal of Physiology*, Vol. 580, No. 3, 859–882, 2007 with permission from the author and Wiley-Blackwell Publishing.'']

recruitment of a delayed-rectifier potassium current that reduces firing frequency (**Figure 2**). In addition, BK channels have a secondary effect that contributes to greater firing frequencies in CA1 cells. BK channels' ability to repolarize action potentials, and then quickly deactivate, effectively removes sodium channel inactivation without interfering with the depolarization phase of subsequent action potentials. This mechanism is ideal for supporting high frequency action potential firing and is analogous to the role that fast-gated Kv3 channels serve in fast-spiking interneurons. Indeed, in adrenal chromaffin cells, BK channel blockade also reduces firing frequencies through a mechanism that is attributed to increased sodium channel inactivation.

The examples given earlier indicate that fast-gated BK channels may be incompatible with some firing patterns that require broadened action potentials to recruit other potassium currents. The BK channel accessory β4 subunit is one example where evolution has designed a mechanism to prevent BK channel's activation during a spike. **Figure 3** demonstrates how β4 may prevent BK channel activation. Utilizing a square wave voltage stimulus (**Figure 3(a)**), we observe that BK/β4 channels have a greater than ten-fold slower activation than BK channels lacking β4 subunits. Relative to the short times scales of an action potential (<2 ms in most neurons), β4 at moderate calcium concentrations (7 μM) slows channel activation to rates that would preclude significant BK current during an action potential. Indeed, utilizing an action potential-shaped stimulus waveform (see **Figure 3(b)**), fast-gated BK channels in 7 μM calcium have a significant current, whereas BK/β4 channels do not. However, as calcium is increased to 100 μM, activation rates are similar for both channel subtypes (**Figure 3(a)**) and both can contribute to action potential repolarization (**Figure 3(b)**, right panel). Thus, the role of BK/β4 channels is strictly dependent on the local calcium environment. One may speculate that BK channels are strongly inhibited by the β4 subunit in the absence of very high calcium concentrations. However, at some sites where calcium rises to high concentrations, such as occurs at presynaptic sites, β4 would be permissive for BK channel contribution to action potential repolarization.

Methods

To investigate the role of the BK channel β4 subunit in neurons, the first exon of the β4 gene was deleted in embryonic stem cells by homologous recombination with the enhanced green fluorescent protein cDNA (eGFP). The eGFP replacement serves dual functions.

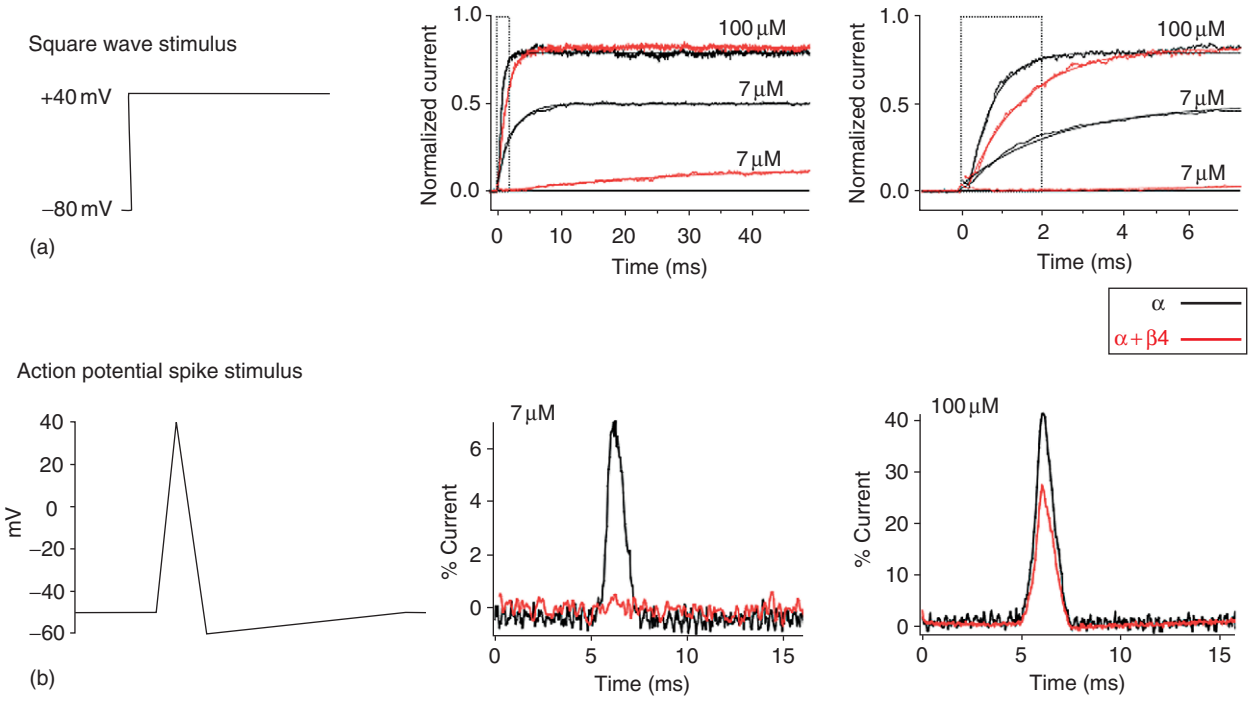


Figure 3 Effects of the β4 subunit on BK channel gating. (a) BK channel activation using a step potential to +40 mV (left panel) and resultant currents shown at two timescales (right panels). Time constants for activation in 7 μM calcium are 7.3 ± 1.7 ms for α and 244 ± 93 ms for α + β4. Time constants for activation in 100 μM calcium are 0.7 ± 0.03 ms for α and 1.5 ± 0.20 ms for α + β4 The dashed box indicates the theoretical time window for potassium currents to influence action potential repolarization. (b) BK currents (right panels) evoked by a 2.5 ms duration spike-shaped stimulus (left panel). Red traces are α + β4 and black traces α alone channels. Calcium concentrations are indicated near the traces. Currents were measured from mouse slo and mouse slo/β4 BK channels expressed in transfected HEK293 cells using the inside-out patch clamp configuration and physiological ionic concentrations.

First, it deletes a critical first exon and, by inclusion of a transcriptional terminator, precludes expression of other downstream β4 exons. In addition, the eGFP serves as a reporter for assaying distribution of the normal β4 gene expression. The gene-targeted embryonic stem cells were used to generate mice that carry the targeted β4 allele in the germline. Staining for eGFP in the transgenic mice showed that β4 was most abundant in the dentate gyrus of the hippocampus, an observation that consequently directed us to focus on these cells for further studies. As discussed above, EEG recordings indicated that β4 knockout mice had generalized, non-convulsive seizures that appeared to initiate from the temporal lobe. This feature of these mice was also consistent with the possibility that a defect in the hippocampus may contribute to the seizure phenotype.

Our study of the β4 subunit was investigated in brain slices of β4 knockout and wild type mice, using single channel recording of BK currents from excised patches and whole cell recording under current clamp conditions to observe effects on action potential firing patterns. For current clamp studies, the intracellar/pipette solution was weakly buffered to near resting calcium levels with 0.1 mM EGTA (resulting in ∼50 nM free calcium concentration) to allow calcium to accumulate during action potential firing. This technical approach allowed us to study how calcium-activated potassium currents regulate action potential shape and firing patterns in wild type and knockout granule cells.

Recent Results

The β4 Knockout as a Model for BK Channel Gain-Of-Function

Given that β4 slows BK channel activation, one may expect that the knockout would be a gain-of-function, allowing a greater BK channel activation during action potential firing. Consistent with this hypothesis, the β4 knockout indeed causes a sharpening of action potentials (**Figure 4(a)**) that is sensitive to the BK channel blocker, paxilline. In the wild type mouse, where β4 presumably reduces BK channel activation, action potentials are relatively broad and paxilline has little effect. Most importantly, the gain-of-function BK channels enhance firing frequency, and this effect can be reversed by BK channel blockade with paxilline (**Figure 4(b)**).

How does action potential broadening by β4 decrease firing frequency in the dentate gyrus? As discussed above, blocking BK channels and action potential broadening in CA1 cells secondarily recruits a slow-gated delayed

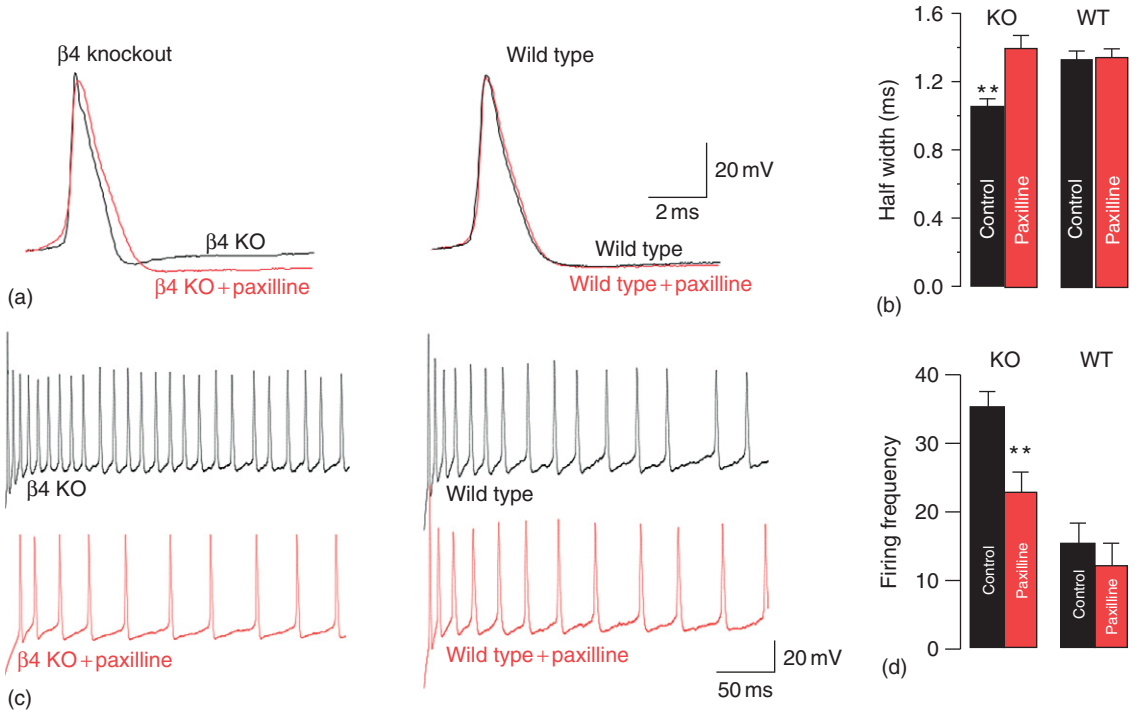


Figure 4 Firing properties of dentate gyrus granule cells indicate that gain-of-function of BK channels sharpens action potentials but increases firing frequencies. Action potentials are evoked by current clamp in the absence (black) or presence of BK channel blocker paxilline (5 μM, red). In knockout cells action potentials are sharp and have a more sustained afterhyperpolarization (a). Block of BK channels in the knockout sharpens action potentials (a, b) but increases firing frequencies (panels c and d). In wild type cells the β4 subunit reduces BK channel activation during action potential firing. This is evidenced by the fact that paxilline block has little effects on action potential waveform (a, b) or firing frequency (c, d). Representative data is shown from the 10th action potential waveforms during a 300 pA current injection. Representative firing patterns are during the first 300 ms during the trace.

rectifier potassium current that moderates spike frequency (Figure 2). In dentate gyrus granule cells, it appears that action potential broadening instead recruits SK channels and results in spike frequency adaptation. The mechanism is likely to be due to increased calcium influx during the broadened action potentials. Consistent with this hypothesis, a small concentration of cadmium, which non-selectively blocks many voltage-dependent calcium channels, increases firing frequency similar to that seen in cells from the β4 knockout/BK channel gain-of-function model (data not shown). The role of SK channels can be evaluated utilizing the SK channel blocker, apamin. In wild type neurons, apamin reduces the interpulse interval (an indicator of spike frequency adaptation) and increases firing rates (Figure 5). However in the β4 knockout, where BK channels are active, SK channel blockade does not affect the interpulse interval or firing frequency. These findings suggest a mechanism whereby β4 confers slow gating kinetics to BK channels to restrict their contribution to action potential repolarization. The broader spike width allows the necessary recruitment of other potassium currents during the interspike that moderate firing frequencies. In contrast, gain-of-function of BK channels in the β4 knockout reduces SK channel recruitment and enhances excitability.

Regulatory Mechanisms to Prevent BK Channels Activation

The human D434G mutation and the β4 gene knockout have uncovered an important glimpse into the regulation of channel expression that must underlie the finely tuned electrical properties of neurons. The relatively large potassium conductance of BK channels, unless appropriately regulated, can have antagonistic effect on activation of other potassium channels, such as delayed rectifier channels in CA1 cells or SK channels in dentate gyrus granule cells, that may otherwise reduce excitability. In hindsight, we can see how other potential regulatory mechanisms, like the β4 subunit, preclude BK channel activation during action potential spiking in neurons where β4 is not expressed. Indeed, in CA1 cells BK channels facilitate high frequency spiking only during early components of a spike train. Inactivation of BK channels at later components of the spike train, perhaps mediated by the accessory B_2 Subunit, allow action potential broadening and spike frequency adaptation. As well, an alternative splice product of BK channels that is expressed in pituitary and adrenal gland, the so called 'STREX' exon, promotes BK channel activation to similar levels as the proepileptic D434G mutation. However, expression of this

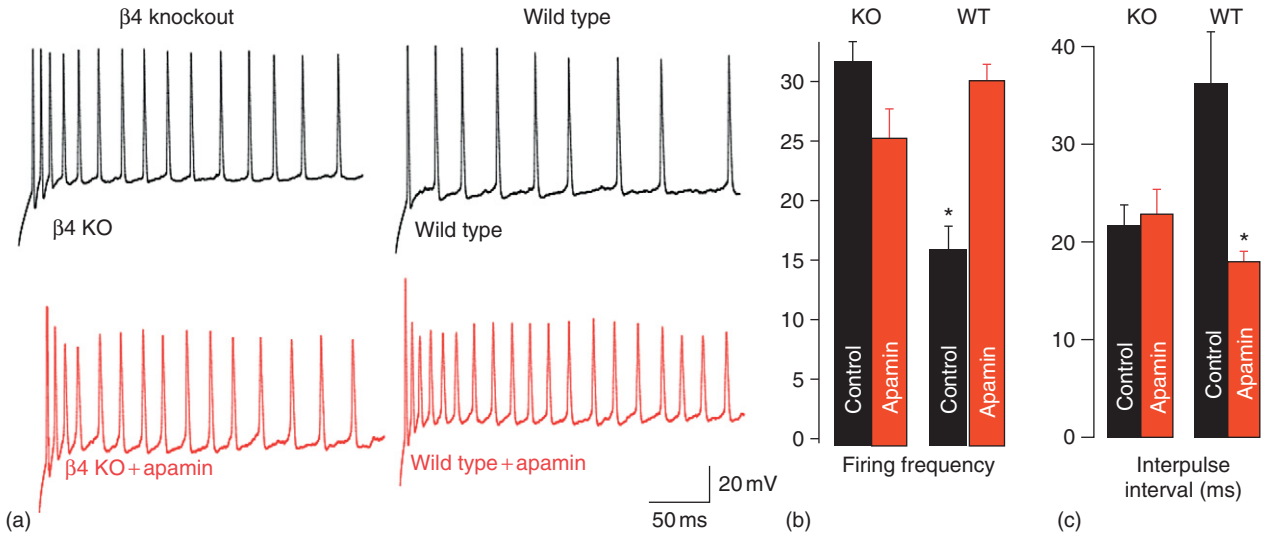


Figure 5 Loss of SK channel activation in the b4 knockout (BK channel gain-of-function) underlies increased firing frequency. The SK channel blocker apamin (100 nM) increases firing frequency in wild type neurons, (a,b) and reduces the interpulse interval (c). In BK channels gain-of-function (β4 KO), blocking SK channels is without significant effect (a–c). This suggests that the β4 KO reduces SK channel recruitment. Action potentials were evoked by a 250 pA current injection and example traces are the first 300 ms during the trace.

exon is downregulated in late neuronal development and is largely absent from most regions of the adult CNS.

From a standpoint of epilepsy, the dentate gyrus granule cells of the hippocampus have been regarded as a filter, positioned between strong excitatory input from the entorhinal cortex and the easily excited pyramidal cells of the hippocampus. The filter mechanism is attributed to strong inhibitory input from local interneurons that converge on dentate gyrus granule cells. These findings suggest that the intrinsic membrane properties of dentate gyrus granule neurons also have an important role. In this role, the β4 knockout studies have uncovered SK-type calcium-activated potassium channels as a key player. By mediating spike-frequency adaptation, SK channels reduce firing frequency and the likelihood of excitation of other cells in the hippocampus circuit. New classes of SK channel agonists indicate that this may be the case. These drugs include a low affinity agonist 1-ethyl-benzimidazolinone (1-EBIO, $EC_{50} \sim 500\,\mu M$), as well as recently identified higher affinity agonists DCEBIO ($EC_{50} = 27\,\mu M$) and NS309 ($EC_{50} = 0.6\,\mu M$). 1-EBIO is protective in a variety of rat brain slice models of epileptiform activity. In vivo effects of 1-EBIO indicate that SK channel activation affords protection against several rat seizure models (including electrically- and pentylenetetrazole-induced models), but little protection against pilocarpine-induced seizures.

Challenges for the Future

The mechanism by which BK channels negatively affect the recruitment of SK channels requires further study. The hypothesis that BK channels restrict calcium influx is not new; similar concepts have been developed in studies of smooth muscle and neurosecretory cells. SK and BK channels are frequently colocalized with specific calcium channels. We expect that this relationship occurs only in neurons where BK channels regulate a calcium influx pathway that SK channels utilize for activation. Indeed, it appears that there are examples where activation of BK channels, through leptin signaling or through BK channel agonists, reduces rather than enhances epileptiform activity of CA1 cells (in a rat brain slice model). Whether BK channel couple differently to SK channels in CA1 cells than granule cells requires further studies. Also, it is possible that leptin activation of BK channels occurs in CA3 presynaptic terminals, where BK channels have an established role in reducing excitability and neurotransmitter release.

Epilepsy is a neuronal circuit phenomenon. To cause seizures, changes in excitability must be coupled with increased synchronization within the circuit. Although this review has focused on BK channel effects on dentate gyrus granule cells, it is not certain that defects in these cells serve as the linchpin within the hippocampal circuit responsible for seizures. Future studies utilizing more sophisticated genetic techniques, such as tissue-specific and temporal-specific knockouts, will allow us to consider the role of these channels within the broader context of the neuronal circuit. This approach will allow us to address key questions, such as the followings:

- Are BK channel gain-of-function in dentate gyrus sufficient for temporal lobe epilepsy or are other cell types also involved?

- Are changes in intrinsic membrane properties sufficient to confer seizures, or do BK channels affect synaptic reorganization (such as mossy fibre sprouting) that contributes to the seizure phenotype?

From a clinical perspective, BK channels have been a target for drug design owing to their antagonist effects on calcium influx and the resultant relaxation of smooth muscle. BK channels being expressed in smooth muscle but not cardiac muscle has meant that agonists for BK channels could be used for many diseases associated with increased smooth muscle constriction (such as hypertension, incontinence and asthma) without side-effects associated with cardiac function. In the CNS, studies have investigated the use of BK channel agonist to control calcium-induced damage in neurons following stroke. However, these studies have taught us that BK potassium channel roles in the CNS are much more complex than anticipated in attempts at rational drug design. The possibility that BK channel gain-of-function may be pro-epileptic lends considerable caution to utilizing BK channel agonists that can cross the blood-brain barrier.

See also: **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Sodium Channel Mutations that Cause GEFS+; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury.

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Sodium Channel Mutations that Cause GEFS+

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Introduction

Generalized epilepsy with febrile seizures plus (GEFS+) is an autosomal dominant disorder with a complex and heterogeneous clinical phenotype. Although febrile seizures occur in ~5% of all children under the age of 6 years, they are not typically associated with increased risk of epilepsy in adolescence and adulthood. In contrast, patients with GEFS+ demonstrate febrile seizures that either persist beyond 6 years of age or are associated with generalized epilepsies such as absences, myoclonic seizures, atonic seizures, and myoclonic-astatic epilepsy.

- Are changes in intrinsic membrane properties sufficient to confer seizures, or do BK channels affect synaptic reorganization (such as mossy fibre sprouting) that contributes to the seizure phenotype?

From a clinical perspective, BK channels have been a target for drug design owing to their antagonist effects on calcium influx and the resultant relaxation of smooth muscle. BK channels being expressed in smooth muscle but not cardiac muscle has meant that agonists for BK channels could be used for many diseases associated with increased smooth muscle constriction (such as hypertension, incontinence and asthma) without side-effects associated with cardiac function. In the CNS, studies have investigated the use of BK channel agonist to control calcium-induced damage in neurons following stroke. However, these studies have taught us that BK potassium channel roles in the CNS are much more complex than anticipated in attempts at rational drug design. The possibility that BK channel gain-of-function may be pro-epileptic lends considerable caution to utilizing BK channel agonists that can cross the blood-brain barrier.

See also: **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Sodium Channel Mutations that Cause GEFS+; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury.

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Sodium Channel Mutations that Cause GEFS+

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Introduction

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GEFS+ has been shown to result from mutations in five different ion channel genes, three of which encode subunits of the voltage-gated sodium channel.

Background

The voltage-gated sodium channel is comprised of a pore-forming α subunit that forms a fully functional channel whose properties can be modulated by association with one or more accessory subunits (β1, β2, β3, and β4). The α subunit is ~260 kDa and consists of four homologous domains termed I–IV, with six transmembrane segments called S1–S6 within each domain (**Figure 1**). The mammalian voltage-gated sodium channel α subunits are encoded by nine genes, resulting in the expression of distinct isoforms called $Na_v1.1$ through $Na_v1.9$. Four isoforms are expressed at high levels in the CNS ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$) and four isoforms are expressed at high levels in the peripheral nervous system ($Na_v1.6$, $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$). The remaining two isoforms are expressed in adult skeletal muscle ($Na_v1.4$) and in embryonic skeletal muscle and heart muscle ($Na_v1.5$).

Mutations in three different CNS sodium channel genes result in two subtypes of GEFS+. GEFS+ type 1 is caused by mutations in the *SCN1B* gene encoding the β1 subunit, and GEFS+ type 2 is caused by mutations in either the *SCN1A* gene encoding the $Na_v1.1$ α subunit or the *SCN2A* gene encoding the $Na_v1.2$ α subunit. Mutations that cause GEFS+ have been identified in two additional genes, *GBRG2* encoding the $GABA_A$ receptor γ2 subunit and *GBRD* encoding the $GABA_A$ receptor δ subunit. The majority of the known GEFS+ mutations, 13 thus far, have been identified in the *SCN1A* gene. Severe myoclonic epilepsy in infancy (SMEI), the most severe form of GEFS+, results from a haploinsufficiency of *SCN1A*, and intractable childhood epilepsy with generalized tonic–clonic seizures is caused by missense mutations in *SCN1A*.

All of the epilepsy syndromes resulting from sodium channel mutations that have been identified thus far are inherited in an autosomal dominant fashion, meaning that mutation of just one copy of the gene is sufficient to cause the syndrome. Most of the mutations involve a change in a single amino acid, so that the channel is still active but has altered functional properties. However, some of the mutations involve the insertion of a nonsense codon and thus terminate the protein early in the reading frame, resulting in complete loss of protein. Missense mutations in the *SCN1A* sodium channel gene are the most common cause of GEFS+, whereas nonsense mutations in the same gene are the most common cause of SMEI. The severity of the epileptic syndrome resulting from mutations in *SCN1A* depends on the type of mutation, with the most severe phenotype being caused by complete loss of sodium channel protein, as occurs in SMEI.

Methods

Characterization of Mutant Sodium Channels by Expression in *Xenopus* Oocytes

Because the $Na_v1.1$ sodium channel is expressed primarily in the CNS, it is not possible to directly determine the effects of mutant channels that cause GEFS+. In addition, neurons typically contain more than one

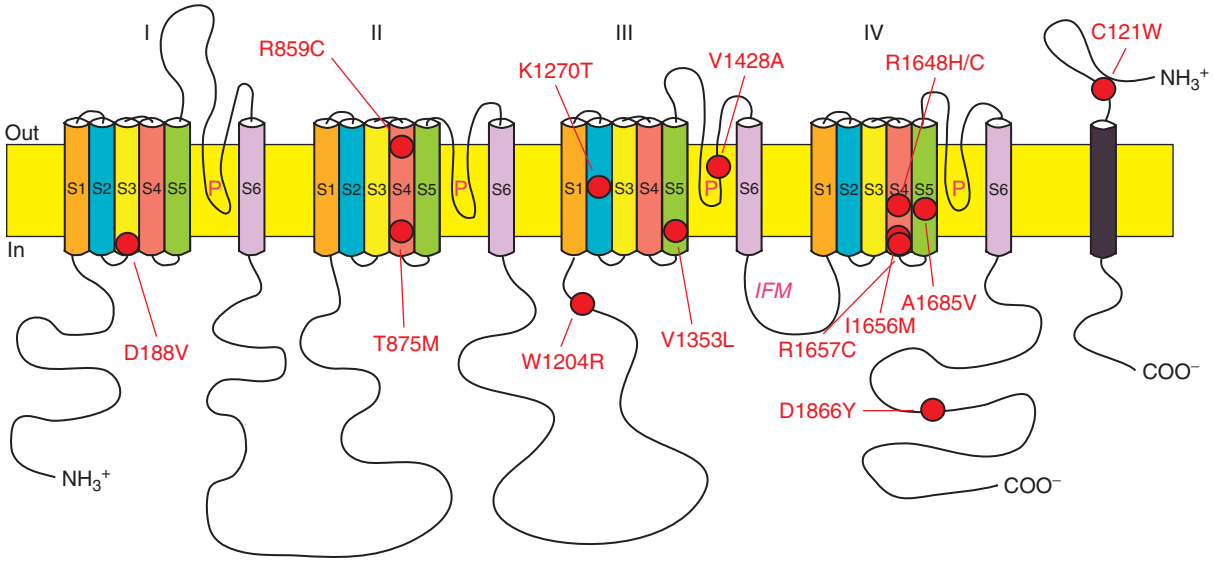


Figure 1 Schematic diagram of the voltage-gated sodium channel α and β subunits showing the positions of the mutations that cause GEFS+ (red circles). The four homologous domains in the α subunit are labeled I–IV, with each domain containing six transmembrane segments labeled S1–S6.

sodium channel isoform, making it difficult to isolate sodium currents through $Na_v1.1$. Therefore, we used a heterologous expression system to determine how the GEFS+ mutations alter sodium channel function. The cells that we used are oocytes from *Xenopus laevis* frogs, which have a number of advantages. First, the oocytes do not express any endogenous sodium channels, and so it is possible to record from the heterologous channels without contaminating currents. Second, the subunit composition can be controlled, making it possible to determine if the mutations alter the interactions between the α and β subunits. Third, there are a variety of electrophysiological recording techniques that can be used to obtain detailed information about channel activity.

We used the rodent cDNA clone for $Na_v1.1$ because our ultimate goal was to examine the function of the mutations in transgenic and knock-in mice, and the human and rat channels are 98% identical in amino acid sequence. The channels were expressed by transcription of RNA in vitro for injection into *Xenopus* oocytes, after which the electrophysiological properties of the channels were determined using the cut-open oocyte voltage-clamp. Each of the mutations was examined in the absence and presence of the β1 subunit. The various properties that were examined included the voltage-dependence of activation and inactivation, the kinetics of inactivation and recovery, use-dependent decreases in current amplitude, and slow inactivation.

Computational Model

The electrophysiological recordings provide data concerning how the mutations affect function of the sodium channel. However, the physiologically relevant property is the effect of the mutations on neuronal and network activity. To determine how the changes in sodium channel function might affect neuronal activity, we designed a computational model using the experimentally defined characteristics of the mutant sodium channels and a generic delayed rectifier potassium channel. Hodgkin–Huxley conductance-based models of spiking neurons were constructed using NEURON simulation software. The models included either wild-type or mutant channels with properties determined from the electrophysiological characterization. Sodium currents were recorded from the model neurons using protocols comparable to those used for the recordings from oocytes to assess the validity of the computational model. Current clamp simulations were then carried out to determine how the mutations might alter action potential threshold, shape, and repetitive firing properties.

Recent Results

All of the sodium channel GEFS+ mutations that have been identified thus far are missense alterations resulting in single amino acid substitutions. In addition, nearly all of them are located in or adjacent to the transmembrane or pore-forming regions of the channel (**Figure 1**), suggesting that the primary effect of the mutations is to alter the functional properties of the channel rather than to alter gene expression, protein processing, or targeting of the channel to the membrane. Our working hypothesis is that alterations in channel function lead to the seizure phenotype. To begin to test this hypothesis and determine how the alterations cause seizures, we and others have analyzed the effects of many of the GEFS+ mutations using heterologous expression systems. The results thus far suggest that most of the mutations alter different aspects of sodium channel function, and that the alterations depend on the expression system.

We have examined the effects of five different mutations in the rat $Na_v1.1$ sodium channel expressed in *Xenopus* oocytes, and each mutation altered sodium channel activity in a different manner. Some of the effects would be predicted to increase sodium channel activity, whereas others would be predicted to decrease activity. The five mutations are R859C, T875M, W1204R, R1648H, and D1866Y, and they are located throughout the sodium channel (**Figure 1**). R859C replaces a positively charged arginine with cysteine, and T875M replaces a threonine with methionine, with both located in the domain II S4 segment that comprises part of the voltage sensor for activation. W1204R replaces a hydrophobic phenylalanine with a positively charged arginine in the cytoplasmic linker between domains II and III. R1648H replaces a positively charged arginine with an uncharged histidine in the S4 voltage sensor of domain IV. Finally, D1866Y replaces a negatively charged aspartate with a tyrosine residue in the cytoplasmic carboxy-terminus of the channel.

The mutations that increased sodium channel activity are W1204R, R1648H, and D1866Y (**Table 1**). The W1204R mutation shifted the voltage-dependence of activation and inactivation in the hyperpolarized direction, causing the channel to open at more negative potentials. The R1648H mutation accelerated recovery from inactivation and decreased use-dependence, both of which would facilitate repetitive firing. The D1866Y mutation decreased modulation of the α subunit by β1, which normally causes a negative shift in the voltage-dependence of inactivation in oocytes. There was less of a shift with the mutant channel, thus increasing the magnitude of the persistent window current, an effect that would increase firing. The other two mutations (R859C and T875M) decreased sodium channel activity. The R859C mutation caused a positive shift in the voltage-dependence of sodium channel activation, slowed recovery from slow inactivation, and decreased levels of current compared with wild-type channels. The T875M mutation enhanced slow inactivation, resulting in fewer channels that are available to be activated. That each mutation

Table 1 Properties of the sodium channel mutations that cause GEFS+ type 2

Mutation	*Channel*[a]	*Cell type*[b]	*Effects*
Increased activity			
D188V	rNa$_v$1.2	HEK	↓ Use-dependence, faster recovery from slow inactivation
T875M	rNa$_v$1.1	*Xenopus* oocytes	↑ Persistence
W1204R	rNa$_v$1.1	*Xenopus* oocytes	Negative voltage-dependence
W1204R	hNa$_v$1.1	tsA201	↑ Persistence
R1648H	rNa$_v$1.1	*Xenopus* oocytes	↓ Use-dependence, faster recovery
R1648H	hNa$_v$1.1	tsA201	↑ Persistence
R1648H	hNa$_v$1.4	tsA201	Faster recovery
R1648C	rNa$_v$1.1	tsA201	↑ Persistence
D1866Y	rNa$_v$1.1	*Xenopus* oocytes	↑ Persistence
Decreased activity			
R859C	rNa$_v$1.1	*Xenopus* oocytes	Positive voltage-dependence, slower recovery from slow inactivation, ↓ current
T875M	rNa$_v$1.1	*Xenopus* oocytes	↑ Slow inactivation
T875M	hNa$_v$1.4	tsA201	↑ Fast and slow inactivation
V1353L	hNa$_v$1.1	tsA201	No sodium current
I1656M	hNa$_v$1.1	tsA201	Positive voltage-dependence
R1657C	hNa$_v$1.1	tsA201	Positive voltage-dependence, ↓ current
A1685V	hNa$_v$1.1	tsA201	No sodium current

[a]Channel in which the mutations were constructed for electrophysiological analysis. The channels are encoded by the following genes: rNa$_v$1.2, rat *Scn2a*; rNa$_v$1.1, rat *Scn1a*; hNa$_v$1.1, human *SCN1A*; hNa$_v$1.4, human *SCN4A*.
[b]Cell type in which the mutations were expressed for electrophysiological analysis.

affected a different aspect of sodium channel function suggests that multiple different alterations in sodium channel activity can cause a similar seizure phenotype.

A number of mutations have also been examined by other laboratories using expression in tsA201 cells, a human embryonic kidney (HEK) cell line, with similar findings of both increased and decreased activity (Table 1). Five mutations were observed to alter the channel in a manner consistent with increased activity. D188V in human Na$_v$1.1 was studied using HEK cells by the laboratories of Ragsdale, Dunn, and Rouleau. That mutation caused decreased use-dependence and accelerated recovery from slow inactivation, similar to our results with R1648H. The laboratories of Lehmann-Horn and Lerche observed similar effects for the R1648H mutation, which they studied in the human Na$_v$1.4 skeletal muscle channel. In contrast, the laboratory of George noted a marked increase in persistent current for the R1648H mutation, and a lesser increase in persistent current for W1204R, R1648C, and T875M. They hypothesized that the persistent current resulted in hyperexcitability and the epileptic phenotype.

However, this prediction about the effects of mutations on persistent current is not consistent with all of the data. T875M decreased sodium channel activity in our experiments and also in those of Lehmann-Horn and Lerche. In addition, four other mutations caused alterations that decreased sodium channel activity. The George laboratory observed positive shifts in the voltage-dependence of activation for I1656M and R1657C, reduced sodium current density for R1657C, and complete absence of sodium current in the heterologous system for V1353L and A1685V. Complete absence of current could be the result of a nonfunctional channel or a failure in protein trafficking to the cell membrane. That all of these mutations decreased sodium channel activity without increasing persistent current indicates that persistent current is not the general cause of seizures in GEFS+. In fact, there does not appear to be any one consistent functional consequence among the many mutations that have been characterized thus far.

Given the wide range of effects of the GEFS+ mutations, with some increasing sodium channel activity and others decreasing activity, it is extremely difficult to predict how the mutations might alter neuronal function. To begin to address this question, we used a simple computer modeling approach to gain insight into how the mutations could affect the firing of action potentials. This approach has a number of advantages. First, action potentials are dynamic events, and so the steady-state biophysical properties of sodium channels determined in heterologous systems may not combine to produce the expected outcomes in vivo. A computational model is a logical tool to predict how changes in steady-state properties might interact and alter the dynamic process of action potential generation. Second, the computer model makes it possible to include both mutant and wild-type channels in the same cell, which is important because GEFS+ is dominantly inherited. Finally, the computational model can help to guide future experiments once an animal model is developed.

To study the effects of GEFS+ mutations on action potential firing, we constructed single compartment neuronal soma models that contained Na$_v$1.1 or GEFS+ mutant sodium channels and delayed rectifier potassium channels, the minimum components necessary for the firing of an action potential. Although the interaction of the wild-type and mutant sodium channels with potassium channels could vary depending on the type of potassium channels that are present, we chose to use a simplified model containing only delayed rectifier potassium channels so that any differences in the firing of action potentials between the mutant and wild-type cells

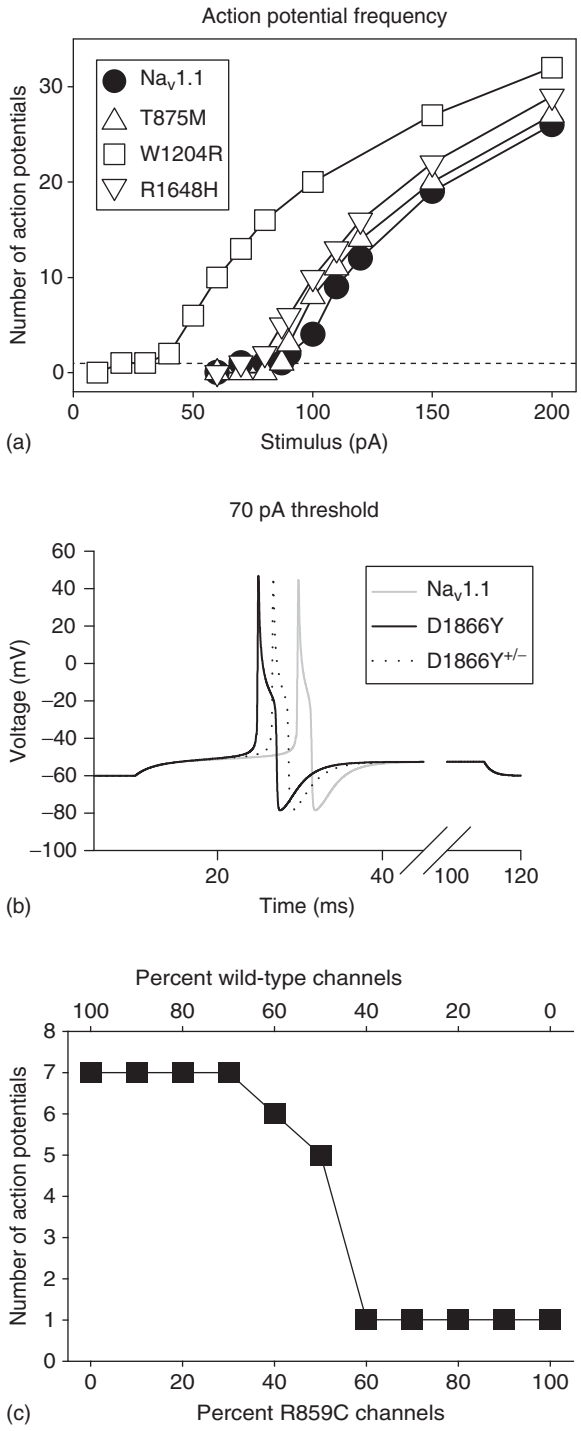


Figure 2 Effects of the GEFS+ mutations in a computational model. (a) The number of action potentials elicited by 200 ms current injections starting with 10 pA and increasing by 20 pA are shown for model neurons expressing wild-type $Na_v1.1$ (circles), T875M (up triangles), W1204R (squares), and R1648H (down triangles). (b) At the threshold stimulus for a single action potential (70 pA), the D1866Y mutant model neuron (black line) fired a single action potential with a shorter onset delay than the wild-type $Na_v1.1$ model neuron (grey line). When the population of channels was mixed in a 1:1 ratio ($D1866Y^{+/-}$, black dotted line), the mutant channels displayed a dominant effect, resulting in a shorter onset delay for firing a single action potential than would be attributed exclusively to changes in the sodium channel conductances. Passive parameters similar to the passive properties of hippocampal principal cells were adapted from previous models.

Using the NEURON simulation software, we conducted a typical input–output neurophysiology experiment on the model neurons. The model neurons were stimulated by injection of current and the resulting changes in membrane potential were generated by the computational model. Beginning with a subthreshold stimulus, we gradually increased the amplitude of the current injection and counted the number of action potentials generated to estimate the threshold for action potential firing. We observed that W1204R decreased the threshold for firing a single action potential, T875M and R859C increased the threshold, and R1648H and D1866Y did not affect the threshold. Therefore, effects on action potential threshold were not consistent with a single mechanism of pathogenesis.

We also examined the propensity of the model neurons containing mutant channels to fire multiple action potentials. Three mutations (T875M, W1204R, and R1648H) resulted in more action potentials being fired compared with a model neuron containing wild-type channels (**Figure 2(a)**). The magnitude of this effect was different for each mutation, with the most severe being W1204R and the least severe being T875M. Although the D1866Y mutation did not alter the number of action potentials fired in response to a given stimulus, it did alter the timing of those action potentials, reducing the latency to firing and the spike-to-spike interval between action potentials, thus increasing the spontaneous firing frequency (**Figure 2(b)**). In contrast, the final mutation (R859C) decreased the propensity of the model neuron to fire multiple action potentials.

Since GEFS+ is an autosomal dominant disorder, affected individuals express a mixed population of mutant and wild-type channels. Although it is difficult to vary the ratio of mutant and wild-type channels in a heterologous

would be expected based on the individual behavior of the two groups. (c) Model neurons containing a mixture of R859C mutant and wild-type channels behaved in an additive fashion. Neurons were assigned a mixed population ranging from 0% to 100% R859C mutant (100% to 0% wild-type) sodium channels, and the number of action potentials elicited during injection of 150 pA was determined and plotted versus the percentage of mutant channels. Reproduced from Spampanato J, Kearney JA, de Haan G, *et al.* (2004) A novel epilepsy mutation in the sodium channel SCN1A identifies a cytoplasmic domain for β subunit interaction. *Journal of Neuroscience* 24: 10022–10034, with permission from the Society for Neuroscience; Barela AJ, Waddy SP, Lickfett JG, *et al.* (2006) An epilepsy mutation in the sodium channel SCN1A that decreases channel excitability. *Journal of Neuroscience* 26: 2714–2723, with permission from the Society for Neuroscience.

expression system, this is a very simple experiment to perform using a computational model. When we varied the percentage of each of the mutant channels compared with the wild-type, the results depended on the mutation. D1866Y and W1204R were dominant in that a 1:1 mixture of mutant and wild-type channels demonstrated properties similar to a neuron with all mutant channels. In contrast, the other three mutations (R1648H, T875M, and R859C) each behaved in an additive fashion, so that the phenotype of a 1:1 mixture was intermediate between that of wild-type and mutant channels (**Figure 2(c)**). These results suggest that the phenotype of individuals with these mutations depends on the ratio of mutant to wild-type channels that is present in the neuron.

Although the computer modeling results suggest that there is no single mechanism of seizure pathogenesis for the GEFS+ mutations, it is difficult to extrapolate from computer modeling to the situation in vivo. The effects of the mutations on network activity depend very strongly on the specific neuronal populations in which the mutant channels are expressed, and there is evidence that $Na_v1.1$ is responsible for a substantial percentage of the total sodium current in interneurons but a very small percentage in pyramidal cells. The same biophysical alteration would have dramatically different effects on network firing if it were present in inhibitory interneurons versus excitatory pyramidal neurons. In addition, $Na_v1.1$ is expressed predominantly in the soma and dendrites of neurons, whereas action potential initiation most likely occurs at the axon hillock, so that the GEFS+ mutations may have a greater effect on signal integration rather than action potential initiation or propagation.

Future Goals

The data from heterologous expression systems and computer modeling suggest that both gain-of-function and loss-of-function mutations in the gene encoding $Na_v1.1$ can predispose the CNS to epileptogenesis. However, this conclusion assumes that the effects of the mutations in heterologous systems are a direct reflection of the effects in native neurons, which may not be the case for a number of reasons. First, sodium channels in the CNS associate with two of four accessory β subunits (β1 or β3 and β2 or β4). The specific β subunits that are present in each type of neuron could alter the properties of the sodium channels, and hence the effects of the mutations. Second, the GEFS+ mutations could alter aspects of channel processing or trafficking that may be unique to neurons, so that the physiologically relevant phenotype cannot be observed in another cell type. Third, neurons express multiple sodium channel isoforms as well as an array of other voltage- and ligand-gated ion channels. The repertoire of channels can have a dramatic effect on the properties of a mutation, as has been demonstrated by the laboratory of Waxman. They observed that a sodium channel mutation in $Na_v1.7$ causing erythermalgia made sympathetic neurons hypoexcitable, but the same mutation made sensory neurons hyperexcitable because of the presence of $Na_v1.8$ in those neurons. For these reasons, it is essential to determine the effects of the GEFS+ mutations in native neurons, which is the goal of our current research.

We are using two approaches for this purpose. First, we are using a Semliki Forest virus (SFV) vector system to obtain high efficiency expression of the sodium channels in neurons. Second, we are constructing both transgenic and knock-in mouse models expressing the GEFS+ mutations. Each approach has advantages and disadvantages.

The primary advantage of using the SFV vector system for expression of sodium channels is that SFV infects neurons with very high efficiency. In addition, it is possible to construct and analyze many different mutations because it is relatively fast to construct a viral vector with each mutation and the neurons can be easily infected. To facilitate these studies, we have inserted the coding region of $Na_v1.1$ into an SFV vector with two modifications. First, sequences encoding the red fluorescent protein (RFP) were inserted at the amino terminal end of the channel, making it possible to identify infected cells by fluorescence. Second, the channel was made relatively resistant to tetrodotoxin (TTX) by the E954Q mutation. The primary CNS sodium channels ($Na_v1.1$, $Na_v1.2$, $Na_v1.3$, and $Na_v1.6$) are all blocked by nanomolar concentrations of TTX, whereas channels containing the E954Q mutation are not blocked until the TTX concentration reaches millimolar levels. Therefore, we can record currents through only the mutant channels by blocking the endogenous neuronal channels with nanomolar concentrations of TTX. We have shown that SFV containing the sodium channel is efficiently expressed in primary neuronal cultures and we are now in the process of recording currents from infected cells.

A mouse model system is the most physiologically relevant approach for examining the effects of the sodium channel mutations that cause GEFS+, but it is impractical to construct mice expressing many different mutations. Therefore, we are using this approach to study a few mutations in depth by constructing both transgenic and knock-in mice. The use of transgenic mice provides some distinct advantages and disadvantages than that of knock-in mice. A major advantage of transgenic mice is that the channel can be easily modified with either a synthetic epitope or a variant of green fluorescent protein at the amino terminus and the TTX resistance mutation. Therefore, we can determine which neurons express the mutant channel, what percentage of sodium current is conducted through the mutant channel, and the biophysical effects of the mutation in isolation. The major disadvantage is that regulation is abnormal. We have mitigated

this problem by using a bacterial artificial chromosome containing the entire gene encoding $Na_v1.1$. Because the chromosome contains the entire sodium channel gene with its native promoter, expression is regulated normally. A second disadvantage of using transgenic expression is that the mutant gene is expressed in addition to the wild-type gene, so that the cells express more than two alleles. The presence of wild-type alleles should not mask the effect of the mutant allele, though, because all of the GEFS+ mutations are inherited in a dominant manner.

The major advantage of knock-in mice is that they are the most physiologically relevant model of the human syndrome – heterozygous mice express one mutant allele and one wild-type allele. A second advantage is that the dose-dependent effects of the mutation can be analyzed by studying both heterozygous and homozygous mice. The major disadvantage is that the mutant channel cannot be distinguished from the endogenous wild-type CNS channels, including $Na_v1.1$, $Na_v1.2$, and $Na_v1.6$, so that these mice cannot be used to determine which populations of neurons express the mutant channels nor to determine the biophysical properties of the mutant channels in neurons.

Our collaborator, Dr. Andrew Escayg of Emory University, has constructed both transgenic and knock-in mice expressing the R1648H mutation, and we are in the process of analyzing both lines of mice. We have used the transgenic mice to determine the relative expression levels and the biophysical properties of the mutant $Na_v1.1$ channel in dissociated cortical neurons. We are using the knock-in mice to examine the effects of the mutation on network properties by recording from hippocampal and cortical slices, which should provide insight into how the altered neuronal activity leads to synchronous firing in the population. The knock-in mice are also being used to determine the phenotypic effects on the animal by characterizing susceptibility to seizures induced by a variety of agents, including heat as a model for febrile seizures.

The data thus far concerning relative levels of expression indicate that the R1648H channel is expressed at higher levels in bipolar inhibitory cortical neurons compared with pyramidal neurons, suggesting that $Na_v1.1$ is preferentially expressed in inhibitory neurons. These findings are consistent with results obtained from two mouse models of SMEI. The Catterall laboratory observed significant loss of sodium current density in heterozygous and homozygous *Scn1a* knock-out mice in inhibitory interneurons but not in excitatory pyramidal neurons from the hippocampus. The Yamakawa laboratory noted that $Na_v1.1$ was expressed predominantly in parvalbumin-positive inhibitory interneurons in knock-in mice expressing a *Scn1a* nonsense mutation. We have also obtained preliminary data concerning the biophysical properties of R1648H mutant channels in these bipolar neurons. The most prominent effect of the mutation was increased use-dependence, leading to decreased sodium channel activity. This effect was not observed for R1648H in any of the heterologous expression systems, all of which demonstrated alterations that increased sodium channel activity.

Our current hypothesis is that loss of sodium channel activity in inhibitory neurons is a general cause of GEFS+ type 2. This hypothesis is based on a number of results. First, the $Na_v1.1$ channel is expressed preferentially in inhibitory neurons in both the cortex and hippocampus. Second, the R1648H mutation decreased sodium channel activity in inhibitory cortical neurons. Third, complete loss of one *SCN1A* allele encoding $Na_v1.1$ causes SMEI, which can be categorized as the most severe form of GEFS+. Finally, decreased inhibition has been postulated to be the cause of a number of different epilepsy syndromes. A consistent model is that loss of sodium channel activity in inhibitory neurons causes epilepsy, with the severity of the syndrome being proportional to the loss of activity. Decreased activity, such as that from missense mutations in GEFS+, causes moderate epilepsy syndromes. Complete loss of one allele, such as that from nonsense and frameshift mutations in SMEI, causes more severe epilepsy syndromes. More detailed analysis of mouse models for the syndromes resulting from sodium channel mutations should make it possible to test the validity of this model.

See also: **Genetics**: Clinical Genetics of Epilepsy; Single Gene Mutations in Inherited and Sporadic Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; Ion Channel Mutations in Familial Eplepsy.

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K

KETOGENIC DIET

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Animal Models of the Ketogenic Diet and Other Dietary Therapies

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Introduction

Therapeutic options for management of medically refractory seizures in young children are limited. Despite the plethora of old and new anticonvulsant medications, up to one-third of children with epilepsy continue to have seizures, and many of these children take multiple medications with concurrent intolerable side effects. Alternative treatments have included the vagus nerve stimulator and dietary approaches. The ketogenic diet (KD), a high fat, low carbohydrate, adequate protein formulation, has been used since early in the twentieth century for seizure control in refractory epilepsy. Clinical studies have verified the effectiveness of the KD. In general, at least half of all patients treated with the KD will exhibit a 50% or greater reduction in seizure frequency. Despite this effectiveness, the mechanisms by which the diet works are still unknown.

Since the resurgence in clinical use of the KD in the 1990s, several laboratories have attempted to develop animal models to discern the mechanism of KD action and improve its formulation and effectiveness. In general, such models have met with modest success. Part of the challenge has been to replicate the effect of the KD in humans. However, the metabolism of rodents (the most commonly used model) differs in important ways from humans. During fasting, ketone bodies supply relatively more brain energy in humans than in rats. In the neonatal period, ketone bodies are used more by suckling rats than by humans. In addition, previous models have employed a wide variety of paradigms of seizure induction (kindling, pentylenetetrazole (PTZ), kainic acid, etc.), KD formulations, outcome measures, and assessments of metabolic parameters. Any mechanistic explanation for KD effectiveness in humans must account for certain clinical observations in animal models (**Table 1**).

When switching from a diet high in carbohydrates to one high in fats and with stringent carbohydrate restriction, the basic metabolic alteration is that fats become the primary energy source. Fat breakdown occurs in the liver, creating ketone bodies (β-hydroxybutyrate, acetoacetate, and acetone), which circulate to the brain and are taken up into cerebral tissue via specific monocarboxylate transporters. In neuronal mitochondria, ketones are metabolized to adenosine triphosphate (ATP) via the tricarboxylic acid (TCA) cycle and oxidative phosphorylation. The challenge has been to understand how this energy shift results in an anticonvulsant effect. Obviously, multiple sites exist in the relevant biochemical pathways in which seizures could be suppressed, and the mechanism (or more likely, mechanisms) by which the KD exerts an anticonvulsant effect likely involves the combination of altered energy homeostasis and regulation of membrane and synaptic excitability.

Early theories of KD mechanism focused on the involvement of ketone bodies. However, several clinical

Table 1 Criteria for an ideal animal model of the ketogenic diet

Seizure type: Broad efficacy against partial and generalized seizures
Age range: More effective at younger ages
Diet formulation: Effective with classic 3:1 and 4:1 ketogenic ratios as well as with KD modifications such as fasting, the medium-chain triglyceride diet, the Atkins diet, and the low glycemic index diet
Calorie restriction: Equally or more effective than classic KD
Ketosis: Necessary but not sufficient
Latency to effectiveness: Seizure reduction during fasting or first few days of KD initiation
Loss of seizure protection with KD discontinuation or addition of carbohydrates: Rapid (within hours)

Not all of these criteria are likely to be met in a single model and useful information can be obtained from animal models not meeting all the criteria (see text).

and experimental studies have shown that the relation between serum or urinary ketone levels and seizure control is imprecise, at best. Direct effects of ketones on neurophysiological parameters, excitatory and inhibitory neurotransmission, and ictal activity in vitro have been essentially negative, although the inclusion of glucose in the bathing medium of some brain slice experiments could have counteracted the influence of the ketones. Recent experiments have shown that, in vivo, rats on the KD or a calorie-restricted normal diet exhibit increased paired-pulse inhibition in the hippocampal dentate gyrus and increased resistance to maximal dentate activation. At present, it may be concluded that some as yet undetermined threshold level of ketosis is required and must be maintained for the KD to be maximally effective. Whether this threshold correlates with ketone levels remains unclear; the concentration of ketones at the synapse is unknown.

Other early theories included an alteration in cerebral energetics induced by the KD, which favor an increased 'energy charge' (a relative increase in the ATP/ADP ratio resulting from metabolic alterations of enzymes involved in glycolysis and the TCA). It was hypothesized that the greater availability of energy reduces cellular excitability, enhancing hyperpolarization. Another theory involved alterations in the inhibitory neurotransmitter, γ-aminobutyic acid (GABA). GABA function could be enhanced through the GABA shunt whereby glial conversion of glutamate to glutamine (a GABA precursor) is enhanced by ketosis. Ketones mimic GABA structurally and could play a direct inhibitory role on cellular excitability by activating GABA receptors or enhancing their action. KD treatment and calorie restriction increase the expression of glutamic acid decarboxylase (GAD), providing a mechanism by which GABA synthesis might increase in a patient on the KD.

Other theories have implicated the quantity or type of fat in the diet. Obviously, the KD involves large excess of fatty acids, usually of the saturated variety in the form of heavy cream or butter. Fatty acids of various chain lengths are typically included in the diet. Early clinical studies have shown a correlation between plasma lipid levels and seizure control in children on the KD. Experimental studies have likewise shown a correlation of plasma lipid levels with seizure control, independent of the level of ketosis.

A neuroprotective effect of the KD has also been proposed. The KD has been shown to increase the expression of mitochondrial uncoupling proteins and thereby reduce neuronal damage induced by formation of injurious reactive oxygen species (ROS).

In summary, there is no lack of theoretical mechanisms by which the KD can exert an anticonvulsant effect. It is doubtful that a single mechanism can account for all of the observed clinical and experimental effects.

Background

The original idea for the KD was derived from the beneficial effect of fasting on seizures. Fasting is obviously impractical except for very short-term treatment. Nevertheless the health benefits of modest calorie restriction are becoming increasingly clear and include an increased lifespan, reduced risk of cancer and cardiovascular disease, and amelioration of the degenerative effects of aging. Restriction of calorie intake can also be neuroprotective. In rats receiving kainic acid, learning defects and cellular damage are reduced when animals are placed on a calorie-restricted diet, possibly as a result of reduced oxidative stress. It is not known how calorie restriction prevents detrimental degenerative effects of aging. Multiple genes that enhance neuronal survival and plasticity are likely to be involved. It is worth exploring the possibility that calorie restriction can limit seizure occurrence.

Children on the KD are typically restricted to 75% of the recommended daily calorie allowance for their ideal weight and height. This calorie level allows linear growth but prevents significant weight gain. Calorie restriction may facilitate ketosis and exert an antiseizure effect, independent of ketosis. In children on the KD, blood glucose levels are usually in the lower end of normal, but patients are rarely hypoglycemic. Therefore, a metabolic adaptation occurs in response to the KD to maintain relatively normal glucose levels.

Recent animal studies from the laboratory of Dr. Thomas Seyfried have provided evidence that calorie restriction alone can reduce seizure occurrence. Calorie restriction increases seizure threshold in a model of genetic epilepsy (the EL mouse), which typically develop seizures at several weeks of age. By restricting calories to

85% of normal, seizure onset in EL mice was delayed by several weeks. It was postulated that the KD works by calorie restriction and altered glucose metabolism.

Another group of researchers, led by Drs. Kris Bough and Douglas Eagles, elicited seizures by tail-vein infusion of the convulsant PTZ in rats on various diets. Thresholds to generalized PTZ seizures were elevated by use of a ketogenic calorie-restricted diet to a greater extent than a normal calorie-restricted diet or a KD ad libitum. That is, rats ingesting a normal diet restricted in calories had a higher seizure threshold than did rats on a KD without calorie restriction. Subsequently, investigators showed that even a high carbohydrate calorie-restricted diet (15% normal calories) afforded PTZ seizure protection, similar to that seen in a less severely calorie-restricted KD (90% normal calories). Calorie restriction and the KD were equally effective in suppressing dentate gyrus excitability and limiting the ictal manifestations of maximal dentate activation.

Taken together, these data suggest that calorie restriction is sufficient to influence seizure threshold and may augment the effects of KD treatment. Despite the beneficial effects of calorie restriction, it should be noted that favorable effects of the KD have been achieved in animal models without using calorie restriction. Therefore, the explanation for the seizure protective effect of the KD is not a simple matter of fewer calories.

A remarkable feature of the KD is that ingestion of even a small amount of carbohydrate can rapidly result in loss ketosis and seizure recurrence in patients who were previously well-controlled on the KD. This clinical observation suggests that glycolysis and carbohydrate metabolism might promote seizure susceptibility and that inhibition or reduction of glycolytic metabolism might have anticonvulsant effects. In support of this hypothesis, we have shown (unpublished data) that isomolar substitution of glucose by alternative energy sources – e.g., pyruvate or lactate – in hippocampal slice bathing medium reduced interictal epileptic burst discharges induced by slice exposure to elevated extracellular potassium levels.

Furthermore, administration of 2-deoxy-D-glucose (2DG), which inhibits glycolysis, reduces the progression of kindling evoked by perforant path stimulation in adult rats. 2DG differs from normal glucose only by the removal of an oxygen atom from the hydroxyl group at the 2-position. 2DG is taken up by glucose transporters and is subsequently phosphorylated into 2-deoxy-D-glucose-6-phosphate (2DG-6P), which cannot be converted to fructose-6-phosphate by phosphoglucose isomerase, thereby preventing catabolism through subsequent steps of glycolysis. The trapping of 2DG-6P in cells after uptake through glucose transporters has enabled investigators to use 2DG as a metabolic tracer for glucose utilization, and its adaptation in positron emission tomography (PET) scanning and human clinical imaging.

In our previous study, intraperitoneal administration of 2DG at a dose of 250 mg kg^{-1} in rats impaired the development of kindled seizures in response to stimulation of the perforant path. The antiepileptic effects of 2DG against kindling were associated with reduction of seizure-induced increases in brain-derived neurotrophic factor (BDNF) and its receptor trkB, which are required for kindling progression. Seizure-induced increases in BDNF and trkB were prevented by 2DG through a mechanism of transcriptional repression dependent on neuron restrictive silencing factor (NRSF) and its NADH-sensitive cofactor carboxy-terminal binding protein (CtBP), which maintain a repressive chromatin environment at the BDNF and trkB promoter regions. These observations suggest that glycolytic inhibition through reduction of glycolytic flux, or inhibition by glycolytic inhibitors such as 2DG, might have anticonvulsant and antiepileptic effects. As this mechanism works through alteration of gene expression, the effect is not immediate but likely occurs over hours to days (Figure 1).

Methodology

Our laboratory has been using multiple approaches to establish the effectiveness, safety, and mechanism of 2DG action in inhibiting seizures. As indicated earlier, we have shown that 2DG is an anticonvulsant, and that it down-regulates BDNF and trkB gene expression. To evaluate the effects of 2DG on metabolism, Dr. Avtar Roopra has employed molecular techniques to study NRSF/CtBP metabolic sensing. Another current focus in our laboratory is the study of the acute anticonvulsant and chronic antiepileptic effects of 2DG in rats, in vitro and in vivo. The acute anticonvulsant actions of 2DG are assessed in vitro in rat hippocampal slices exposed to 7.5 mM extracellular potassium, 4-aminopyridine, or

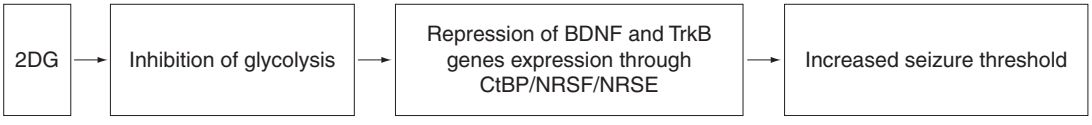


Figure 1 Simplified model for 2DG suppressive effects on kindled seizures. 2DG, 2-deoxy-D-glucose; BDNF, brain-derived neurotrophic factor; TrkB, tyrosine kinase B; CtBP, carboxy-terminal binding protein; NRSF, neuron restrictive silencing factor; NRSE, neuron-restrictive silencing element.

bicuculline. These multiple seizure induction methods are being used to investigate the effects of 2DG on interictal and ictal firing patterns in the hippocampus. In addition, we are studying several in vivo seizure models, including kindling, audiogenic seizures in Fring's mice, 6-Hz stimulation in mice, maximal electroshock seizures in rats, and seizures evoked by subcutaneous PTZ.

Results

In Vitro Anticonvulsant Effects

Removal of glucose and substitution with alternative energy sources such as lactate or pyruvate reduces high potassium-induced interictal burst firing in hippocampal area CA3 by greater than half, an effect that is reversible by restitution of glucose in the bathing solution. Therefore, glucose is necessary for maximal interictal bursting, but lactate and pyruvate are effective substitutes to maintain neural transmission.

In the CA3 hippocampal subfield of rats (postnatal age P28–40), under the conditions of high potassium exposure, addition of 10 mM 2DG reversibly decreases interictal bursts by almost 50%. Ictal bursts in the same region (in younger, P10–13 rats) were reduced markedly by the same concentration of 2DG. These seizure protective effects of 2DG are not limited to the high potassium condition; interictal bursts elicited by 10 mM bicuculline or 50–100 mM 4AP were similarly decreased by 10 mM 2DG, in a reversible fashion. Therefore, in vitro, 2DG exerts acute anticonvulsant effects on both interictal and ictal discharges, induced by a variety of convulsant agents, in the hippocampal CA3 region. It is likely that these rapid effects, occurring over minutes, are mediated by a mechanism different than the chronic effects of 2DG on kindling discussed earlier.

In Vivo Effects of 2DG

We are also investigating the anticonvulsant and antiepileptic effects of 2DG on a variety of in vivo seizure models. Garriga-Canut et al. previously showed that rats treated with 2DG and kindled from the perforant path exhibited unique anticonvulsant effects. Typically (i.e., in saline-treated rats), successive kindling stimulations lead to a gradual decrease in afterdischarge threshold (ADT). However, in 2DG-treated animals, there is a gradual *increase* in ADT as a function of afterdischarge stimulation number. This 2DG effect appears to be at least somewhat specific to the perforant path kindling paradigm, since with kindling from the olfactory bulb, both saline- and 2DG-treated animals exhibit the expected gradual decrease in ADT as a function of after discharge number (unpublished data). Although the effects of 2DG on the ADT differ according to stimulation site, 2DG slowed the rate of progression of kindled seizures evoked from both olfactory bulb and perforant path. That is, rats treated with 2DG required about 50% more afterdischarges (evoked by stimulation of the olfactory bulb) to reach the first class III, IV, or V seizure (compared with saline-treated controls). These data imply that 2DG protects against kindling epileptogenesis.

Studies in several other in vivo seizure models are underway. 2DG has anticonvulsant effects against seizures evoked by 6-Hz stimulation in mice, and against audiogenic stimulation in Fring's mice (unpublished data).

Future Goals

The goal of laboratory investigations into the mechanism of the KD is to improve its effectiveness and develop a formulation that will improve seizure control in children with refractory epilepsy. To this end, a variety of alternative diets have been used clinically, including a modified Atkins diet and a low glycemic index diet. In the Atkins diet, a state of ketosis is induced by providing high fat and little carbohydrate. Because it does not restrict calories, that diet could work by a direct effect of ketosis; the role of calories in the Atkins diet with regard to seizure control has not been elucidated. In the low glycemic index diet, the *quality* of carbohydrate rather than its amount is altered. Early clinical results show that some restriction of carbohydrates may have an anticonvulsant effect. While there are no current animal model studies of the Atkins and low glycemic index diets, such considerations have led to the current research program in which we are attempting to determine whether inhibition of glycolysis can lead to seizure reduction.

Our current experimental results are very promising, showing that inhibition of glycolysis with the glucose analog 2DG has both anticonvulsant and antiepileptic effects in a variety of animal models. A molecular explanation for these results suggests that 2DG potently reduces progression of kindling and blocks seizure-induced increases in the expression of brain-derived neurotrophic factor and its receptor, TrkB. Reduced expression of those genes is mediated by the transcription factor NRSF, which recruits the NADH-binding corepressor CtBP to generate a repressive chromatin environment around the BDNF promoter. We are now further characterizing the behavioral effects of 2DG and determining its safety profile. The cognitive and behavioral consequences of long-term 2DG treatment are being assessed using a variety of cognitive, learning and memory tests. Furthermore, physiological mechanisms by which 2DG acutely alters epileptiform burst discharges (which may be different from its longer term molecular effects on BDNF and TrkB) will be studied using electrophysiological techniques. The variable effects of 2DG on

kindling from two different brain structures raises another intriguing possibility, that metabolic activity in relation to seizure control is site-specific. Finally, alternative inhibitors at different sites within the glycolytic pathway could also be explored as possible antiseizure agents. In summary, 2DG appears to be a safe compound with anticonvulsant and antiepileptic effects, making it an exciting new potential therapy for epilepsy with novel mechanisms of action.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Ketogenic Diet**: Anticonvulsant Mechanisms of a Ketogenic Diet; Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies; Dietary Therapies for Epilepsy; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; **Psychiatric Symptoms**: Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Anticonvulsant Mechanisms of a Ketogenic Diet

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Published by Elsevier Ltd.

Introduction

The ketogenic diet (KD) is a high-fat, adequate-protein, low-carbohydrate diet that has been used as a treatment for epilepsy since 1921. The KD is typically administered in a 4:1 ratio of fats to carbohydrates + proteins, whereby ~90% of calories are derived from fat. Typically, the recommended daily caloric intake is reduced by ~25%. During diet treatment, the liver metabolizes fats into ketone bodies, which in place of glucose then become the primary source of metabolic fuel. Somehow, this switch in metabolism confers an anticonvulsant effect. In general, the number of seizures is reduced by half or more in approximately 50% of those who adhere to the diet. This dietary regimen must be strictly maintained, however, as even small amounts of glucose (or excessive caloric intake) diminish KD efficacy.

We have focused our studies on KD mechanisms for a variety of reasons. First, anticonvulsant drugs do not adequately control seizures for many patients with

kindling from two different brain structures raises another intriguing possibility, that metabolic activity in relation to seizure control is site-specific. Finally, alternative inhibitors at different sites within the glycolytic pathway could also be explored as possible antiseizure agents. In summary, 2DG appears to be a safe compound with anticonvulsant and antiepileptic effects, making it an exciting new potential therapy for epilepsy with novel mechanisms of action.

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Anticonvulsant Mechanisms of a Ketogenic Diet

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Published by Elsevier Ltd.

Introduction

The ketogenic diet (KD) is a high-fat, adequate-protein, low-carbohydrate diet that has been used as a treatment for epilepsy since 1921. The KD is typically administered in a 4:1 ratio of fats to carbohydrates + proteins, whereby ~90% of calories are derived from fat. Typically, the recommended daily caloric intake is reduced by ~25%. During diet treatment, the liver metabolizes fats into ketone bodies, which in place of glucose then become the primary source of metabolic fuel. Somehow, this switch in metabolism confers an anticonvulsant effect. In general, the number of seizures is reduced by half or more in approximately 50% of those who adhere to the diet. This dietary regimen must be strictly maintained, however, as even small amounts of glucose (or excessive caloric intake) diminish KD efficacy.

We have focused our studies on KD mechanisms for a variety of reasons. First, anticonvulsant drugs do not adequately control seizures for many patients with

epilepsy and often cause unwanted side effects. Today the diet is primarily used to treat medically refractory seizures and effectively manages a wide variety of epilepsies. There are few side effects. Perhaps most importantly, however, there are experimental hints that the diet may also limit disease progression and prevent neurodegeneration. Cross comparisons of the KD anticonvulsant profile with the spectrum of activity for other AEDs suggest that the KD acts uniquely to control seizures. Thus, elucidation of KD mechanisms will likely provide unique insights into brain metabolism from which potent new treatment strategies for epilepsy and, perhaps, other neurodegenerative disorders could emerge.

Background

To begin to assess underlying mechanisms of KD, initial experimental studies defined the pharmacodynamic endpoints and when they occur. Behavioral investigations employing a variety of seizure models demonstrated that the KD acts primarily to increase seizure threshold. It thereby decreases seizure frequency but does not prevent seizure spread or attenuate convulsive activity once a seizure begins. In rodents, the KD appears to be most effective in seizures induced by $GABA_A$ antagonists, which has led to speculation that the diet may augment GABAergic inhibition. In rodents, seizure threshold reaches a zenith after 10–14 days on the diet, requiring a similarly prolonged time to return to baseline. This parallels the time course described for KD-fed patients. Although improved seizure control can be noted in patients within a day of diet onset, optimal seizure control is typically not reached until after approximately 2 weeks of sustained diet treatment.

Other studies have provided insights into key variables related to diet treatment. Collectively, both clinical and experimental work indicates that factors such as seizure type, seizure duration, diet composition (e.g., type of fats), and gender do not significantly affect treatment outcome. Although there appears to be no effect of age (at diet onset) on treatment outcome, this variable remains controversial (perhaps due to issues of compliance more than physiology). By comparison, variables such as diet ratio and the degree of caloric restriction (CR) appreciably influence the degree of seizure protection. Reducing caloric intake is the most common factor used to enhance KD efficacy. The importance of caloric intake is perhaps not surprising given that the diet originated from insights into the physiology of fasting. CR treatment itself has been shown to mitigate hyperexcitability in animal studies. In addition, seizure control is generally improved in a dose-dependent manner if the KD ratio is increased above 3:1 (to a maximal of 5:1).

Although there is no ideal model that mirrors all facets of the clinical condition, few studies have systematically explored KD. Because most investigations administered sundry high-fat ketogenic diets for different durations, induced seizures by dissimilar means, and examined animal cohorts of various ages, genera, and gender, meaningful cross comparisons have proven challenging. The vast majority of data are taken from studies employing acute seizure models. More relevant information will likely arise from studies of cellular/network hyperexcitability with concomitant alteration of spontaneous recurring seizures in multiple animal models of epilepsy.

Methods

A standard battery of behavioral tests has been implemented to characterize fully the anticonvulsant and cognitive impairments of KD treatment. Chemoconvulsant drugs have been injected into rats, mice, and rabbits to initiate seizure-like activities and establish different facets of the KD's antiepileptic efficacy. Additional behavioral tests such as Rotarod, water maze, and open field tests have been completed in both mice and rats to evaluate potential behavioral deficits in motor function and/or learning and memory following treatment with a KD.

Limited electrophysiological field recordings have been completed to assess KD-induced effects on network excitability. Field recordings have been completed both in vivo and in hippocampal tissue slices in vitro. Microarray studies have been used to assess KD-induced changes in patterns of gene expression in the hippocampus. Biochemical, histological, immunoblotting, and electrophysiological techniques were completed in parallel in an attempt to correlate transcriptional alterations with translational changes and their potential impact on synaptic function. Additional biochemical techniques (e.g., nuclear magnetic resonance imaging, gas chromatography-mass spectroscopy) have been applied to gain specific insight into the metabolic impact of ketones on neuronal and glial metabolism measured in synaptosomes, cultured astrocytes, and tissue from KD-fed or ketone-injected animals.

A putative antiepileptogenic role for the KD has also been explored. Toward this goal, maximal dentate activation (a 1-day electrical kindling protocol) was employed (while recording in vivo) to determine whether KD treatment limited the prolongation of electrical seizure duration. The antiepileptogenic actions have also been assessed following kainate-induced status epilepticus and in genetically seizure-prone EL mice. Behavioral, electrophysiological, immunohistological/histological, and molecular studies have been completed in parallel to determine the degree of KD-induced effects on traditional markers of epileptogenesis (e.g., mossy fiber sprouting), neuronal cell death, and network excitability, as well as its relation to the number of spontaneous recurring seizures.

Recent Results

Early studies of KD focused on establishing efficacy. Only following a resurgence of interest within the last decade or so have studies begun to focus on how the diet works. Several mechanistic theories have been proposed. Those that have received at least some support include (1) enhanced GABAergic inhibition, (2) modified amino acid metabolism, (3) enhanced energy metabolism, (4) diminished glycolysis, and (5) augmented noradrenergic tone. Because various low-carbohydrate KD protocols all produce some level of seizure control, it has become increasingly clear that anticonvulsant actions of the KD stem from diminished glucose and/or ketonemia. But how might this metabolic switch lead to improved seizure control?

Direct Action of Ketones

Given that ketosis is widely considered the clinical hallmark for KD success, the majority of mechanistic studies have focused on establishing a causative link between ketone bodies and seizure protection. Ketones may act directly to limit seizures. Although no acute effect of ketone bodies on neuronal $GABA_A$, AMPA, and NMDA receptors has been observed, application of either β-hydroxybutyrate or acetoacetate to rat or mouse hippocampal slices activated K_{ATP} channels and reduced the spontaneous firing rate of hippocampal neurons in an activity-dependent manner. In vivo, acute injection of acetone, acetoacetate, or ethyl-acetoacetate diminishes seizure activity in a variety of acute and chronic models of epilepsy; interestingly, however, β-hydroxybutyrate does not.

Indirect Actions of Ketones

Ketones may also act indirectly to limit hyperexcitability. This effect could occur in three main ways. First, ketones appear to improve seizure control via a modification in amino acid metabolism. Numerous studies indicate that ketones and KD treatment increase cerebral concentrations of glutamate and GABA, while decreasing aspartate levels. Because ketone body metabolism is thought to diminish the transamination of glutamate to aspartate, these data support the notion that increased glutamate concentrations would be available to enhance GABA levels and increase synaptic inhibition (**Figure 1**).

In synaptosomes, the addition of either β-hydroxybutyrate or acetoacetate increases levels of glutamate and GABA, and decreases levels of aspartate. Although elevated levels of GABA have not been observed throughout the brain, clinical studies are generally consistent with experimental findings; that is, GABA levels are elevated in patients maintained on a KD and there is a positive correlation between GABA levels and improved seizure control. Ketones and/or KD treatment also increase brain glutamine levels. It is thought that elevated astrocytic glutamine is then transported from the brain into the circulation, diminishing overall brain glutamate levels. In view of many studies indicating elevated glutamate during KD, however, this possibility seems somewhat at odds with the data.

Second, ketone bodies and KD enhance energy metabolism. Microarray studies indicate a coordinate upregulation of genes related to ATP production after KD treatment. Notably, KD also induces considerable mitochondrial biogenesis (**Figure 2**), with a parallel increase in energy reserves. For example, KD has been shown to

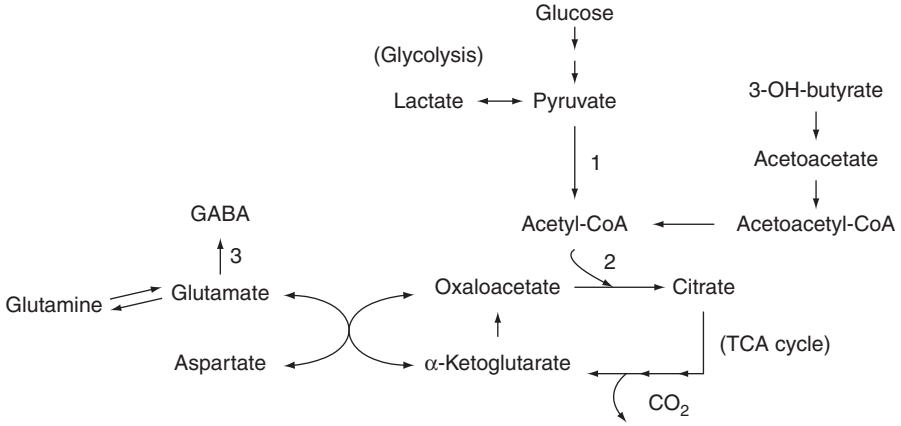


Figure 1 *Modifications in amino acid metabolism after treatment with the ketogenic diet (KD) may favor the production of GABA.*<? tfqs- As a result of a switch from D-glucose to ketone bodies (e.g., β-hydroxybutyrate and acetoacetate) as a primary energy source, a fraction of pyruvate (1) is no longer converted to acetyl-CoA. In contrast, all ketone bodies are converted to acetyl-CoA and thus enter the tricarboxylic acid (TCA) cycle (2) via citrate synthase. In the process, oxaloacetate is used preferentially within the TCA cycle to generate energy, and becomes less available for the transamination reaction of glutamate to aspartate. (3) More glutamate then becomes available for GABA synthesis via glutamic acid decarboxylase (GAD), which increases brain GABA concentrations (Adapted from Yudkoff *et al.*, 2004).

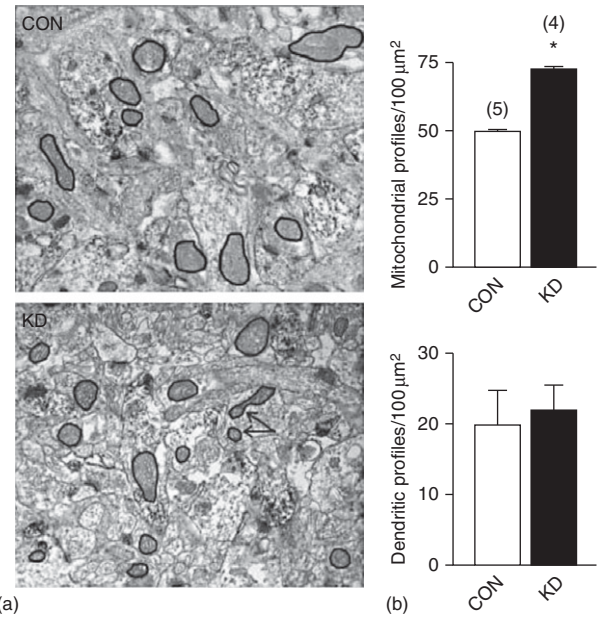


Figure 2 *Mitochondrial biogenesis in the hippocampus of rats maintained on a ketogenic diet (KD).* (a) Representative electron micrographs taken from control-(CON) or KD-fed animals (magnification 20 000; calibration bar 0.5 μm). The number of mitochondrial profiles in each micrograph was counted. An arrow indicates a single mitochondrial profile. (b) There were significantly more mitochondrial profiles in the dentate gyrus of KD-fed animals than in controls. Mitochondrial counts were taken from four KD-fed animals representing a total of 2680 μm^2 and five control rats representing 3080 μm^2. Data are presented as mean of averaged counts ± SEM per 100 μm^2 ($n = 4$ or 5). *$p < 0.001$ by *t*-test. By comparison there was no difference in the density of dendritic profiles in the same micrographs, which guards against the potential differential shrinkage of tissue in KD versus control brains (Adapted with permission from Bough *et al*., 2006).

elevate brain glycogen, boost brain ATP concentrations, and increase the PCr/Cr ratio. In accordance with this finding, tissue from KD-fed animals was more resistant to metabolic challenge with low glucose. KD-fed patients exhibited similar elevations in the PCr/Cr ratio, suggesting that similar enhancements in energy metabolism also occur clinically.

Third, ketone bodies and KD may limit neuronal dysfunction by reducing the generation of excitotoxic mitochondrial reactive oxygen species (ROS). KD increases the expression and activity of mitochondrial uncoupling proteins. Similarly, ketones diminish ROS production and increase the mitochondrial respiratory rate. In this way, the ketones produced during the KD may prevent neuronal dysfunction and help alleviate oxidative stress that could contribute to seizure onset.

Numerous studies now indicate that ketones potently diminish ROS generation and prevent neuronal cell death in a variety of in vitro models of disease. There is some evidence that KD also prevents neuronal cell loss after kainate-induced status epilepticus. Thus, ketones produced during KD may help to stabilize neuronal and/or glial metabolism to reduce hyperexcitability and limit neurodegeneration.

Of related interest is the role for norepinephrine (NE) in KD. Several lines of evidence indicate that greater noradrenergic tone increases seizure resistance. When NE-deficient (Dbh−/−) mice were treated with a KD, they failed to exhibit an anticonvulsant response, suggesting that NE was required for the anticonvulsant effects of the diet. KD may increase NE levels in hippocampus. Moreover, elevation of synaptic levels of NE (via a genetic knockout of the NE transporter) in KD-fed mice led to enhanced seizure resistance (compared to mice fed with KD alone). Together, these findings suggest that NE is a seminal component of the anticonvulsant underpinnings of the KD.

Diminished Glycolysis

The antiepileptic actions of the KD may not solely arise from ketones, however. KD treatment also diminishes significantly blood concentrations of glucose. Findings from several studies point toward a KD-induced inhibition of glycolysis. One report has recently suggested that 2-deoxygluose (2DG), an inhibitor of glycolysis, could prevent both seizures (anticonvulsant) and disease progression (antiepileptic). Injection of 2DG elevated afterdischarge threshold in the olfactory bulb of perforant path of kindled rats; it markedly reduced the progression of kindling, and it prevented seizure-induced increases in the expression of BDNF and TrkB, its cognate receptor. In vitro, epileptiform burst frequency was reduced by 20–75% in rat hippocampal slices exposed to elevated extracellular potassium. Because the activation of TrkB receptors has been integrally linked to processes of epileptogenesis, diminished glucose-induced modifications in gene expression and related modifications in hyperexcitability seem to indicate that the low-glucose KD may not only limit seizures but also prevent disease progression.

A summary of potential mechanistic pathways is depicted in **Figure 3**.

Future Goals

We have begun to define key features of efficacy, but the mechanisms of the KD remain largely unexplored. One of the foremost questions that remain to be determined is as follows: Which aspects of the KD – ketonemia or diminished glucose – are responsible for its effects? Because these two metabolic states are so tightly intertwined, it is difficult to ascertain which feature (if not both) is most critical to diet efficacy. Identification of those factor(s) of diet treatment that most significantly impact(s) its

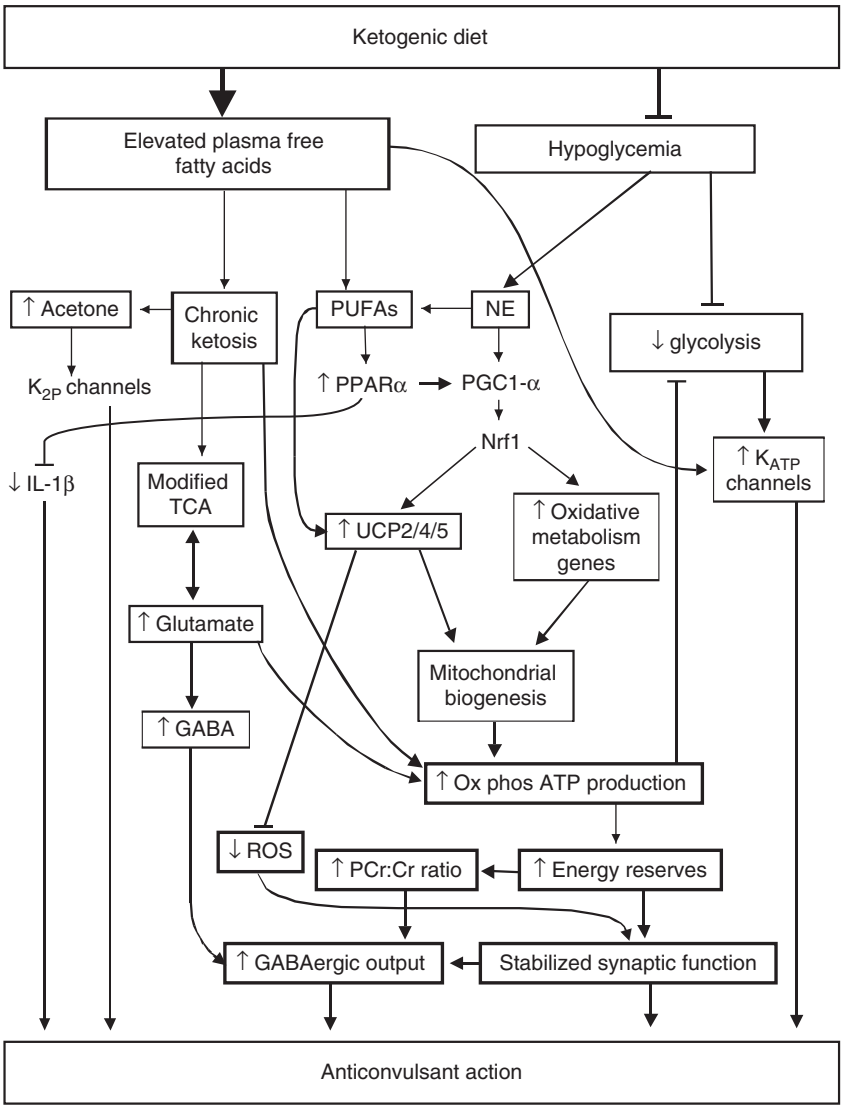


Figure 3 *Hypothetical pathways leading to the anticonvulsant effects of the ketogenic diet (KD).* Boxed variables depict findings taken from KD studies. Up (↑) or down (↓) arrows indicate the direction of the relationship between variables as a result of KD treatment. Elevated plasma free fatty acids result in chronic ketosis as fatty acids are metabolized into ketone bodies and increased concentrations of polyunsaturated fatty acids (PUFAs) in the brain. Chronic ketosis is predicted to lead to elevated levels of acetone; although not discussed in detail, acetone may activate K_{2P} channels to hyperpolarize neurons and limit neuronal excitability. Chronic ketosis is also anticipated to modify the tricarboxylic acid (TCA) cycle. This metabolically favors the production of glutamate and, subsequently, GABA synthesis in brain. PUFAs are expected to boost the activity of brain-specific uncoupling proteins (UCPs); this is expected to limit reactive oxygen species (ROS) generation to diminish neuronal dysfunction, stabilize neuronal functioning, and increase the resistance to hyperexcitability. Acting via the nuclear transcription factor peroxisome proliferator-activated receptor-α (PPARα), PUFAs would be expected to induce the expression of UCPs and coordinately upregulate several dozen genes related to oxidative energy metabolism. PPARα expression is inversely correlated with interleukin-1β (IL-1β) cytokine expression; in view of the role of IL-1β in hyperexcitability and seizure generation, diminished expression of IL-β cytokines during KD treatment could also contribute to improved seizure control. Mitochondrial biogenesis is predicted to increase ATP production capacity and enhance energy reserves, leading to stabilized synaptic function and improved seizure control. In particular, an elevated phosphocreatine/creatine (PCr/Cr) energy-reserve ratio is predicted to enhance GABAergic output, perhaps in conjunction with the ketosis-induced elevated GABA production, leading to diminished hyperexcitability. Reduced glucose coupled with elevated free fatty acids is proposed to reduce glycolytic flux during KD, which would further be feedback inhibited by high concentrations of citrate and ATP produced during KD treatment. This would activate metabolic K_{ATP} channels. Opening of K_{ATP} channels would hyperpolarize neurons and diminish neuronal excitability to contribute to the anticonvulsant nature of the KD. Reduced glucose is also expected to downregulate brain-derived neurotrophic factor (BDNF) and TrkB signaling in brain. As activation of TrkB pathways by BDNF have been shown to promote hyperexcitability and kindling, it has been suggested that these potential KD-induced effects may not only limit seizures, but also the progression of epilepsy (Adapted from Bough and Rho, 2007).

anticonvulsant action will lead to the optimization of KD treatment and more focused study of underlying mechanisms.

The 2DG model presents us with an opportunity to examine the role of low glucose in the mechanism of the KD. For example, if low glucose is a critical feature of KD treatment, then it would be of interest to see whether 2DG glycolytic inhibition recapitulates all the effects observed for the KD – i.e., a similar anticonvulsant profile, diminished hippocampal network excitability, modifications in amino acid metabolism, and enhancements in aerobic respiration (e.g., mitochondrial biogenesis).

In teasing apart a mechanistic function for ketones, we need to determine the levels of acetone, acetoacetate, and β-hydroxybutyrate in human brain during KD treatment. Despite results from many in vitro studies suggesting that ketone bodies limit (directly and/or indirectly) hyperexcitability, we do not know if the concentrations applied in many of those studies are physiologically relevant. Not only would it be of value to correlate measures of ketone levels with improved seizure control clinically, but it would help to better define the cerebrospinal fluid on which future in vitro studies could be based.

Something that was not described in great detail in the earlier discussion, but also needs to be addressed, is a putative role for hyperlipidemia during KD. Polyunsaturated fatty acids (PUFAs) such as eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) modulate voltage-gated sodium channels and L-type calcium channels in hippocampal tissue. Although these PUFAs do not cross the BBB freely, plasma levels of PUFAs are increased in KD-fed children and correlate positively with improved seizure control. Thus, KD-induced hyperlipidemia may act directly to increase seizure resistance and should be explored further.

Several biochemical and molecular studies indicate that regional differences in KD effect throughout brain. Many studies have focused on hippocampus initially because of its well-defined role in epilepsy models. However, restriction of experimental studies to this one structure is clearly insufficient. Additional studies will be required to ascertain the global nature of KD effects, which in turn will help to focus future investigations.

Evidence is beginning to support several possible mechanisms consistent with the anticonvulsant action of the KD; however, all the potential mechanisms remain incompletely defined. Whereas numerous genetic, molecular, and biochemical studies indicate that KD diminishes glycolysis and enhances aerobic respiration, it remains to be determined how these metabolic alterations lead to improved seizure control during diet treatment. Given that metabolism is so closely linked to neuronal activation, a substantial area of need is the characterization of synaptic function in tissue from KD-fed animals. In view of the unique metabolic relationship between neurons and glia, these studies would help overcome a major issue in sorting out how much of a role glia may play in influencing neuronal excitability during KD. Obtaining appropriate electrophysiological recordings in vitro, however, is not without problems. As indicated earlier, glucose is thought to rapidly diminish some of the KD-induced clinical effects – but most in vitro brain slice studies use artificial cerebrospinal fluid that contains high concentrations of glucose. Thus, KD-induced changes on network and/or synaptic hyperexcitability remain largely unexplored. Whereas underlying transcriptional and translational changes should also be linked with electrophysiological modifications, of course, these changes will ultimately need to be associated with improved seizure outcome and tied together with clinical experience.

An issue of major importance is the putative antiepileptogenic role induced by KD. It is obvious that this claim will need to be corroborated in multiple models of epileptogenesis. Further characterization of disease-limiting effects and identification of KD-induced modifications of known antiepileptogenic mechanisms are needed. For example, identification of a similar diminution of BDNF and TrkB receptors, along with a concomitant decrease in spontaneous recurring seizures (as was demonstrated in the 2DG studies), would represent an important first step in confirming a putative antiepileptic role for the KD. If true, support for this effect in further studies could lead to a paradigm shift in the way we approach the treatment of epilepsy, enabling physicians to help prevent the disease, and not just treat the symptom.

Observations that the KD can have such a profound influence on so many diverse intractable epilepsies, and few side effects, underscore the importance of discovering how the diet works. The elucidation of KD mechanisms will likely provide the foundation from which we can gain an improved understanding of basic brain metabolism, its relationship to both normal and abnormal activity (e.g., seizure initiation), and susceptibility to multiple epilepsies. These insights, in turn, will likely lead to profound new treatment strategies for epilepsy and, perhaps, other neurological diseases.

See also: **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies; Dietary Therapies for Epilepsy; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation; **Psychiatric Symptoms**: Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies

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Introduction

Our research focuses on the use of diet as a therapy for neurological and neurodegenerative diseases. Although epilepsy is among the oldest known neurological disorders, seizures continue to be unmanageable in many patients with epilepsy despite intensive research into antiepileptic drug (AED) development. In addition, although many available AEDs provide seizure control, their use is often associated with unanticipated and adverse side effects that diminish quality of life, particularly when used in combination as polytherapy. Regardless of whether seizures have a symptomatic or idiopathic etiology, it is our contention that epilepsy, as a neurological disorder involving dysregulated brain energy metabolism, is potentially manageable through principles of metabolic control theory (analysis). Lennox also considered disrupted energy metabolism as a focal point of epilepsy. Metabolic control theory applies principles of bioenergetics and genome flexibility for the control or management of complex phenotypes such as epilepsy. Since energy metabolism is a fundamental process that underlies all phenotypes, modification of metabolism can lead to the modification of the phenotype. Metabolic control analysis is based on the premise that compensatory genetic and biochemical networks, operating through flexible biological systems, are capable of modulating energy metabolism. Moreover, this dynamic system can potentially produce a normal phenotype through altered energy metabolism, despite the continued presence of the genetic or environmental factors that produce the epileptic phenotype. Our goal is to test this hypothesis in epileptic mice and to eventually identify underlying mechanisms.

Background

Diet Therapies for Epilepsy

Diet therapies for the control of epilepsy are as old as the disease itself, with the type or composition of the diet being adapted over time to reflect new perspectives on the etiology of epilepsy. Antiepileptic diets were used during the time of the ancient Greeks and Romans as well as during the Renaissance. These diet therapies were designed to rid the brain of toxic agents that were believed to underlie the development of epileptic seizures. It was thought that a strict diet regimen was essential for managing or curing the disease. These early antiepileptic diets, however, often consisted of vile concoctions of raw animal organs and extracts that induced nausea or vomiting, and were frequently administered with purgatives and enemas. Restricted food intake or reduced uptake of

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nutrients over days or weeks would often be an unintended consequence of such dietary therapies. This restriction would produce the physiological conditions of fasting, during which circulating blood glucose levels are reduced and ketone levels are elevated. Hence, altered brain energy homeostasis would be expected following antiepileptic dietary therapies that lead to a change in circulating levels of glucose and ketone bodies.

Fasting and the Ketogenic Diet

Fasting has long been recognized as an effective antiepileptic therapy for a broad range of seizure disorders. Reference is often made to Biblical accounts of fasting as a cure for epileptic seizures. Biblical scholars interpret these passages to mean that the afflicted individuals could not be cured unless the *healer* engaged in prayer and fasting. Modern clinical findings indicate that fasting can significantly reduce seizure susceptibility in the majority of patients with epilepsy. Using Conklin's water-only diet, Lennox and coworkers demonstrated an impressive management of epilepsy in patients who were having as many as five seizures per day. Although seizure incidence actually increased over the first couple of days of fasting, seizure incidence decreased markedly after three days in most patients. This finding is interesting because it generally requires about three days for cerebral energy metabolism to gradually transition from glucose to ketone utilization during fasting. Seizure control through fasting was also associated with reduced blood glucose and increased blood ketone levels. When the fast was broken through food intake, seizure protection was lost in most patients. Some patients, however, remained seizure free for extended periods after fasting, indicating individual variability for the antiepileptic effects of fasting. The return of seizures was often associated with rising blood glucose levels and falling blood ketone levels.

Although effective in managing seizure disorders, periodic fasting is impractical for the long-term seizure management due largely to issues of compliance. Consequently, the high fat low carbohydrate ketogenic diet (KD) was developed to mimic the physiological effects of fasting (ketosis) without severe food restriction or starvation. The KD is effective in managing intractable seizures in children and can also be effective in managing seizure disorders in adults. In addition, the KD is effective in reducing epileptogenesis and seizures in animal models of epilepsy. Although the mechanisms by which the KD inhibits seizure susceptibility remain unresolved, alterations in brain energy metabolism are likely involved.

The role of ketone bodies (β-hydroxybutyrate and acetoacetate) and glucose in the seizure protective effects of fasting or the KD is under active investigation. Elevated plasma ketone levels have been associated with seizure protection in some studies, but not in other studies. Since ketone utilization in brain can depend on the plasma levels of ketones, glucose, and other metabolites, associations between seizure protection and plasma ketone levels may be obscured. Likewise, blood glucose levels remain constant during KD treatment in some studies, but are reduced during the treatment in other studies. Few studies have investigated the relationship between ketones, glucose, and seizure susceptibility under long-term antiepileptic diet therapies.

Caloric Restriction

Caloric restriction (CR) is a natural dietary therapy that improves health, extends longevity, and reduces the effects of neuroinflammatory diseases in humans and rodents. CR involves a reduction of total dietary energy intake while maintaining adequate levels of vitamins and minerals. In contrast to starvation, which is a pathological state, CR lowers glucose levels and raises ketone body levels within normal physiological ranges. The gradual transition in energy production from glucose to ketone bodies enhances vitality and metabolic homeostasis. We recently showed that in addition to the KD, CR alone could reduce seizure susceptibility in epileptic mice. It is important to mention that CR in the mouse is not the same physiologically as CR in humans since basal metabolic rate is about seven times greater in mice than in man. CR in mice is similar to therapeutic fasting in humans since a 40% CR in inbred C57BL/6J mice produces changes in serum lipids similar to those seen in humans following therapeutic fasting or very low calorie dieting (below 500 kcal day^{-1}). Hence, CR in mice can be an effective model system for investigating the anticonvulsant and antiepileptogenic mechanisms of therapeutic fasting in humans.

The Epileptic EL Mouse: A Natural Model of Human Idiopathic Generalized Epilepsy

Although many animal models of epilepsy can provide insight on anticonvulsant therapies, only those models that exhibit naturally recurring seizures are valuable. The epileptic EL mouse has long been recognized as a natural model of human epilepsy. Seizures in EL mice originate in or near the parietal lobe, quickly spread to the hippocampus and to other brain regions, and commence with the onset of sexual maturity (50–60 days of age). The seizures are accompanied by EEG abnormalities, vocalization, incontinence, loss of postural equilibrium, excessive salivation, and head, limb, and chewing automatisms (**Figure 1**). The seizure disorder in EL mice also models Gowers' dictum where each seizure increases the likelihood of recurrent seizures. Indeed, the EL mouse is the only known mouse model of this hallmark of progressive epilepsy. Adult EL male mice also experience a sexual dysfunction similar to that described in men with



Figure 1 Epileptic EL mouse. The EL mouse models multifactorial idiopathic epilepsy. The mouse expresses excessive salivation, and head, limb, swallowing, and chewing automatisms. The arching Straub tail is indicative of seizure spread to spinal cord. Reproduced from Seyfried TN *et al.* (2004), with permission.

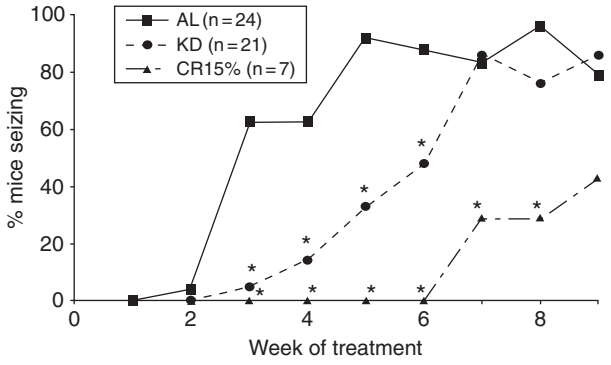


Figure 2 Influence of CR and the KD on seizure susceptibility in juvenile EL mice. Asterisks indicate that the number of mice seizing was significantly lower in the CR and KD groups than in the AL group at $^*p < 0.01$. Reprinted from Greene *et al*. (2001) Caloric restriction inhibits seizure susceptibility in epileptic EL mice by reducing blood glucose. *Epilepsia* 42(11): 1372–1378, with permission.

temporal lobe epilepsy. EL mice express abnormalities of excitatory and inhibitory neurotransmission and develop a hippocampal gliosis with seizure progression. The gliosis in EL mice, however, is not associated with obvious hippocampal neuronal loss or synaptic rearrangements (e.g., mossy fiber sprouting). Gene–environmental interactions play a significant role in the determination of seizure frequency and onset in EL mice, as is the case with multifactorial human idiopathic epilepsies. Hence, the EL mouse is a good model for evaluating the effects of dietary therapies for generalized idiopathic epilepsies.

Methods and Recent Results

Influence of the KD and CR on Seizure Susceptibility in Juvenile EL Mice

The implementation of diet therapies in mice, although simple in principle, can be difficult in practice. Prior to the initiation of diet therapy, it is essential that all mice be matched for age, weight, and sex. All mice should be individually housed and body weight along with food intake recorded 3–4 times prior to any dietary modification. Baseline blood glucose and ketone body levels should also be obtained at least once prior to diet initiation. These practices are important since any dietary manipulation can cause some mice to lose or gain weight relative to the weight they had before diet implementation, and these changes can influence data interpretation. We also found that it is better to use body weight, rather than food intake, as the independent variable when instituting CR or dietary restriction experiments. This is because physiological differences exist among individual mice for food intake even when they are matched for age, sex, and genetic background. Body weight is therefore the preferred independent variable for evaluating the physiological effects of dietary manipulations.

Our initial studies evaluated the influence of the KD on seizure onset in juvenile (about 30 days old) EL mice (Figure 2). The fat in this diet was derived from lard and the diet had a ketogenic ratio (fats: proteins + carbohydrates) of 4.75:1. The KD contained a balance of mouse nutritional ingredients and according to the manufacturer's specification (Purina or Zeigler Bros., Inc. Gardners, PA, USA), the diet delivered 6.85 kcal g^{-1} gross energy, where fat, carbohydrate, protein, and fiber comprised 90%, 0%, 10%, and 3% of the diet, respectively. The mice were deprived of food and water for 14 h prior to initiating the KD therapy. The mice in both the treatment (KD) and control (AL) arms were fed either the high fat diet or the high carbohydrate diet ad libitum (unrestricted), respectively. Mice in the AL arm received the PROLAB RMH3000 chow diet (LabDiet, Richmond, IN). This standard rodent food pellet diet (SD) contained a balance of mouse nutritional ingredients. According to the manufacturer's specification, this diet delivered 4.4 kcal g^{-1} gross energy, where fat, carbohydrate, protein, and fiber comprised 6%, 62%, 27%, and 5% of the diet, respectively. Mice in the KD treatment arm were matched for body weight throughout the study. The KD temporally delayed the onset of seizures relative to the AL group, suggesting an effect on epileptogenesis. Although the KD-fed mice had elevated blood ketone levels, glucose levels were similar in AL and KD groups. We also compared the effects of the KD with that of a calorie restricted SD arm (CR) on seizure susceptibility in the juvenile EL mice. We found that a mild 15% CR diet was more effective than the AL-fed KD in delaying seizure onset and in reducing seizure susceptibility

(Figure 2). Although body weight was about 8% lower in CR mice than in AL-SD mice, the rate of body weight gain was similar in AL-SD and CR groups. This observation is extremely important, since delay or stunting of growth is an adverse effect that can confound data interpretation. In contrast to the mice fed with KD ad libitum, which elevated plasma ketone bodies without lowering glucose levels, the CR fed mice showed elevated ketone levels with significantly lower glucose levels. Mild CR was more effective in preventing seizures than was the KD fed ad libitum. The KD may inhibit epileptogenesis in the juvenile EL mice by prolonging the preweaning period, thereby causing the brain to metabolize ketones for energy despite glucose availability. We suggest that CR enhances this antiepileptogenic effect by lowering blood glucose levels in addition to elevating blood ketone levels.

The Relationship of the KD and CR in Seizure Management in Children

The KD is most effective in reducing seizure susceptibility in children when administered with fasting or under restricted caloric intake. Clinical studies have also shown that the anticonvulsant efficacy of the KD is associated with body weight and blood glucose reductions of about 10%. Our findings in EL mice on the CR diet are consistent with these findings in humans. It is interesting that the anticonvulsant effects of the KD can be lost in patients who experience a rise in blood glucose levels, as in the case of those who gain weight on the diet or who consume carbohydrates. On the basis of our studies with EL mice, we suggest that the seizure protective effects of the KD would be best when administered in reduced amounts, so that blood glucose levels and body weight are also reduced. It is also important to recognize that consumption of the KD in unrestricted amounts is unhealthy because of the high fat content of the diet. Consumption in restricted amounts provides maximum therapeutic benefit against epilepsy while reducing the adverse effects of the high fat content of the diet.

Influence of the KD and CR on Seizure Susceptibility and Plasma Energy Metabolites in Adult EL Mice

Although the KD is used largely for the management of refractory epilepsy in children, the KD can also be effective in managing seizures in adults. In addition to evaluating the effects of the KD and CR on seizure susceptibility in juvenile EL mice, we evaluated the effects of these diet manipulations on seizure susceptibility in adult EL mice (about 210 days of age) that had already developed multiple recurrent seizures. For these studies, we reduced body weight to a similar degree (about 22%) in both the mice fed a high carbohydrate standard diet (SD) and the KD, and measured seizure susceptibility once/week over a 9-week period (Figure 3(a)). Seizure susceptibility remained high in the adult EL mice fed either the KD or SD under unrestricted feeding (UR groups) throughout the study. The KD, when administered in unrestricted amounts, was unable to significantly lower seizure susceptibility in the EL mice. In marked contrast, seizure susceptibility was significantly reduced to a similar degree in both mouse groups fed the restricted SD or the KD diets (Figure 3(b)).

Our findings clearly showed that seizure management in EL mice associated more with caloric intake than with the origin of dietary calories. We also found that seizure susceptibility was strongly correlated with blood glucose levels (Figure 3(c)). Plasma glucose levels were similarly high in both UR groups (mice exhibited high seizure susceptibility), but were similarly low in both restricted calorie groups (mice exhibited low seizure susceptibility). A less strong correlation was found, however, between seizure susceptibility and plasma ketone bodies (β-hydroxybutyrate) (Figure 3(d)). Although circulating ketone bodies were significantly elevated in the KD-UR group, the mice in this group were highly seizure susceptible. The highest plasma ketone levels were observed in the restricted KD mice, but this elevation was not associated with better seizure management relative to that found in the restricted SD mice. Finally, we used binary logistic regression analysis to determine the relationship between seizure susceptibility, plasma glucose, and plasma ketone levels in mice fed the SD or the KD. We found that regardless of diet composition, glucose could predict seizure susceptibility with a 75% degree of accuracy. As blood glucose levels dictate blood ketone levels under normal physiological conditions, glucose is the key energy metabolite for predicting seizure susceptibility in the epileptic EL mice.

Evidence that the KD Suppresses Seizure Susceptibility through CR

In contrast to the KD, CR elevates blood ketone levels naturally through a gradual reduction in blood glucose levels. Although both the KD and CR elevate blood ketone levels, circulating ketone levels alone do not explain the anticonvulsant effects of these diets in either humans or animal models. Indeed, the brain does not generally metabolize ketones for energy unless blood glucose levels are reduced. We suggest that it is the reduction of blood glucose levels together with an elevation of blood ketone levels that predicts the anticonvulsant efficacy of both the KD and CR. Blood glucose also

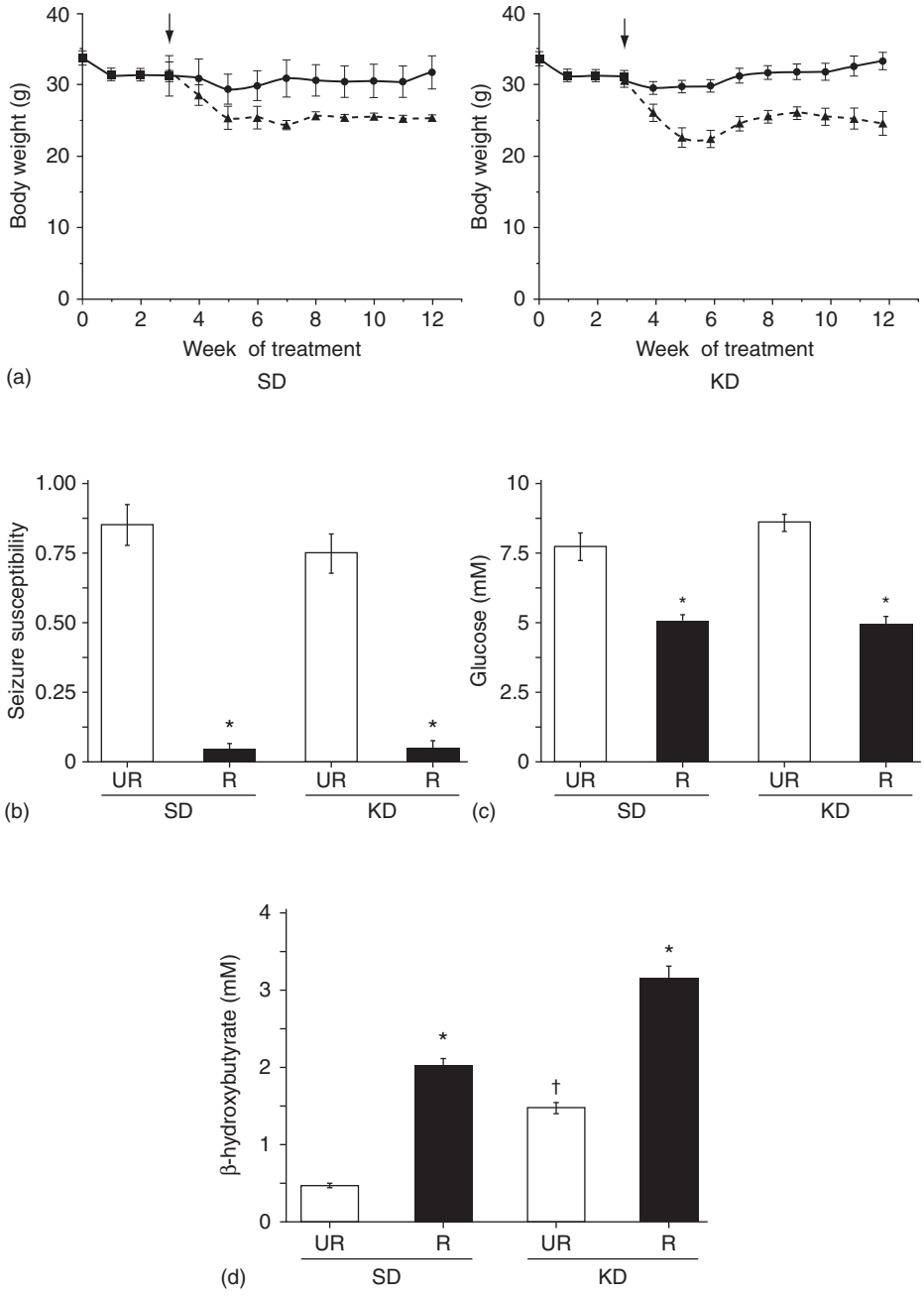


Figure 3 Influence of CR and KD on body weights (a), seizure susceptibility (b), plasma glucose levels (c), and plasma β-hydroxybutyrate levels (d) in adult EL mice. In (a), squares represent the pretrial period when all mice were fed the SD-UR. Circles and triangles represent the UR-fed and R-fed groups, respectively. The arrow indicates initiation of CR. In (b-d), the asterisks indicate that the values were significantly lower ($p < 0.001$) in the R-fed groups than in the respective UR-fed groups. The cross in (d) indicates that the plasma β-hydroxybutyrate levels were significantly higher ($p < 0.001$) in the KD-UR group than in the SD-UR group. Values were pooled from treatment weeks 5–12 (see (a)) and are expressed as the mean ± SEM ($n = 6$ mice per group). Reprinted from Mantis *et al*. (2004) Management of multifactorial idiopathic epilepsy in EL mice with caloric restriction and the ketogenic diet: Role of glucose and ketone bodies. *Nutrition and Metabolism* 1: 11, with permission.

positively correlates with susceptibility to clonic and tonic-clonic flurothyl-induced seizure in rats. High glucose may also exacerbate human seizure disorders. These observations suggest that the KD may be most effective in reducing seizure susceptibility when blood glucose levels or total calories are also restricted. The relationship of blood glucose and ketone body levels to seizure management in EL mice is illustrated in **Figure 4**.

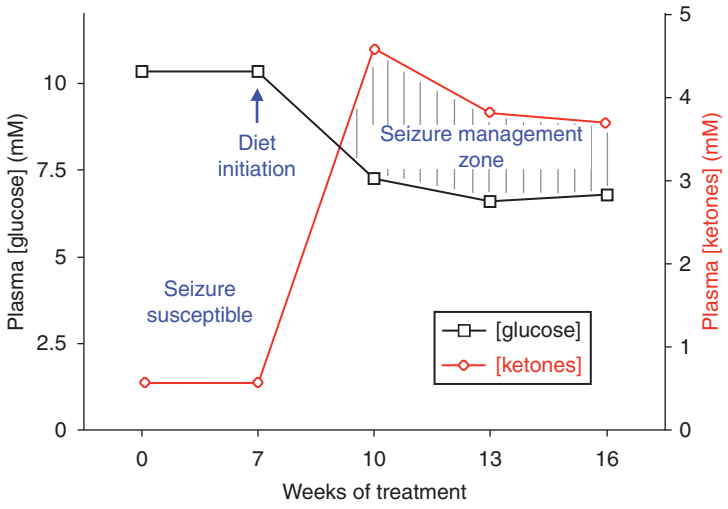


Figure 4 Relationship of circulating glucose and ketone levels to seizure management in epileptic EL mice.

The association between blood glucose levels and seizure susceptibility is less clear in humans with epilepsy than in mice with epilepsy. The reduction of body weight and restriction of calories in children on the KD implies reduced glucose levels relative to the levels prior to the initiation of the diet. In order to determine the relationship between glucose and seizure protection under the KD, it would be necessary to monitor glucose levels in children prior to the initiation of the KD. In other words, glucose levels should be monitored both before and during the KD, with each child serving as his/her own control. Body weight or weight gain measurements, however, might be more accurate predictors of diet efficacy than blood glucose levels in children. Hence, further studies with epilepsy animal models are needed to assess the relationship among blood glucose levels, body weight changes, and seizure protection with antiepileptic diets.

Perspectives on the Antiepileptic Mechanism of CR

Glucose uptake and metabolism increase more during an epileptic seizure than during any other brain activity. This increase appears to be independent of seizure etiology and occurs with either generalized or focal seizures, in either idiopathic or symptomatic epilepsies. Glycolytic energy is crucial for maintaining synaptic activity and enhanced glycolysis is associated with seizure activity. Brain lactate levels also increase significantly during seizures. The seizure-associated lactate increase reflects the rapid increase in glycolytic rate over the cerebral metabolic rate of oxygen. Pyruvate dehydrogenase fails to metabolize pyruvate to acetyl-CoA at the same rate that pyruvate is produced, thus leading to lactate accumulation. It is not yet clear, however, to what extent enhanced glycolysis is related to the cause or to the effects of seizure activity. In addition to providing immediate energy for synaptic activity enhanced brain glycolysis during seizures may be necessary to provide a rapid and large amount of ATP for maintaining or restoring neuronal membrane potentials during the seizure itself. This energy would be needed to protect the brain from glutamate toxicity and to help restore brain homeostasis (i.e., the balance of inhibitory and excitatory systems).

We suggest that CR alone, or the calorically restricted KD, inhibits seizure susceptibility by transitioning brain energy metabolism from glucose to ketone bodies. This transition could maintain brain energy homeostasis through enhanced energy production from respiration. According to Veech, ketone bodies are a 'super fuel' that provide more energy equivalents than glucose for metabolic activities. It is generally recognized that metabolic homeostasis and energy efficiency is better in the fasted state than in the 'well fed' state, when ketone bodies displace glucose as a major metabolic fuel.

We propose that epilepsy involves disruptions of brain energy homeostasis and that seizure disorders are potentially manageable through metabolic control, where a gradual transition from glucose to ketone body metabolism controls the seizure disorder by restoring energy homeostasis. Furthermore, these transitions in glucose and ketone levels occur within physiological ranges that maximize metabolic homeostasis (**Figure 4**). Transitions with a physiological range are important, since shifts that are too extreme can produce the pathological complications associated with hypoglycemia or ketoacidosis. The key to treatment is to maintain seizure management within a zone defined by a effective ratio of circulating blood glucose to ketone bodies. Since the metabolism of ketone bodies is thermodynamically more efficient than the metabolism of glucose, a transition in brain energy metabolism from

glucose to ketones will produce a homeostatic state that is capable of restoring the physiological balance of excitation and inhibition. This balance comes from more efficient activities of membrane pumps, which are major consumers of cellular ATP, and from more efficient neurotransmitter uptake, which is coupled to pump activity. The restoration of a nonepileptic (normal) phenotype can be obtained in this way, despite the continued presence of the idiopathic (genetic) or symptomatic (acquired) defects responsible for the disease. New experimental approaches involving biological system state analyses will be necessary to evaluate the mechanisms by which these gene and metabolic networks interact to manage epilepsy.

Future Goals

In summary, we propose that the metabolic management of epilepsy occurs through two distinct processes. The first involves a reduction in circulating glucose levels. The second involves an elevation in circulating ketone body levels that supply the body with energy that would otherwise not be generated during conditions of decreased glucose metabolism. These alterations would produce a shift in the neurotransmitter pools that modulate neuronal membrane potential, thus restoring the physiological balance of excitation and inhibition. This restoration of the neurotransmitter pool and membrane potential is based on the inherent genetic and metabolic flexibility of the brain to reorganize and adapt to changes in the availability of brain energy metabolites. Future goals will require identification of those neurochemical and neurophysiological changes that accompany seizure management in association with changes in glucose and ketone levels. A better understanding of the genetic and neurochemical networks of compensatory brain energy metabolism may lead to novel antiepileptic therapies that are more effective and less toxic than those currently available.

Acknowledgements

The work was supported by the Boston College research fund, the Epilepsy Foundation, and grants from the National Tay-Sachs and Allied Diseases Association (NTSAD) and NIH (HD39722).

See also: **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; Anticonvulsant Mechanisms of a Ketogenic Diet; Dietary Therapies for Epilepsy; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation; **Psychiatric Symptoms**: Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Dietary Therapies for Epilepsy

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Introduction

As the search for new treatments for epilepsy continues, the ketogenic diet (KD) maintains an enduring stance as an effective treatment option, notably for medically intractable patients. Since its initial use in the 1920s, at a time when few anticonvulsant medications were available, the KD has seen episodic resurgence, most recently in the 1990s. Even with the advent of more anticonvulsant medications, dietary therapy for epilepsy has continued to offer responder rates that rival or exceed the new medications. Epilepsy patients who have failed multiple antiepileptic drug trials sometimes experience dramatic responses to dietary therapy, and the KD has proven invaluable for certain epilepsy syndromes. Both clinical and basic mechanistic research in the field of dietary therapies for epilepsy continues to grow. Further, efficacious variations of the traditional KD – including the Atkins diet and low-glycemic index treatment – are being investigated and may offer useful and better-tolerated alternatives to the more restrictive classic formulation.

Background - History of the KD

The beneficial effect of fasting on seizure control had been noted as early as Hippocrates' time, with later references found in the Bible. After Jesus healed a boy with seizures, he told his disciples that the 'evil spirit' could only be exorcised with prayer and fasting. Centuries later, Rawlin Geyelin reported in 1921 that a 3-week fast was effective in treating epilepsy. His work was based on a 'water diet,' proposed by the osteopathic physician Hugh Conklin, which deprived patients of food, but allowed water for a period of 3 to 4 weeks. Geyelin reported high cure rates that persisted even after the diet was discontinued, providing the first hint of the possible anticonvulsant effects of the KD.

Based on these and other historical observations, Wilder proposed that the ketosis produced from fasting may explain the improved seizure control. He devised a diet with a fat to carbohydrate plus protein ratio of at least 3:1 (by weight) to mimic the ketosis during fasting, a formulation that is very similar to that used today. During the 1920s, Peterman at the Mayo Clinic modified the diet originally proposed by Wilder and studied its efficacy in the treatment of epilepsy. Peterman reported that 50% of patients became seizure-free with dietary therapy.

Following the introduction of phenytoin in 1938, the KD became less popular as more anticonvulsant medications became available for clinical use. The group at Johns Hopkins Hospital reinvigorated research into the diet in the 1990s with the successful treatment of Charlie, the son of a Hollywood producer. This success led to increased public awareness of the efficacy of the diet through mainstream media, and introduced the KD to many practitioners previously unfamiliar with its use. Of particular importance is the fact that the KD appeared to have considerable efficacy against seizures/epilepsies that did not respond to any of the available medical therapies. Subsequently, there has been mounting interest in optimizing clinical implementation and elucidating underlying mechanisms of the KD.

Methodological Issues

Initiation of the KD

The typical protocol used by many centers is borrowed from the experience of the Johns Hopkins group (see Table 1). Prior to initiation of the KD, patients undergo a comprehensive medical evaluation, involving a multidisciplinary team composed of a neurologist, dietician, and nursing staff, all experienced in the use of the KD. Dietary preferences and calorie counts are recorded, and this information is used to develop daily menus for each patient. Baseline laboratory studies usually include a comprehensive metabolic panel, lipid panel, and complete blood count. A careful examination of medications being taken is required, as liquid medications often contain hidden carbohydrates – which could easily break the systemic ketosis; such medications are changed to tablet forms. Encouraging families to bring to the hospital some recreational and/or educational activities for their children helps ensure a more manageable hospital stay. Several available books geared toward the lay audience address many of the questions that arise when considering or initiating the KD. Important to note, however, is the fact that the KD is fraught with certain adverse side-effects. For example, patients with an elevated urinary calcium-to-creatinine ratio may be at risk for nephrolithiasis and should be followed closely.

Patients are usually hospitalized for 4 days, although recently, outpatient initiation of the diet has been shown to be a safe and effective alternative. Hospitalization is advocated for a number of reasons, including (1) observation for hypoglycemia and other potential

Table 1 Typical regimen for initiation of the KD

Before diet
Nutrition history obtained
Minimize carbohydrate intake for 1 day
Fasting begins after dinner the evening prior to admission
Day 1
Admission to the hospital
Conversion to carbohydrate-free medications
Basic laboratory obtained if not done previously (metabolic profile, urine calcium, urine creatinine, fasting lipid profile, antiepileptic drug levels)
Check fingerstick glucose every 6 h; if <40 mg dl^{-1}, check every 2 h
If symptomatic, or glucose <25 mg dl^{-1}, give 30 ml orange juice, measure blood glucose again
Parents begin classes
At dinner, one-third of the calculated ketogenic meal given as 'eggnog'(e.g., if the full meal is calculated as 150 ml give 50 ml at this meal)
Blood glucose checks discontinue after dinner
Day 2
At breakfast and lunch, one-third of the calculated ketogenic meal given as 'eggnog'
Symptomatic ketosis (e.g., nausea, vomiting) can be relieved with small quantities of glucose
Parent classes continue
At dinner, two-thirds of the calculated meal given as 'eggnog'
Day 3
At breakfast and lunch, two-thirds of the calculated meal given as 'eggnog'
Parent classes conclude
At dinner, the first full ketogenic meal is given (not 'eggnog')
Day 4
After breakfast (full ketogenic meal), the patient is discharged home
Prescriptions written for carbohydrate-free medications, urine ketone test strips, a sugar-free, fat-soluble
Multivitamin and calcium supplements, citrate salts (if indicated)
Clinic follow-up appointment arranged

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side-effects, (2) gradual initiation of the diet under dietary supervision to ensure that the diet is well tolerated by each patient, and (3) close personal education for the families to help with comfort level and understanding of the diet. Prior to entering the hospital, some centers advocate fasting, while other centers have found that nonfasting may allow improved tolerance of the diet and lowers the risk of dehydration.

The diet is typically calculated to yield a ketogenic ratio of 4:1 (i.e., 4 g of fat to 1 g of protein plus carbohydrate). The total calories are calculated to achieve ideal body weight, while total fluid intake is often restricted. Fluid restriction had been advocated in the belief that such restriction would enhance ketosis, and presumably seizure control. However, there is little information that fluid restriction is a necessary condition for clinical efficacy. Protein intake at 1 g per kilogram of body weight per day is provided to ensure linear growth. The diet is often started at one-third strength on day 1, and gradually increased by one-third strength each day to the full targeted ketogenic ratio. The dietician works closely with the family to individualize meals that are both palatable and also compliant with the strict requirements of the diet. Engaging children in their meal selection and preparation can help improve compliance. KetoCal (Nutricia N.A., subsidiary of SHS International, Liverpool, England) can provide the complete enteral nutritional needs for patients fed via feeding tubes, and is easily used by parents.

It is important to follow certain metabolic parameters while on the KD. Blood glucose is measured via fingerstick, three times per day, or when the patient experiences signs or symptoms of hypoglycemia. Glucose levels above 25 mg dl^{-1} are usually well tolerated and do not require treatment. If the patient becomes symptomatic or drops below 25 mg dl^{-1}, 30 mL of orange juice is generally sufficient to improve the hypoglycemia. To assess ketosis at home, family members are taught to use urinary test strips (i.e., Ketostix, Bayer AG), which specifically measure acetoacetate levels. This home intervention adds to the empowerment that families feel when administering the KD. Since urine dipstick measurements do not correlate well with ketonemia, practitioners can alternatively measure serum β-hydroxybutyrate levels to assess the degree of ketosis and compliance.

There are other important variables in the clinical management of patients. Vitamin supplementation is recommended because the diet lacks fat-soluble vitamins and calcium. Proper counseling of the family on the need for carbohydrate restriction – including consideration of the carbohydrate content of over-the-counter and prescribed liquid medications – may help prevent breakthrough seizures following loss of ketosis. If the diet is successful, patients are traditionally maintained on the KD for a minimum of 2 years (though it is unclear if early responders require such prolonged treatment), after which consideration is given to discontinuation of the diet. Patients may not respond initially, but families are encouraged to maintain the diet for at least 2–3 months before determination of failure and eventual weaning. During this initial period, modifications to the ratio of the diet may help improve seizure control.

Safety of the KD

Contrary to popular belief, the KD is not a 'natural' treatment for epilepsy, and as such, is not without side-effects. Fortunately, most side-effects are mild and can be managed successfully. These side-effects can be grouped into acute and chronic complications of the diet. Initiation of the diet forces the body's metabolism to undergo multiple systemic changes, which may be difficult for

the patient to tolerate. Gastrointestinal side-effects, such as nausea and vomiting (which can lead to dehydration), is common, particularly when the diet is initiated with fasting. Constipation is more often seen with the classic KD, while diarrhea is more common when using the medium-chain triglyceride (MCT)-oil. Laxatives to treat constipation are often needed by patients on the classic KD. Symptomatic hypoglycemia is not uncommon, but is easily treated with a glucose load (such as 30 ml of orange juice). Elevations in serum lipid levels may be seen as an early or late complication of treatment. Hypertriglyceridemia and hypercholersterolemia may occur, but often stabilize and do not require intervention. The long-term consequences of this dyslipidemia are not well understood, but a reduction in the ketogenic ratio often causes a decrease to acceptable levels. Nephrolithiasis appears to be a relatively common side-effect, seen in ~5% of patients. Alkalinization of the urine, in patients with elevated urinary calcium-to-creatinine ratios or those with a history of nephrolithiasis, can significantly reduce this risk. The relatively low protein and caloric intake of the KD may also impact the linear growth of patients, particularly younger children, and thus needs to be closely followed. Osteopenia from poor calcium absorption and increased osteoclastic activity may lead to an increased incidence of fractures. There have been rare case reports describing potentially fatal side-effects such as hepatitis, cardiomyopathy, and pancreatitis, but the cause-and-effect relationships are unclear. The KD also can provoke a secondary carnitine deficiency, as with medications such as valproic acid. As such, there has been great concern that there may be an increased susceptibility to hepatotoxicity when valproic acid is coadministered with the diet. However, this combination can be safely administered if patients are carefully followed. Overall, the side-effects of the KD are relatively mild and certain modifications and interventions can ensure increased tolerability.

Results

Efficacy of the KD

With the resurgence of interest in the KD, there are a growing number of studies evaluating clinical efficacy and tolerability, and special attention has been given toward identifying which subgroups of patients may represent more favorable responders. The Johns Hopkins experience revealed a very favorable response rate in patients with medically refractory epilepsy. Thirty-six percent of patients who were on the KD at one year exhibited a >90% reduction in seizures, and 13% were seizure-free at the 1-year follow-up mark. Seventy-two percent (8/11) of fully controlled patients remained seizure-free after discontinuing the diet. Sixteen percent (12/72) of patients who continued to have seizures at 1 year of follow-up became seizure-free at the 3–6 year follow-up time-point. An additional benefit was that 36% of children no longer took anticonvulsant medications, and another 34% took only one medication (compared with a previous mean number of medications of 1.97). These findings demonstrate the anticonvulsant efficacy of the KD, but also suggest a more enduring (and possibly, antiepileptogenic) effect.

Recently, the first randomized controlled trial of the KD in the treatment of pediatric patients with medically refractory epilepsy was completed in the United Kingdom. This study showed a statistically significant difference in responder rates between KD-treated vs. control groups. Twenty-eight children (38%) in the diet-treated group had a greater than 50% seizure reduction compared with only four (6%) controls ($p < 0.0001$).

While some larger studies have suggested that seizure type is not a predictor of treatment success, patients with complex partial seizures are less like to have an early, dramatic response to the KD. The diet has been used in a wide variety of epileptic syndromes, including Lennox–Gastaut syndrome, tuberous sclerosis complex, Landau–Kleffner syndrome, myoclonic–astatic epilepsy, Rett syndrome, and infantile spasms, often with very positive results. Dravet syndrome (severe myoclonic epilepsy of infancy) seems particularly responsive to the KD and should be considered early. Patients with glucose transporter-1 deficiency and pyruvate dehydrogenase deficiency are ideally treated with the KD, since this treatment can bypass the underlying biochemical defects. Specifically, ketone body transport and utilization through monocarboxylic acid transporters and by the tricarboxylic acid (TCA) cycle, respectively, can compensate for these metabolic deficiencies. Interestingly, despite data suggesting neuroprotective properties of the KD, Lafora body disease was not appreciably altered by such dietary intervention.

Modifications to the KD

The classic 4:1 formulation of the KD has always been hampered by the degree of carbohydrate restriction necessary to induce ketosis. This restriction has limited the palatability of the diet, and consequently its clinical use and acceptance by patients. Investigators have long sought modifications of the diet that might circumvent this problem, while retaining clinical efficacy.

The MCT Diet

The first major modification of the diet was advanced in the 1960s. Medium-chain triglycerides (MCT) were used as a supplement to the traditional KD to enhance ketosis. Medium-chain triglycerides are passively absorbed from the gastrointestinal tract and are strongly ketogenic;

they are more ketogenic than long-chain trigylcerides, and can allow for a higher carbohydrate and protein intake than the classic diet. Initiation of the MCT variation was described by Huttenlocher in 1971. Children were admitted to the hospital and started on a 50 cal day^{-1} fast for about 48 h until they became strongly ketotic. They were then started on the diet with 60% of their calories provided as MCT oil. The oil was served as milkshakes made with skim milk. The remaining calories came from protein and carbohydrate, depending on the individual tastes of the patient, to ensure the minimal daily requirements to promote growth. Four of six patients with myoclonic seizures responded well, while patients with complex partial seizures seemed to have a lower response rate. Multiple patients demonstrated a marked improvement in their electroencephalogram (EEG) and many were noted to have improved behavior and alertness. However, gastrointestinal side-effects, including cramping, nausea, and diarrhea, are commonly seen with this diet. These symptoms may improve with time or with using only small sips of the MCT oil throughout each meal, rather than ingestion all at once. Unfortunately, the traditional MCT oil diet tends to be unpalatable and can thus limit overall acceptance of this diet. Recently modified formulations employing less MCT oil content, however, have been shown to be better tolerated.

Modified Atkins Diet

In 1972, Dr. Robert Atkins introduced a modification of the classic KD to promote weight loss. His diet allows for more liberal carbohydrate content than do the classic KD; fiber and sugar alcohols are not counted toward the daily carbohydrate count, as they do not alter blood sugar levels significantly. The popularity of the Atkins diet has led to a wide variety of foods that conform to this diet and allow for greater selection. The diet does not significantly restrict protein or caloric intake as does the classic KD, and this factor can diminish weight loss as a side-effect.

The Atkins diet has been modified and implemented for the treatment of epilepsy, based on the recommendations of the Johns Hopkins group. The Atkins diet typically consists of 60% fat, 30% protein, and 10% carbohydrates by weight. When patients are started on the Atkins diet, carbohydrate content is limited to 10 g day^{-1} for the first month, corresponding to the induction phase. Urinary ketones are followed semiweekly through the use of Ketostix (see **Table 2**). Laboratory studies to evaluate baseline nutritional status are checked prior to starting the diet and are later compared with the 3 and 6 month values. Carbohydrate intake may be gradually liberalized if the child finds the diet too unpalatable. Low-carbohydrate multivitamins are also instituted, as in the classic KD. Families find that the popular book, Dr. Atkins New Diet Revolution, can answer many of their questions regarding this diet and provides useful tips and recipes.

Despite the increase in carbohydrate content, ketosis with the Atkins diet can clearly be achieved, although the degree and consistency of ketosis may be variable. In one study of epileptic patients, seizure freedom at 6 months was 20%; 35% of patients achieved a >90% reduction of seizures, and 65% of patients had a >50% reduction in seizures. This reduction in seizure frequency is especially noteworthy, given that the patient population had failed an average of 6.5 AEDs and averaged 163 seizures per week. Overall, the side-effects of the Atkins diet were typically mild and consisted primarily of gastrointestinal complaints. Further, changes in cholesterol levels were not as apparent as those seen in the classic KD.

Low-Glycemic Index Diet

In another effort to enhance the palatability of the classic KD, other researchers have advocated a diet with a low-glycemic index as an alternative. The glycemic index is a value that describes the extent to which a carbohydrate is absorbed and elevates blood glucose. This rise in blood

Table 2 Modified Atkins' diet protocol

- Copy of Dr. Atkins' New Diet Revolution provided
- Carbohydrates described in detail and restricted to 10 g day^{-1} for the first month
- Fats (e.g., 36% heavy whipping cream, oils, butter, mayonnaise) encouraged
- Clear, carbohydrate-free fluids not restricted
- Low-carbohydrate multivitamin (Unicap M) and calcium (Calcimix) supplementation prescribed
- Calendar provided to document seizures daily, urine ketones semiweekly, and weight weekly
- Medications unchanged for at least the first month, but changed if necessary to tablet or sprinkle (nonliquid) preparations
- Low-carbohydrate, store-bought products (e.g., shakes, candy bars, baking mixes) discouraged for at least the first month
- Children evaluated in clinic 1, 3, and 6 months on the diet
- Complete blood count, complete metabolic profile (SMA-20), fasting lipid profile at baseline, 3, and 6 months; urine calcium and urine creatinine obtained at 3 and 6 months

Reproduced from Kossoff EH, McGrogan JR, Bluml RM, Pillas DJ, Rubenstein JE, and Vining EP (2006) A modified Atkins diet is effective for the treatment of intractable pediatric epilepsy. *Epilepsia* 47: 421–424, with permission from Blackwell Publishing.

glucose is typically compared with the change noted following glucose absorption itself; the higher the value, the faster the carbohydrate is released into the bloodstream as glucose. In general, foods with a low-glycemic index induce a slower insulin response compared to glucose.

There are few studies describing the use of a low-glycemic index diet in epileptic patients, but the preliminary results noted by Pfeifer and colleagues are very encouraging. Four of 11 medically intractable patients (36%) became seizure-free, while another 4 experienced a >50% reduction in seizure frequency. The low-glycemic index diet caused some elevations in the β-hydroxybutyrate levels of patients, but to a degree much less than that seen with the classic KD. Overall, the diet was well tolerated, and common side-effects included somnolence and gastrointestinal discomfort. Clearly, additional studies are necessary to demonstrate the effectiveness and safety of this promising dietary therapy for epilepsy.

Mechanism(s) of Action

The mechanisms underlying the clinical effects of the KD are not readily understood, but likely involve multiple parallel (and possibly) synergistic mechanisms resulting in an anticonvulsant effect (see **Figure 1**). β-Hydroxybutyrate is the major ketone body formed by the liver during ketogenesis and is followed clinically to assess the degree of fatty acid oxidation. While clinical efficacy of the KD does not always correlate well with serum levels of ketone bodies, in animal models of acute seizures, acetoacetate and acetone have been shown to be directly anticonvulsant when administered intraperitoneally. Interestingly, β-hydroxybutyrate – which is rapidly interconverted to acetoacetate by a dehydrogenase enzyme – is ineffective in these models. Despite these tantalizing observations, in vitro experiments have failed to confirm that any ketone body can reduce neuronal hyperexcitability and/or hypersynchrony of discharge. These negative findings would suggest that the effects of the KD may not be a direct anticonvulsant effect of ketone bodies, but rather a reflection of a larger systemic adaptation – marked by ketosis – as patients shift their metabolism to reduced glycolytic flux and increased fatty acid oxidation.

Notwithstanding these negative results, ketone bodies have recently been reported to modulate the activity of cellular membrane-bound ATP-sensitive potassium (K_{ATP}) channels. K_{ATP} channels are inwardly rectifying ligand-gated receptors that link metabolic alterations to cellular membrane excitability; specifically, K_{ATP} channels are opened when intracellular ATP levels fall. It was reported that ketone bodies, in a use-dependent manner, reduced the spontaneous firing of GABAergic neurons in rat substantia nigra – a putative subcortical 'seizure gate' – by opening K_{ATP} channels. Intriguing as this finding appears, one must reconcile an inherent discrepancy when implicating K_{ATP} channels as mediators of an anticonvulsant action. It is well known that the KD can increase levels of ATP and other bioenergetic substrates through enhanced mitochondrial respiration. Since high ATP levels block K_{ATP} channel activity, it is unclear how opening of these channels be achieved by infusion of ketone bodies in the SNr.

Glucose restriction alone, however, may play an important role in an anticonvulsant action of the KD. Calorie restriction in rodents has been shown to reduce seizure susceptibility, and a lowered level of blood glucose is correlated with retardation of epileptogenesis in a genetic model of epilepsy (i.e., the El mouse). These data suggested that less readily available glucose may limit the brain's ability to generate and promote seizure activity. In further support of the glucose restriction theory, it was recently reported that fructose-1,6-bisphosphate – a biochemical intermediate that shifts cellular metabolism of glucose from glycolysis to the pentose phosphate shunt – exerted broad-spectrum anticonvulsant activity in several animal seizure models. Investigators have also demonstrated that 2-deoxyglucose – an inhibitor of phosphoglucose isomerase – potently inhibits seizure progression in the rat kindling model of temporal lobe epilepsy, and decreases the expression of brain-derived neurotrophic factor (BDNF) and its principal receptor, TrkB. These effects were linked to the induction of the transcription factor NRSF (neuron restrictive silencing factor), an important negative regulator of neuronal genes. This study indicates that pharmacological inhibition of glycolysis alone can lead to downstream physicochemical modulation of gene transcription that critically interferes with epileptogenesis (at least in the kindling model).

Polyunsaturated fatty acids (PUFAs) have also been studied intensely with respect to KD action. PUFAs are known to block voltage-gated sodium and L-type calcium channels, and hence, represent potentially important mediators of an anticonvulsant effect. Intriguingly, the KD-induced elevations in both arachidonic acid (AA, C20: 4ω-3) and docosahexaenoic acid (DHA, C22: 6ω-3) in serum and brain of patients and animals, respectively, suggesting that these substrates might exert anticonvulsant effects by inhibiting sodium and calcium channels, much like many anticonvulsant drugs. Interestingly, the rise in serum AA and DHA during KD treatment closely paralleled observed seizure control in clinical studies, but this relationship has not been consistently observed. Does dietary supplementation with PUFAs actually result in an anticonvulsant action? Unfortunately, at this time, both human and animal data addressing this question are conflicting.

Nonetheless, PUFAs may exert a neuroprotective action through upregulation of mitochondrial uncoupling protein activity and expression, which decreases the production of reactive oxygen species (ROS). Excessive ROS

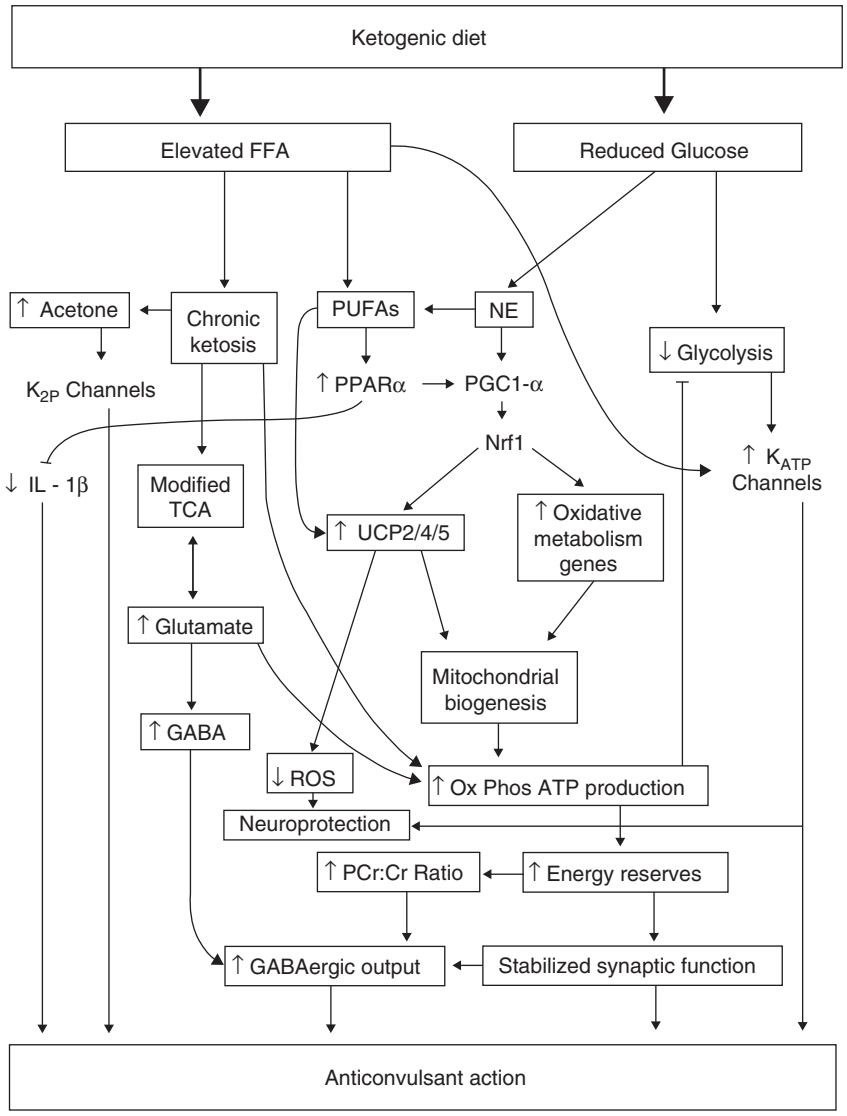


Figure 1 Hypothetical pathways leading to the anticonvulsant effects of the ketogenic diet (KD). Elevated free fatty acids (FFA) lead to chronic ketosis and increased concentrations of polyunsaturated fatty acids (PUFAs) in the brain. Chronic ketosis is predicted to lead to increased levels of acetone; this might activate K2P channels to hyperpolarize neurons and limit neuronal excitability. Chronic ketosis is also anticipated to modify the tricarboxcylic acid (TCA) cycle. This would increase glutamate and, subsequently, GABA synthesis in brain. Among several direct inhibitory actions, PUFAs boost the activity of brain-specific uncoupling proteins (UCPs). This is expected to limit ROS generation, neuronal dysfunction, and resultant neurodegeneration. Acting via the nuclear transcription factor peroxisome proliferator-activated receptor-α (PPARα), PUFAs would induce the expression of UCPs and coordinately upregulate several dozen genes related to oxidative energy metabolism. PPARα expression is inversely correlated with IL-1β cytokine expression; given the role of IL-1β in hyperexcitability and seizure generation, diminished expression of IL-β cytokines during KD treatment could lead to improved seizure control. Ultimately, PUFAs would stimulate mitochondrial biogenesis. Mitochondrial biogenesis is predicted to increase ATP production capacity and enhance energy reserves, leading to stabilized synaptic function and improved seizure control. In particular, an elevated phosphocreatine:creatine (PCr:Cr) energy-reserve ratio is predicted to enhance GABAergic output, perhaps in conjunction with the ketosis-induced elevated GABA production, leading to diminished hyperexcitability. Reduced glucose coupled with elevated free fatty acids are proposed to reduce glycolytic flux during KD, which would further be feedback inhibited by high concentrations of citrate and ATP produced during KD treatment. This would activate metabolic K_{ATP} channels. Opening of K_{ATP} channels would hyperpolarize neurons and diminish neuronal excitability to contribute to the anticonvulsant (and perhaps neuroprotective) action of the KD. Reduced glucose is also expected to downregulate brain-derived neurotrophic factor (BDNF) and trkB signaling in brain. As activation of TrkB pathways by BDNF have been shown to promote hyperexcitability and kindling, these potential KD-induced effects would be expected to limit the symptom (seizures) as well as the progression of epilepsy. Boxed variables depict findings taken from KD studies; up (↑) or down (↓) arrows indicate the direction of the relationship between variables as a result of KD treatment. Reproduced from Bough KJ and Rho JM (2007) Anticonvulsant mechanisms of the ketogenic diet. *Epilepsia* 48: 43–58, with permission from Blackwell Publishing.

levels have been implicated in a variety of neurodegenerative conditions, including Alzheimer's and Parkinson's diseases, and amyotrophic lateral sclerosis. A high-fat KD has also been reported to increase mitochondrial biogenesis and thus enhance overall availability of bioenergetic substrates (such as adenosine triphosphate or ATP), which may help stabilize cellular membrane function in the face of the metabolic stress that accompanies seizure activity. Specifically, elevations in ATP may help the functioning of the Na^+–K^+-ATPase, which would stabilize the neuronal cell membrane, hence limiting neuronal hyperexcitability and increasing the seizure threshold.

Altered neurotransmitter function may play another critical role in the anticonvulsant effects of the KD. Norepinephrine (NE) levels are increased in brains of rats fed a KD, while mice lacking the ability to produce NE (i.e., dopamine-β-hydroxylate knockout mice) lose the protective benefits of the KD in the setting of seizures acutely provoked by fluorothyl, a volatile convulsant. Perhaps most relevant to seizure control, γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the mammalian central nervous system, may also play an important role in the underlying mechanisms of the KD. Animal models of epilepsy induced by GABA antagonists have the strongest response to the KD, and the diet seems to increase GABA levels, either through increased substrate availability for GABA synthesis or through enhancing the expression of the biosynthetic enzyme, glutamic acid decarboxylase. Further supporting the GABA hypothesis of KD action, clinical studies have demonstrated increased GABA levels in the cerebrospinal fluid level of patients, as well as with magnetic resonance spectroscopy. In vivo electrophysiologic studies in rodents have revealed increased seizure threshold and decreased network hyperexcitability following treatment with a KD. Although clinical data clearly demonstrate that the KD works, the underlying anticonvulsant (and possibly antiepileptogenic) mechanisms remain elusive.

Future of the KD

The KD clearly can be effective in the treatment of intractable epilepsy patients. Accurate identification of suitable epilepsy candidates and refinement in the implementation of the diet continue to be major foci of research. The true nature of the antiepileptic effects of the diet remains unclear and it is hoped that further research may unlock alternative treatments to prevent or control epilepsy. Given the present evidence that the KD may be neuroprotective, there is growing interest into expanded uses of the diet outside of epilepsy. For example, the KD has been used to treat brain cancer, where this intervention may make certain cancers more susceptible to the effects of chemotherapy. Additionally, the wide-ranging effects of the diet may provide a useful treatment for other diseases (such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and perhaps migraine) that affect the brain. Further clinical studies in these areas may unlock further insights into a multiplicity of neurological disease processes and treatments, not just within the realm of epilepsy.

See also: **Antiepileptic Drugs**: Antiepileptic Drug Mechanisms; **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Ketogenic Diet**: Anticonvulsant Mechanisms of a Ketogenic Diet; Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; **Psychiatric Symptoms**: Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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METABOLIC CHANGES

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Cell Signaling by Polyunsaturated Fatty Acids in Epileptogenesis

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Introduction

Synaptic Activity and Seizures

Glutamate, the most abundant excitatory neurotransmitter in the mammalian central nervous system (CNS), plays a central role in learning and memory formation. Fast excitatory glutamate neurotransmission is mediated by the ionotropic receptors AMPA/kainate and *N*-methyl-D-aspartate (NMDA), while the slow glutamate responses are mediated through its interaction with metabotropic receptors (mGluRs) coupled to G proteins. During seizures, release of glutamate may lead to excitotoxic neuronal damage because of an overflow of calcium in postsynaptic neurons, mediated primarily by NMDA-gated channels. In epileptogenesis, as the brain becomes prone to chronic, spontaneous seizures associated with circuitry rearrangements and individual synapse abnormalities, enhanced glutamate-mediated neurotransmission, and decreased GABA-modulated synaptic inhibition takes place. Postsynaptic events regulated by synaptic activation include enhanced phospholipase A_2 induction, which results in the cleavage of acyl chains of membrane phospholipids. Both arachidonoyl and docosahexaenoyl chains are esterified at the C_2 of the glycerol backbone of membrane phospholipids, and phospholipase A_2 cleaves that bond, releasing free arachidonic acid (AA) and free docosahexaenoic acid (DHA). The subsequent activation of other enzymes, in turn, leads to the synthesis of eicosanoids, docosanoids, and platelet-activating factor (PAF) (**Figure 1**). The bioactivity of these lipid mediators during seizures and in epileptogenesis are not well understood, as are the biochemistry, cellular, and subcellular localization of specific enzymes involved in their biosynthesis, degradation, and regulation. The complexity of these events is highlighted by the fact that there are more than 20 known phospholipases A_2. Some lipid mediators have been shown to affect the activities of ion channels, receptors, and enzymes; when released into the extracellular space, they contribute to the modulation of presynaptic neurotransmitter release/re-uptake, and/or affect other neighboring neuronal/glial cells. The significance of bioactive lipids in the intricate mesh of signaling pathways converging to the nucleus that connects upstream to downstream synaptic events is the focus of many research efforts. This article describes seizure-induced glutamate release in the activation of the hydrolysis of membrane arachidonoyl- and docosahexaenoyl-phospholipids via phospholipases A_2, which initiates pathways and/or cascades of bioactive lipids that impinge upon neurotransmission and seizure-induced brain damage.

Epileptogenesis

Mechanisms underlying kindling epileptogenesis are incompletely understood, but it is generally assumed that

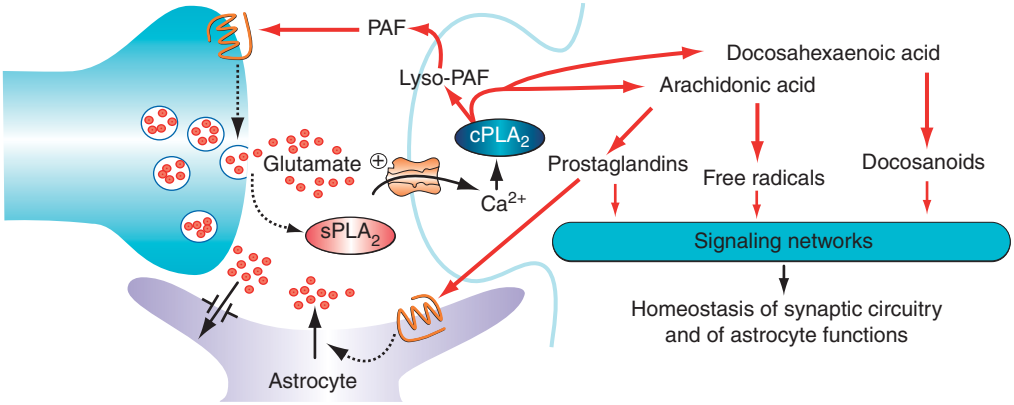


Figure 1 Bioactive lipids formed from membrane phospholipids at the synapse. In addition to pre- and postsynaptic membranes, membranes from astrocytes that surround synapses are sources of bioactive lipids. Seizures trigger glutamate release and, in turn, this activates glutamate receptors. Glutamate binds to the *N*-methyl-D-aspartate receptor and as a consequence an influx of calcium in the postsynaptic neuron occurs. Calcium-mediated activation of a PLA_2 cleaves arachidonoyl- or docosahexaenoyl-acyl chains of membrane phospholipid, releasing arachidonic acid (AA), docosahexaenoic acid (DHA), and lyso-PAF, the PAF precursor. The precise enzymes (PLA_2s, phospholipase C (PLC) followed by diacyglycerol lipase, etc.) as well as the precise localization are not well understood. Lyso-PAF is acetylated at C_2 and, in turn, forms PAF. Although PAF has a short biologic half-life, on repeated stimulus, sufficient PAF accumulates to diffuse back across the synaptic cleft. Evidence for this was provided by injecting PAF into the postsynaptic neuron and monitoring neurotransmitter release. PAF binds to its presynaptic receptor and enhances glutamate exocytosis. During synaptic plasticity events, PAF may also activate gene expression that in turn is probably involved in long-term alterations of synaptic function (not shown in this diagram). Cell surface PAF-receptor antagonists confer neuroprotection during ischemia reperfusion and inhibit PAF-induced glutamate release from hippocampal neurons and CA1 LTP formation, presumably through the same mechanism. The inhibitory effects of this antagonist on glutamate release could account in part for its neuroprotection in ischemia reperfusion. Secretory PLA_2 ($sPLA_2$) may be released from the presynaptic terminal: $sPLA_2$ binding sites are present in neurons, and $sPLA_2$ promotes active AA remodeling in neurons in culture and may also promote DHA release. DHA may also be released by $cPLA_2$. In fact, seizures increase the pool size of both arachidonic and docosahexaenoic acid. Free DHA may subsequently follow enzyme-mediated oxygenation pathways and lead to the synthesis of docosanoids (novel mediators). Free radicals would accumulate during seizures and oxidative stress. Downstream lipid signaling modulates neuronal function and survival. Synapses are intimately surrounded by astrocytes, which express glutamate transporters that remove the excitatory neurotransmitter from the vicinity of the synaptic cleft. Astrocytes also respond to prostaglandins by releasing glutamate through a Ca^{2+}-dependent mechanism. For the sake of simplicity, endocannabinoids, the CB1 receptor, and other bioactive lipids are not included. Modified from Bazan NG (2003) Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *Journal of Lipid Research* 44: 2221–2233, with permission from ASBMB.

an imbalance between GABA inhibitory input and glutamate is involved. Glutamate release is thought to contribute to the reorganization of neuronal connections favoring a permanent hyperexcitable state, often associated with mossy fiber sprouting. Synthesis of new proteins is thought to form the basis for sustained seizure-induced physiological and/or pathological changes in synaptic reorganization and apoptotic/necrotic neuronal death. We postulate that seizure-induced synthesis of docosanoids, derived from docosahexaenoyl chains of phospholipids, downregulates these changes in synaptic restructuring and neuronal death.

Background

Hippocampal Kindling Epileptogenesis

Hippocampal kindling, a model of mesial temporal lobe epilepsy, is developed through repetitive stimulation of the hippocampus and leads to increased after-discharges (as measured by an electroencephalogram (EEG)) and an enduring seizure-prone state. Stimulus-induced phosphorylation of JNK, but not of p38, is enhanced in the CA1 subregion of the hippocampus of kindled animals compared to their naïve counterparts. Immunofluorescent staining confirmed this region-specific pattern of JNK activation, and revealed that reactive astrocytes mediate this effect. Astrocyte proliferation and hypertrophy, as well as upregulation of vimentin protein levels (common markers of astrogliosis), were present after four days of kindling. The consequence of these phosphorylation events suggest a selective response to aberrant synaptic activity – signaling that may modulate kindling progression and/or neuronal damage and death. Seizure-induced plasticity by electrical kindling has several limitations when compared with epilepsy. Perhaps most importantly, in most kindling models, recurrent spontaneous seizures rarely occur.

Synergy by Secretory Phospholipase A_2 ($sPLA_2$) and Glutamate on Inducing Neuronal Cell Death and Sustained AA Metabolic Changes

Secretory phospholipase A_2 ($sPLA_2$) and glutamate induce sustained changes in neuronal arachidonoyl–phospholipid

metabolism, and $sPLA_2$ plus glutamate exert synergistic mobilization of AA and subsequent neurotoxicity. Since $sPLA_2$ type II is present in synaptic vesicles, it is possible that glutamatergic neurotransmission involves the co-release of glutamate and $sPLA_2$. Thus, excitotoxicity may involve not only glutamate, as currently assumed, but may also involve $sPLA_2$ at the synaptic cleft. In this connection, it is relevant that the synthesis of PAF, a retrograde messenger of long-term potentiation (LTP), may be enhanced by $sPLA_2$ at the synapse. 'Cross-talk' between cytosolic PLA_2 ($cPLA_2$) and $sPLA_2$ has recently been suggested in signal transduction events in macrophages, and a complex interplay between glutamate-activated $cPLA_2$ and $sPLA_2$ could be envisioned at the synapse (**Figure 1**). These events remain to be defined in epileptogenesis.

Cyclooxygenase and Models of Epileptogenesis

Cyclooxygenase (COX, or prostaglandin H synthase (PGHS)), catalyzes PGH_2 synthesis from AA, the precursor of prostaglandins. COX comprises two isoforms, COX-1 (constitutive) and COX-2 (inducible). COX-2 participates in the inflammatory response, neuronal death, and neuronal hyperexcitability. Seizures activate cytosolic phospholipase A_2 ($cPLA_2$), induce expression of $cPLA_2$ and COX-2, and promote AA accumulation and PG synthesis. Free arachidonic and docosahexaenoic acids accumulate in synaptic endings isolated during seizures (**Figures 2** and **3**).

Recurrent and spontaneous seizures in epilepsy result from poorly defined cell signaling aberrations thought to include synaptic and extracellular matrix remodeling. Using a rat hippocampal kindling model, COX-2 gene expression in epileptogenesis was studied. COX-2, encoded in an early-response gene, has been shown to increase in a synaptic activity-dependent fashion and also during kainic acid-induced hippocampal damage. We found that during kindling, COX-2 induction occurred initially only in hippocampal neurons, and then spread to neocortical neurons. When rats were rekindled 34 days later, this spreading of COX-2 expression persisted. Induction of hippocampal and neocortical $cPLA_2$, an enzyme that catalyzes the release of COX-2 substrate AA, occurred after 4 days of stimulation during kindling and rekindling (**Figure 4**). Moreover the COX-2 selective inhibitor nimesulide attenuated kindling development indicating that COX-2 activity is involved in kindling epileptogenesis. Failure of nimesulide to fully inhibit kindling development may be due to an incomplete access of this drug to the COX-2 protein to achieve full inhibition or/and to redundancy of signaling pathways. COX-1, which is not inhibited by nimesulide, may synthesize an increased amount of prostaglandins due to accumulation of AA as a result of COX-2 inhibition, thereby minimizing the effect of nimesulide. The mechanism through which COX-2 inhibition slows kindling progress is not clear. However, it is likely that COX-2 inhibition may reduce synthesis of PGs and/or PAF. Both products may involve synaptic facilitation. Thus, the spreading of kindling-induced COX-2 induction from hippocampal neurons to neocortical neurons and attenuation of kindling by COX-2 inhibition suggests that kindling permanently

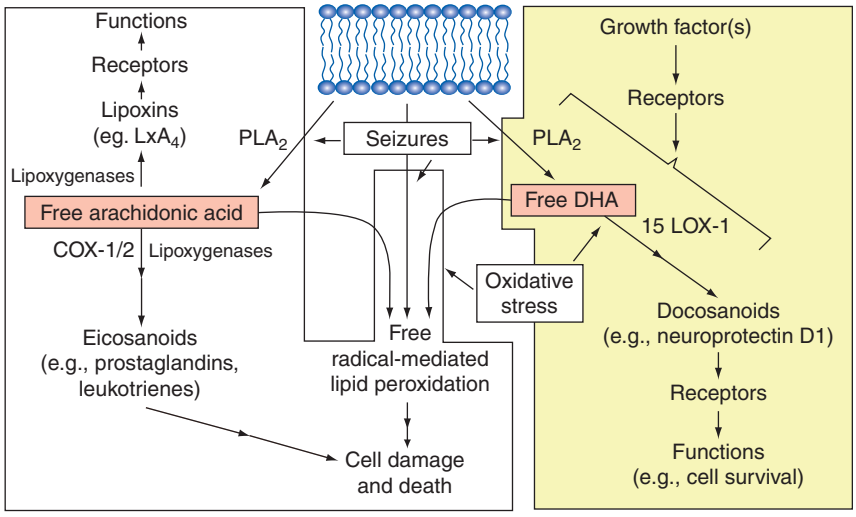


Figure 2 Fate of polyunsaturated acyl chains of membrane phospholipids. PLA_2 = phospholipase A_2. The central arrows indicate that free radical-mediated lipid peroxidation may attack esterifed arachidonoyl or docosahexaenoyl chains, as well as free arachidonic or docosahexaenoic acids (left and right arrows). The evolving lipid peroxidation products are highly reactive and promote further cell injury. On the left of the diagram, phospholipase A_2 is depicted releasing free AA and leading to the eicosanoid cascade that modulates cell functions through specific receptors. On the right side of the diagram, it is suggested that growth factor-mediated activation of the synthesis of docosanoids leads to biologically active mediators such as neuroprotectin D1, which in turn operate through receptors. Oxidative stress by itself activates both docosanoid synthesis (to counteract cytotoxic actions) and free radical-mediated lipid peroxidation.

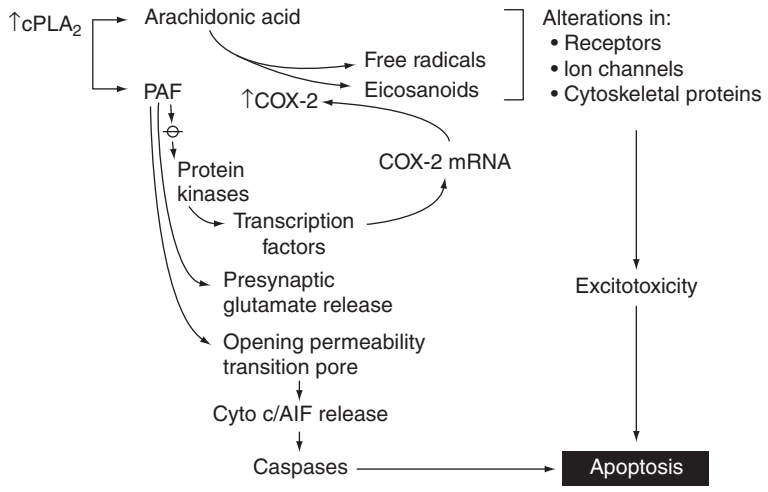


Figure 3 Involvement of phospholipid-derived second messengers in neuroexcitotoxic damage. Modified from Bazan NG, Tu B, and Rodriguez de Turco EB (2002) What synaptic lipid signaling tells us about seizure-induced damage and epileptogenesis. *Progress in Brain Research* 135: 175–185, with permission from Elsevier.

facilitates hippocampus–neocortex functional connectivity and that COX-2 is involved in epileptogenesis. We conclude that neuronal COX-2 gene induction and $cPLA_2$ activation are key signaling events in seizure-induced plasticity by kindling.

Platelet-Activating Factor

PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is another lipid mediator derived from membrane phospholipids (**Figures 1, 3,** and **5**), and is known to exert potent proinflammatory and neuromodulatory bioactivity in the CNS. Under physiological conditions, PAF participates in LTP associated with learning and memory through stimulation of its G-protein coupled receptor (PAFR). When concentrations are elevated under pathological conditions, PAF becomes neurotoxic – and is a key mediator of neuronal death following ischemia, encephalitis, epileptic seizures, meningitis, and HIV-1 dementia.

Docosahexaenoic Acid in Synaptic and Other Neural Membranes

Docosahexaenoic acid (DHA, 22:6,n-3) belongs to the essential omega-3 fatty acid family and is concentrated as an acyl group of phospholipids at synapses and in other cellular membranes of the nervous system. During postnatal development, DHA accumulates in the brain in parallel with synaptogenesis and dendrite formation (and photoreceptor biogenesis). A salient feature of DHA in the CNS is that it displays a very unusual retention ability. Only prolonged dietary deprivation of omega-3 fatty acids is effective in reducing DHA content, and only then do learning impairments or deficiencies in visual acuity occur. These functional impairments are selectively ameliorated by feeding omega-3 fatty acids. The cellular and molecular mechanisms involved in the high retention property of DHA in the nervous system are not well understood. After ingestion of food containing linolenic acid (18:3,n-3, the DHA precursor), the liver elongates and desaturates this fatty acid and, by stimuli that are not understood, releases DHA-containing phospholipids with lipoproteins into the blood stream (and to the CNS). Overall, DHA is involved in brain and retinal development, aging, memory formation, synaptic membrane function, photoreceptor biogenesis and function, and neuroprotection. Epidemiologic studies suggest that diets rich in DHA slow down cognitive impairments and progression of Alzheimer's disease. A major question to date has been: What are the molecular signaling mechanisms through which DHA confers neuroprotection? There are some suggestions that phosphatidylserine containing DHA acyl chains may be involved in the DHA bioactivity.

The free (unesterified) DHA pool size increases in the brain, and is higher in synaptosomes, of animals undergoing seizures. In bicuculline-induced status epilepticus, both free DHA and AA increase (as seen in rapidly-isolated synaptosomes), suggesting the activation of phospholipases A_2 that hydrolyze membrane phospholipids containing these polyunsaturated fatty acids. Since these membrane fatty acids are targets for free radical-mediated lipid peroxidation, and since this degradation process is engaged in cell injury, it has been suggested that these events are linked to brain damage – e.g., in brain ischemia-reperfusion and status epilepticus. Moreover, the deletion of phospholipase $A_2\alpha$, an enzyme that mediates the release of free DHA and AA, results in increased resistance to middle-cerebral artery occlusion-induced brain damage. Clearly, sustained brain injury or status epilepticus fosters an enhancement of both release of DHA, AA and lipid

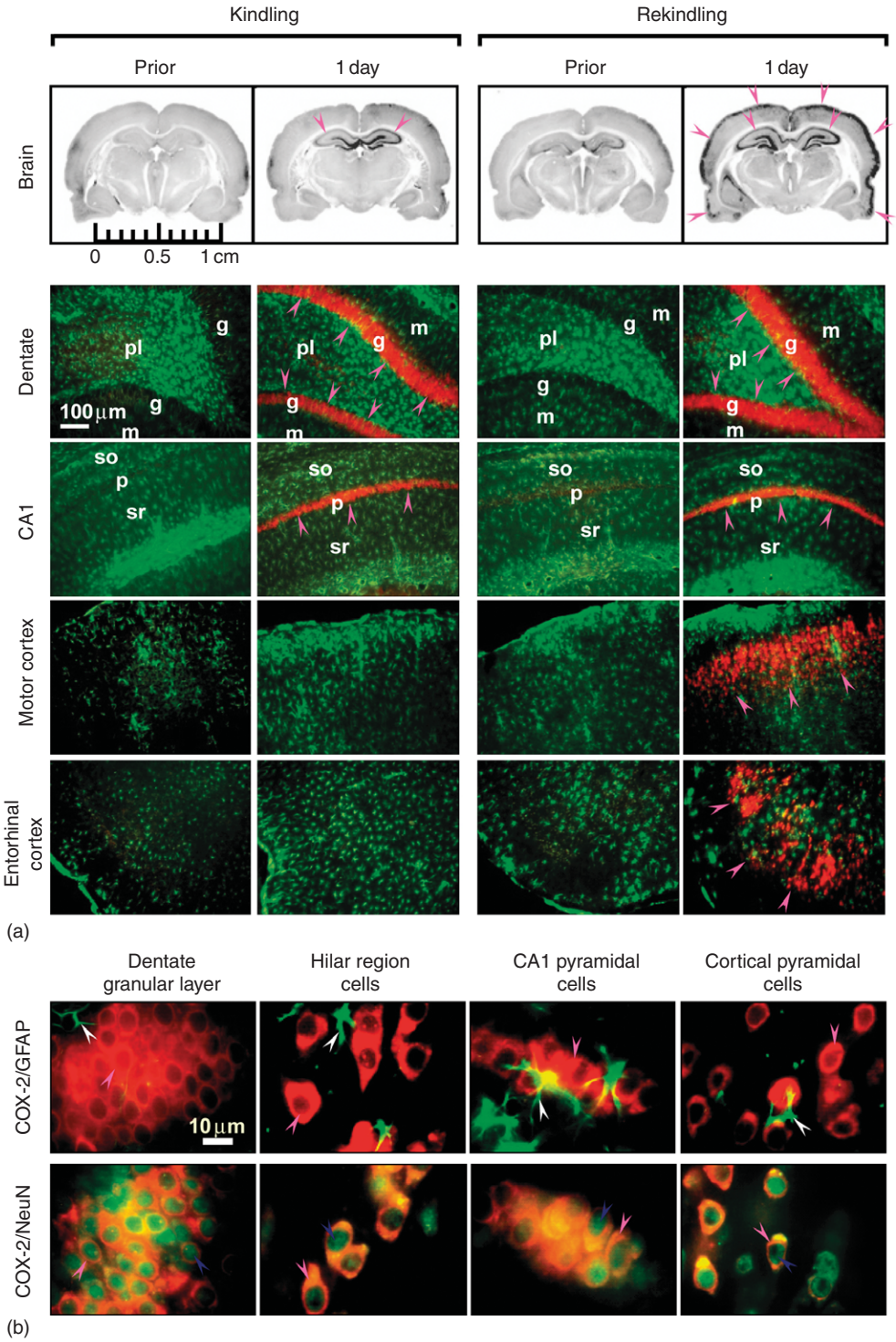


Figure 4 COX-2 induction in kindling is neuronal. (a) COX-2 immunoreactivity labeled with DAB shows low expression in controls and 1 month after kindling. After 1 day of kindling, COX-2 was induced (pink arrowheads) only in the hippocampus, while after 1 day of rekindling (34 days after kindling) the induction appears in the neocortex. Low-magnification (×100) images of COX-2 (red) and GFAP (green) double labeling showed that COX-2 immunoreactivity increased in the dentate granule cell layer and CA1 pyramidal cell layer after 1 day of kindling; however, the increase spread to the neocortex after 1 day of rekindling. g, granule cell layer; m, molecular layer; pl, polymorphic layer; p, pyramidal cell layer; sr, stratum radiatus; so, stratum oriens. (b) Higher-magnification (×1000) images of COX-2 (red)/GFAP (green) or COX-2 (red)/NeuN (green) labeling after 1 day of rekindling. The increase of COX-2 immunoreactivity is located only in cytoplasm (pink arrowheads) of neurons (blue arrowheads), and not in astroglial cells (white arrowheads). Modified from Tu B and Bazan NG (2003) Hippocampal kindling epileptogenesis upregulates neuronal cyclooxygenase-2 expression in neocortex. *Experimental Neurology* 179: 167–175, with permission from Elsevier.

peroxidation. The discovery of neuroprotectin D1 (NPD1), the finding that this lipid mediator is generated in early stages of brain ischemia-reperfusion, and the discovery of its ability to potentially inhibit ischemic brain damage, offer an alternative interpretation – that the cleavage of DHA and the subsequent synthesis of NPD1 during injury constitute a neuroprotective response (**Figure 2**). Recently, we found that NPD1 also increases in the hippocampus during seizures. In the initial phases of injury, it may be that synthesis of NPD1 is a mechanism aimed at maintaining neural cellular integrity and homeostasis.

Methods

LC–MS–MS-Based Mediator Lipidomics

Quantitative analysis of docosanoids by liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS) was performed in hippocampi from mice (C57/BL-6, 20–25 g body weight) killed by head-focused microwave radiation at different time points after the onset of reperfusion. The hippocampi were rapidly dissected (20–70 mg wet tissue weight), homogenized in cold methanol, and kept under nitrogen at −80 °C until purification. Purification was performed by solid-phase extraction technique. In short, samples pre-equilibrated at pH 3.0 were loaded onto C18 columns (Varian) and eluted with 10 ml of 1% methanol in ethyl acetate (EM Science). Samples were concentrated by a nitrogen stream evaporator before LC–MS–MS analysis. Samples were loaded into a Surveyor MS pump (Thermo-Finnegan) equipped with a C18 discovery column (Supelco), 10 cm × 2.1 mm inner diameter, 5 μm internal phase. Samples were eluted in a linear gradient (100% solution A (60:40:0.01 methanol/water/acetic acid) to 100% solution B (99.99:0.01 methanol/acetic acid)) and run at a flow rate of 300 μl min^{-1} for 45 min. LC effluents were diverted to an electrospray ionization probe (ESI) on a TSQ Quantum (Thermo-Finnegan) triple quadrupole mass spectrometer running on negative ion detection mode. Docosanoid standards were used for calibration and optimization. The instrument was run on full-scan mode to detect parent ions and selected reaction monitoring for quantitative analysis to detect daughter ions simultaneously. The selected parent ions were 327 for DHA and 359 for NPD1; daughter ions were 325.1 and 297, respectively.

Kindling

Adult male Wistar rats (250–350 g, 2–3 months old) were anesthetized with 1 ml kg^{-1} anesthetic mixture (ketamine 100 mg ml^{-1} and xylazine 10 mg ml^{-1}) through intraperitoneal injection. Tripolar electrode units (Plastic One, Inc., Roanoke, VA) were used; a pair of twisted polyimide-insulated electrodes (diameter 0.2 mm, tip separation ~0.06–0.08 mm) was implanted stereotaxically in the right ventral hippocampus (from bregma: AP −3.5, ML 5.2, DV −6.5 from dura), and a ground electrode attached to a stainless-steel screw in the left frontal bone. Three more screws were cemented together with the electrode unit using dental acrylic to secure the electrode. To record secondary afterdischarge, a hexa-polar electrode unit (Plastic One, Inc.) was used. Three of the electrode tips were implanted as with the tripolar electrode units, and the remaining electrodes were implanted in the left ventral hippocampus (AP −3.5, ML −5.2, DV −6.5 from dura) and both sides of the motor cortex (AP −1.0, ML ±5.2, DV −1.5 from dura). Two weeks after surgery, kindling was achieved by stimulating rats 12 times daily for 4 days with a subconvulsive electrical stimulation (a 10-s train containing 50-Hz biphasic pulses of 1-ms width and 400-μA amplitude) at 30-min intervals. Thirty-four days later, rats were rekindled for 1 day. Seizure behaviors evoked by stimulation were graded according to Racine's Scale: stage 1, standing still, wet-dog shaking, mouth and facial movements; stage 2, head nodding or jerking; stage 3, forelimb clonus; stage 4, rearing; stage 5, falling. Behavioral seizures greater than stage 1 were considered to be convulsive. EEG was recorded through electrodes, amplified 1000 times, filtered 0.5–40 Hz (3 dB per octave), digitized at a sampling rate of 200 Hz, and analyzed using Enhanced Graphics Acquisition for Analysis software (Version 3.63.00, R. C. Electronics Inc., Santa Barbara, CA). To test afterdischarge threshold, animals were stimulated at 1-h intervals using the same parameters as in kindling stimulation except that the intensity (μA) was gradually raised as follows: 10, 20, 30, 40, 50, 60, 80, 100, 120, 160, 200, 300, 400.

Immunohistochemistry

Rats were deeply anesthetized and perfused transcardially with 200 ml 10 mM PBS (PH 7.4) and then with 200 ml fixative (4% paraformaldehyde, 15% saturated picric acid, and 10 mM PBS). The brains were removed and postfixed in fixative for 1 day. Coronal blocks (3-mm thick) were cryoprotected overnight in 30% sucrose and sectioned into 40-μm-thick slices on a cryostat. Floating slices were treated with blocking buffer (2% BSA, 2% normal goat serum, and 0.2% Triton X-100 in PBS) and incubated overnight with COX-2 antibody (1:1000, Caymen Chemicals, Inc., Ann Arbor, MI). COX-2 was labeled with avidinbiotin complexes (ABC) and visualized with diaminobenzidine tetrahydrochloride (DAB) using a rabbit ABC kit and a DAB substrate kit (Vector Laboratories, Burlingame, CA) or was labeled with Texas red conjugated anti-rabbit antibody (1:100, Caymen Chemicals, Inc.) in double immunostaining. Besides COX-2 antibody, double immunostaining used primary antibodies directed to either glial fibrillary acidic protein (GFAP, 1:200, Chemicon) or neuron-specific nuclear protein (NeuN, 1:100, Chemicon) and an antimouse

fluorescein isothiocyanate (FITC)-conjugated secondary antibody (1:100, Vector Laboratories) to label astroglial cells or neurons. Double labeling was achieved by incubating the sections with a mixture of both primary antibodies followed by incubation with a mixture containing both fluorescent secondary antibodies. COX-2 immunostaining was confirmed by blocking COX-2 labeling with a COX-2 peptide (5 μg ml^{-1}, Caymen Chemicals, Inc.). All animal-use procedures were in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and were approved by the Louisiana State University Medical Center Institutional Animal Care and Use Committee (IACUC).

Results

DHA Effects on Excitability

Several researchers have attempted to use electrophysiological methods to characterize the actions of DHA bioactivity at the synapse. DHA added to hippocampal slices may elicit anti-epileptogenic activity. It has been proposed that DHA modulates ion channels and neurotransmitter receptors, and in turn regulates synaptic plasticity. The slice recordings have yielded conflicting observations on the effects of DHA in hippocampal slices. DHA seems to facilitate NMDA responses and block delayed-rectifier K^+ channels. As a consequence, DHA would enhance neuronal excitability and worsen seizure activity. In contrast, DHA added to neonatal rat ventricular myocytes may stabilize the neuronal membrane through suppression of voltage-gated Ca^{2+} currents and Na^+ channels. Moreover, DHA inhibits the induction of long-term depression (LTD), further supporting its potential role in learning and memory. DHA does not seem to exert actions on multiple spikes induced by bicuculline or in Mg^{2+}-free medium, indicating it does not directly interact with NMDA or non-NMDA glutamate receptors. Moreover, DHA may attenuate synaptic transmission and epileptiform activity in rat hippocampus by frequency-dependent Na^+ channel blockade. Most of these electrophysiologic studies have used added DHA at high concentrations (e.g., 50 μM). Therefore, it is important to note that in vivo, under resting conditions, brain unesterified DHA is present in undetectable amounts. The onset of seizure triggers the release of esterified DHA from phospholipids (e.g., as seen in bicuculline-induced status epilepticus, which promotes the accumulation of unesterified DHA in rapidly-isolated hippocampal synaptosomes) in the low nanomolar range.

DHA-Derived Stereospecific Neuroprotectin D1 Synthesis in the Nervous System

Early on during ischemia-reperfusion damage, enzyme-mediated oxygenation pathways are activated and a stereospecific neuroprotective mediator – neuroprotectin D1 (NPD1, 10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid) – is generated (in reinal cells and in the human brain). NPD1 was found to be reduced in Alzheimer's disease (AD) CA1, but not in other brain regions. These results open a new approach to define the significance of DHA at the synapse, and particularly in epileptogenesis, and offer a different explanation for the significant involvement of DHA in epilepsy.

PAF-Mediated Plasticity and Cell Injury

PAF plays a key role in transducing neuronal death in neurodegenerative conditions, yet therapeutic strategies targeting PAF signaling have not been successful. In rodents, PAFR is primarily expressed by activated microglia with neuronal expression restricted to subpopulations of cells in the hippocampus, notably the CA3 pyramidal cell field, cortex, and cerebellum. Presynaptic receptor-mediated PAF actions potentiate glutamergic transmission, and downstream of postsynaptic NMDA receptors, PAF is a messenger of glutamate actions that lead to gene expression and neuronal plasticity. Postsynaptic neuronal activity activates PAF synthesis, which in turn acts as a retrograde messenger, stimulating presynaptic glutamate release, thus contributing to LTP and memory formation; studies have shown that animals deficient in the PAF receptor displayed attenuated LTP. PAF-mediated effects through an intracellular receptor are linked with PAF-mediated effects on gene expression in the CNS (**Figures 3** and **5**). Electroconvulsive shock- and kainic acid-induced seizures activate early gene expression in the hippocampus, including expression of the inducible COX-2, and this induction is inhibited by PAF-receptor antagonists.

Glutamate triggers the activation of the MAPK signaling pathway, and PAF is the mediator of glutamate–NMDA activation of JNK, p38, and ERK MAPK. The MAPK cascade is also activated in the hippocampus during kainic acid-induced seizures and transient cerebral ischemia. Because PAF–MAPK activation may stimulate $cPLA_2$ and also may be upstream of PAF-induced COX-2 expression, both effects may converge in potentiating the AA cascade and eicosanoid synthesis. In addition, PAF can be the trigger for the COX-2–PGE_2–EPI receptor pathway at the nuclear level, leading to a late gene-transcription activation.

Cyclooxygenase Involvement in Epileptogenesis

Hippocampal kindling epileptogenesis selectively upregulates neuronal COX gene expression in the neocortex. COX-2 is an inducible proinflammatory protein that catalyzes prostaglandin synthesis. Interestingly, COX-2 is also present in a 'constitutive' mode in postsynaptic spines and has been linked to synaptic plasticity. The kindling-induced changes in COX-2 may reflect aberrant synaptic circuitry

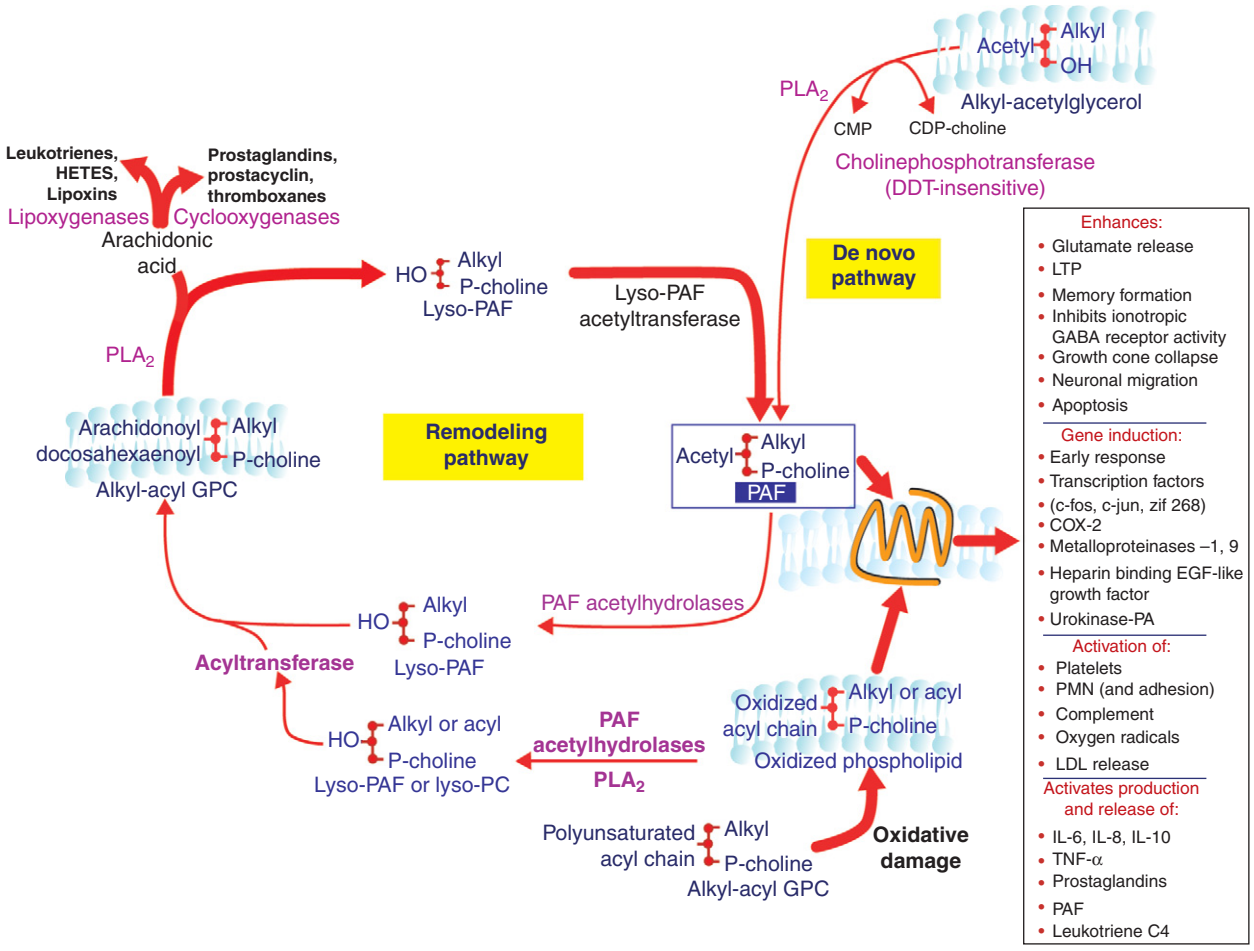


Figure 5 Seizures-stimulated PAF synthesis. In the center of this figure is the remodeling pathway or 'PAF cycle' from the membrane phospholipid PAF precursor, alkyl-acyl-glycerophosphorylcholine (GPC, on the left) to the biologically active PAF (at right). The remodeling route includes the production of lyso-PAF, which is generated from the PAF precursor alkyl-acyl-GPC either directly by the action of phospholipase A_2 or by the transfer of the *sn*-2 acyl moiety to a 'donor' lyso-plasmalogen (top), which is itself mobilized from membrane plasmalogen by phospholipase action. The de novo route of PAF synthesis (upper right) involves the direct transfer of a choline moiety to alkyl-acetylglycerol. Note that PAF acetylhydrolase (bottom) inactivates all PAF molecules, regardless of their biosynthetic route, and additionally inactivates oxidatively damaged phospholipids (shorter peroxidated acyl group at C2) that possess biological activity at the PAF receptor. Seizure activated steps are depicted with ticker red arrows. Modified from Bazan NG (2003) Synaptic lipid signaling: significance of polyunsaturated fatty acids and platelet-activating factor. *Journal of Lipid Research* 44: 2221–2233, with permission from ASBMB.

rearrangements that enhance proinflammatory signaling that, in turn, may foster neuronal excitability.

The COX-2 gene induction in neocortical neurons coincides with intensified behavioral seizures, suggesting that COX-2 induction is an indicator for neuronal seizure activity during kindling. Unlike COX-2, detectable induction of $cPLA_2$ does not occur after a single stimulation; rather, during kindling, a pattern of induction similar to that of COX-2 is found. This observation suggests that endogenous $cPLA_2$ is sufficient to deal with the free AA requirements created by newly induced COX-2 as a result of a single stimulation. Sustained COX-2 induction triggered by kindling epileptogenesis may involve additional, newly induced $cPLA_2$ to generate more free AA. Thus, it appears that only sustained epileptic activity induces $cPLA_2$. Kainic acid injected into one side of the brain causes significantly higher COX-2 induction in ipsilateral cerebral cortex than in contralateral cortex, and is associated with local kainic acid toxicity. The involvement of COX-2 induction in astrocytes by kainic acid adds to the complexity of COX-2 induction in the kainate model, introducing effects of activity in non-neuronal cell types.

Kindling induces brain COX-2 and $cPLA_2$, suggesting that released AA is converted into eicosanoids during epileptogenesis. The COX-2 inhibitor, nimesulide, attenuates kindling, supporting the hypothesis that COX-2 activity is involved in kindling epileptogenesis. Nimesulide fails to fully inhibit kindling development, perhaps because of incomplete access of this drug to the COX-2 protein to achieve full inhibition, and/or because of the 'redundancy'

of other signaling pathways with the COX-2 system. For example, COX-1, which is not inhibited by nimesulide, may synthesize an increased amount of prostaglandins (because of accumulation of AA) as a result of COX-2 inhibition, thereby minimizing the effect of nimesulide. The mechanism through which COX-2 inhibition slows kindling progress is not clear. However, it is likely that COX-2 inhibition may reduce synthesis of PGs and/or PAF (platelet-activating factor). Both lipid mediators may involve synaptic facilitation. Studies of other COX-2 inhibitors and related compounds should provide further insight into the significance of the COX-2 pathway in experimental epilepsy. In summary, COX-2 induction takes place during kindling epileptogenesis and this induction is restricted to neurons. Moreover, kindling induces $cPLA_2$, and a COX-2 inhibitor attenuates kindling development. The spread of kindling-induced COX-2 induction from hippocampal neurons to neocortical neurons, and the attenuation of kindling by COX-2 inhibition, suggest that kindling permanently facilitates hippocampus–neocortex functional connectivity and that COX-2 is involved in epileptogenesis.

Future Goals

Future studies will define the significance of omega-6- and omega-3-derived mediators in the ictal and post-ictal period. In addition, since epileptogenesis is thought to involve, in many cases, an initial injury, another goal is to define the significance of NPD1 in the initiation and progression of epileptogenesis. Some omega-6-derived mediators (e.g., prostaglandins) may represent cell-damaging signaling events, whereas others (e.g., lipoxins) are proinflammatory. Therefore, a balance between mediators derived from essential fatty acids may play a critical role in hyperexcitability and seizures. Furthermore, studies are needed for defining the significance of COX-2 at the dendritic spines, and of COX-2 induced by kindling epileptogenesis (as described here). Elucidation of the modulators of COX-2 induction will contribute to our understanding of the role of this inducible gene (and its bioactive products, prostaglandins) in synaptic circuitry function. Finally, because a balance between excitatory and inhibitory synaptic transmission is likely involved in epileptogenesis, and since GABAergic neuron dropout often is seen in models of epileptogenesis, studies should examine the possibility that NPD1 is a promoter of cell survival and plays a neuroprotective role in epilepsy.

In summary, phospholipids of excitable membranes and of other nervous system cells, including astrocytes, are richly endowed in acyl groups containing AA and DHA. Seizures, including plasticity-induced seizures, activate the cleavage of these acyl groups and their release from phospholipids. These events represent switches that open signaling pathways. The details of these signaling pathways remain to be defined, as is the determination of their significance in clinically relevant models of epilepsy.

Acknowledgements

Supported by National Institutes of Health, National Center for Research Resources grant P20 RR016816 and the NIH, National Institute of Neurological Disorders and Stroke grant R01NS23002.

See also: **Cell Damage/Excitotoxicity**: Molecular Mechanisms of Cell Death After Seizures; **Epileptogenesis**: Role of Transporter Proteins and Lipid Peroxidation in Epileptogenesis; **Neurotransmitter Function**: Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Plasticity**: Inositol Lipid Signaling in Synaptic Activity, Neuronal Plasticity and Epileptogenesis.

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The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy

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Introduction

To date only a minority of epilepsy research has been focused on mitochondria. However, recent advances provide compelling evidence of mitochondrial involvement in the epilepsies. Direct mitochondrial dysfunction is associated with a small percentage of inherited epilepsies – e.g., mitochondrial encephalopathies. A role of mitochondria in acquired epilepsies, which account for ~60% of all epilepsy cases, remains to be fully explored. Temporal lobe epilepsy (TLE) is the most notable example of acquired epilepsy usually preceded by injury such as episode(s) of prolonged seizures or status epilepticus (SE), childhood febrile seizures, hypoxia, or trauma. These initial insults are thought to set in motion complex molecular, biochemical, and structural changes that culminate in the development of spontaneous recurring seizures, or 'epileptogenesis.' Since injury per se can set in motion molecular events that trigger the epileptic condition, it is important to understand the biochemical causes and consequences of epileptic seizures. Identifying these biochemical mechanisms may lead to therapies that interrupt the process of epileptogenesis.

Background

Several major features of the epileptic phenotype suggest that mitochondrial involvement may not be a mere epiphenomenon, but rather a key event contributing to the initiation and progression of TLE. Mitochondria are intimately involved in important cellular events that are leading candidate mechanisms of hyperexcitability and/or its consequences. These potential mechanisms include metabolic and bioenergetic changes associated with seizure activity, the involvement of mitochondria in the processes underlying seizure-induced cell loss (i.e., excitotoxicity and apoptosis), mitochondrial regulation of amino-acid cycling, neurotransmitter biosynthesis, and ionic homeostasis, particularly calcium buffering. Another important reason to consider mitochondrial mechanisms, particularly the involvement of oxidative stress in epilepsy, is the knowledge that the incidence of epilepsy abruptly rises with advancing age; mitochondrial oxidative stress is a leading mechanism of aging and age-related degenerative disease.

The most compelling evidence for mitochondrial (dys) function in epilepsy is the recognition that dramatic metabolic and bioenergetic changes occur following acute seizures and during different phases of chronic epilepsy (**Figure 1**). The acute consequence of seizures associated with SE is an increase in cellular glucose uptake and metabolism that is unparalleled by most conditions. Cerebral blood flow is increased to match this hypermetabolism; since the increased rate of glycolysis exceeds pyruvate utilization by pyruvate dehydrogenase, there is an increased lactate buildup. While *hyper*metabolism occurs in human epileptic foci during ictal phases (seizure episodes), *hypo*metabolism is prevalent during interictal phases (between seizure episodes). A bioenergetic defect is also evident in the measurement of ^{31}P and the loss of mitochondrial *N*-acetyl aspartate in human epileptic tissue, suggesting the involvement of mitochondria in altered neurotransmitter metabolism. Severe metabolic dysfunction characterized by biphasic abnormal NAD(P)H fluorescence transients and changes in mitochondrial membrane potential (dsm) have been observed in ex vivo preparations from both chronically epileptic rats and human subjects. The mechanisms of both hypometabolism and hypermetabolism in seizure-induced brain damage, and in subsequent development of epilepsy, need to be determined.

Methodological Approaches and Recent Results

Oxidative Stress and Mitochondrial Dysfunction: Acute Consequences of Epileptic Seizures

ROS and reactive nitrogen species (RNS) are thought to play important roles in diverse nervous system disorders

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Glucose Metabolism and GABA$_A$ Receptor Dysfunction in Human Partial Epilepsy

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Introduction

Phosphorylation of synaptic receptors modulates several neurotransmission systems in the vertebrate central nervous system. It has been shown, in particular, that glutamate and γ-aminobutyric acid (GABA) ionotropic receptors can be phosphorylated on the intracellular loops of several of their subunits by known kinases. These kinases primarily phosphorylate the β or γ subunits – but not the α subunits – of the type A GABA receptor (GABA$_A$R).

A different phosphorylation mechanism was recently described for this receptor, and termed 'endogenous,' since it does not require exogenous kinase or kinase activators. The endogenous kinase must then be closely related to the receptor at the neuronal membrane. This phosphorylation mechanism prevents rundown of the GABAergic response of acutely dissociated hippocampal or cortical neurons. In the paragraphs below, we will briefly describe the characteristics of this mechanism and then show that this mechanism is deficient in the cortical zones epileptic seizures originate in patients with partial epilepsy.

Background

A New Mechanism: Glycolysis-Dependent Modulation of GABAergic Inhibition

The first step was to identify the GABA$_A$R endogenous kinase. The protein that was isolated after purification and phosphorylation tests of the receptor was not a known kinase, but rather a key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A kinase activity of GAPDH, independent of its role as a dehydrogenase in glycolysis, has been described in rabbit muscles, where it was shown that GAPDH autophosphorylates using glycolytic ATP and then transfers the phosphate group to other muscle proteins – thus playing the role of a kinase. We showed that GAPDH is physically associated with the receptor and is partially colocalized with GABA$_A$R α1 subunits at the plasma membrane of neurons in culture.

We also showed that local synthesis of ATP occurred in washed cerebral cortex membranes when glyceraldehyde-3P (G3P), the specific glycolytic substrate of GAPDH, was supplied. These results indicate that the enzyme that follows GAPDH in the chain of glycolytic reactions, the phosphoglycerate kinase, is also present at the neuronal membrane and that it uses the product of the reaction catalyzed by the GAPDH to produce glycolytic ATP, very likely without diffusion. The ATP is then used for GAPDH autophosphorylation, probably also without diffusion. Next, the phospho-GAPDH phosphorylates the associated GABA$_A$R on the I2 intracellular loop of its α1 subunit, at two identified serine and threonine residues. The endogenous phosphorylation of the GABA$_A$R is thus a glycolysis-dependent mechanism. These results establish a direct link between glucose metabolism and GABAergic neurotransmission (**Figure 1**).

Does GABA$_A$R endogenous phosphorylation have a functional role? We showed, on acutely dissociated pyramidal neurons from rat cortex, that any factor supporting GABA$_A$R endogenous phosphorylation retards rundown of GABA$_A$ currents in whole-cell patch-clamp recordings, and that any factor preventing GABA$_A$R endogenous phosphorylation reinforces rundown. The addition of G3P to the

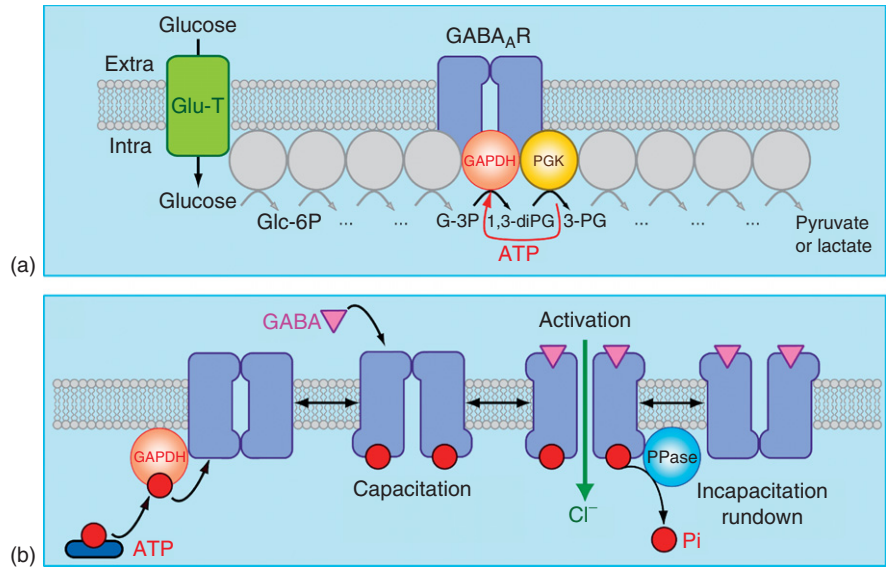


Figure 1 Glycolysis-dependent phosphorylation of $GABA_A$ receptor on the α1 subunit. The maintenance of the $GABA_AR$ in a functional state is ensured by a kinase we have identified as being a key glycolytic enzyme, glyceraldehyde-3P dehydrogenase (GAPDH), closely associated to the receptor at the plasma membrane. GAPDH fulfills successively the two following roles. (a) Glycolytic role: Once taken up by the neuron, glucose enters the glycolytic pathway whose enzymes may chain to each other for a greater efficiency. During the reaction catalysed by GAPDH, inorganic phosphate (Pi) is incorporated into the glycolytic product. At the next step, catalyzed by phosphoglycerate kinase (PGK), an ATP production occurs at the membrane. This ATP is channeled back to GAPDH allowing a supplementary role for this enzyme as the $GABA_AR$ kinase. Glu-T: glucose transporter; Glc-6P: glucose-6P; G-3P: glyceraldehyde-3P; 1,3-diPG: 1,3-diphosphoglycerate; 3-PG: 3-phosphoglycerate. (b) Endogenous kinase role: GAPDH uses the glycolytic ATP for autophosphorylation. This phospho-enzyme transfers two phosphates to the long intracytoplasmic loop of the α1 subunit of the $GABA_AR$, on identified threonine and serine residues. Such phosphorylation induces receptor 'capacitation' by allowing opening of the associated channel when GABA binds on its extracellular sites. This α1-phosphorylation is counteracted by an atypical membrane-bound phosphatase, leading to 'incapacitation' of the receptor and resulting in rundown of $GABA_A$ responses. Fast inhibition is thus fuelled by glucose consumption.

intracellular medium thus maintains the GABAergic responses over time, but a further addition of iodoacetamide – which inactivates GAPDH – rapidly induces a rundown as large as that observed in the absence of the glycolytic substrate. The endogenous glycolytic ATP produced at the neuronal plasma membrane is highly preferred to the exogenous ATP present in the patch-pipette solution.

In addition, an atypical membrane-bound phosphatase dephosphorylates the $GABA_AR$ α1 subunits and opposes endogenous phosphorylation. Addition to the intracellular medium of a phosphatase, broad-range inhibitor (orthovanadate) prevents rundown of GABAergic responses by maintaining the receptor in its phosphorylated state. Under these conditions, further addition of iodoacetamide does not alter the time course of $GABA_A$ responses. We propose that glycolysis-dependent phosphorylation maintains the $GABA_A$ currents, and that an atypical membrane phosphatase opposing the endogenous phosphorylation accounts for rundown of GABAergic currents.

Methods

Patient Populations

The study described below was performed on cortical nervous tissue excised from 50 patients (32 men and 18 women) suffering from temporal or frontal pharmacoresistant epilepsy, who underwent curative neurosurgery in the Epilepsy Centers of Sainte-Anne (Paris, France) and Pontchaillou (Rennes, France). Informed consent was given by all patients, according to the guidelines of the institutional committees. Details of the presurgical and surgical procedures have been described elsewhere (see Further Reading). Most of our samples were taken from the temporal pole of patients with epilepsy. In eleven cases, we obtained cortical tissue from nonepileptic patients, in which resection of extralesional tissue was dictated by the technical imperatives of the surgical approach to a tumor. These samples were considered as 'control' tissue.

Biochemical Studies

Washed membranes were prepared from epileptogenic and control cortex tissue excised from patients undergoing neurosurgery. Endogenous receptor phosphorylation of $GABA_AR$ was performed in presence of Mg^{2+} and $\gamma[^{33}P]$-ATP in a buffered medium at pH 7.3 (kinase or activator not added) and measured by phosphoimaging on protein electrophoresis gels (PAGE). This reaction is catalyzed by the glycolytic enzyme GAPDH, and labeling of the $GABA_AR$ α1 subunit predominates (as previously

demonstrated). The receptor endogenous phosphorylation was compared to the expression of α1 subunits quantified at the protein level by photoaffinity labeling with [^{3}H]flunitrazepam.

Functional Approach

Rundown of GABA$_A$ currents is prevented by endogenous phosphorylation. We have shown that the endogenous kinase is a glycolytic enzyme (GAPDH), and therefore we investigated the role of glycolytic factors on the rundown of GABA$_A$ responses. Whole-cell currents were recorded by repeated (1 s every 3 min) GABA (100 μM) applications on acutely dissociated pyramidal neurons from human epileptogenic and control cortex. The effects of glycolytic factors added in the pipette milieu on rundown of GABA$_A$ responses were compared with the effects obtained under standard conditions.

Recent Results

Deficiency in Endogenous Phosphorylation of GABA$_A$R in Human Epilepsy

Many reports have suggested that a deficiency of GABAergic neurotransmission may be instrumental in the triggering or the propagation of seizures in patients with epilepsy. However, our own results, as well as those of several other teams, have shown that GABAergic inhibition is functional in the nervous tissue excised (for therapeutic reasons) from the cortical zones where seizures originated in patients with epilepsy. Is there a mechanism for rapidly – and transiently – decreasing the effectiveness of inhibitory neurotransmission in these cortical zones? A mechanism involving phosphorylation-dephosphorylation cycles would have these characteristics.

We hypothesized that GABA$_A$R endogenous phosphorylation could be defective in the cortical zones where seizures originate or which are very quickly invaded by epileptic discharges. The results of our study supported this hypothesis: the phosphorylation of GABA$_A$Rs by GAPDH was significantly reduced in washed cortical membranes obtained from epileptogenic tissue than from control tissue (**Figure 2(a)**). This alteration did not result from a reduction in the expression of the α1 subunits, as shown by photoaffinity labeling with the benzodiazepine [^{3}H]flunitrazepam, but was due to an intrinsic deficiency of the endogenous phosphorylation. Indeed, immunochemical labeling showed that the expressions of α1, α2, β2/3, and γ2 GABA$_A$R subunits remained unchanged, while that of α3 subunit was decreased in the neocortex of temporal lobe epilepsy patients.

The next step was to test whether the GABA$_A$R function, as reflected in the rundown of responses recorded using the whole-cell patch-clamp technique, was altered in acutely dissociated pyramidal neurons obtained from epileptogenic cortical tissue. Rundown of the GABAergic responses was significantly faster and greater when the recordings were carried out on cortical neurons isolated from epileptogenic samples compared with the responses of neurons obtained

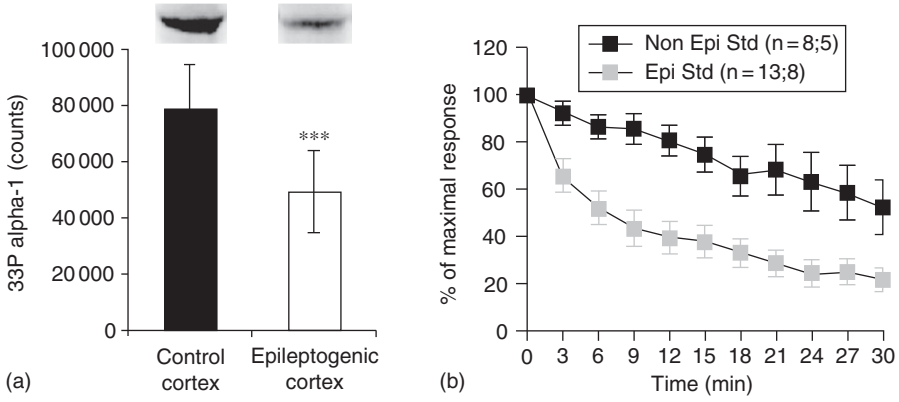


Figure 2 Endogenous phosphorylation of GABA$_A$R and GABA$_A$ currents in epileptogenic cortical tissue from patients with epilepsy and a control group. (a) Endogenous phosphorylation of GABA$_A$R α1 subunit in washed membranes prepared from epileptogenic and control tissue. *Upper part:* Autoradiography of the polyacrylamide gel after endogenous phosphorylation in a membrane preparation from tissue of a nonepileptic control patient and of an epileptic patient. The predominating ^{33}P-labeling at the 51-kDa band corresponding to the GABA$_A$R α1 subunit was clearly decreased in the tissue from epileptic patients. *Lower part:* Quantification of the ^{33}P-phosphate incorporation (in cumulated counts) into the α1 subunit of GABA$_A$R by endogenous phosphorylation of control nonepileptogenic cortex (n = 5 patients) and epileptogenic cortex (n = 23 patients); the error bars represent standard error of the mean (SEM); $P < 0.001$ for the Student's t-test (***). (b) GABA$_A$ currents measured by whole-cell patch clamp on acutely dissociated cortical neurons following GABA (100 μM) applications (for 1 s every 3 min). The responses progressively decreased (rundown) in standard condition (Std). Rundown of averaged normalized currents is compared in neurons from epileptogenic (Epi) and nonepileptogenic (NonEpi) cortex. For each curve, the parentheses indicate, respectively, the number of neurons and the number of patients; the error bars represent SEM. Modified from **Figs. 2** and **4** of Laschet JJ, Kurcewicz I, Minier F, *et al*. (2007) Dysfunction of GABA$_A$ receptor glycolysis-dependent modulation in human partial epilepsy. *Proceedings of the National Academy of Sciences USA* 104: 3472–3477.

from control tissue (Figure 2(b)). The rundown measured in the epileptogenic tissue could be reduced by adding to the pipette milieu either the glycolytic GAPDH substrate (G3P) or by inhibiting with orthovanadate, the membrane-bound phosphatase counteracting the α1-phosphorylation. Conversely, the protective effect of G3P against rundown was abolished by addition to the bath of iodoacetamide (500 μM), an irreversible inhibitor of GAPDH. The deficiency of endogenous phosphorylation thus induced considerable functional lability of the GABAergic currents. Interestingly, in the neurons dissociated from human tissue, the locally-produced glycolytic ATP (in small amounts) was very largely preferred to the ambient ATP (in large amounts) present in the patch-pipette solution.

We propose that, in the patients with partial epilepsies, any fortuitous increase in metabolic demand can induce a transient reduction in the effectiveness of inhibitory neurotransmission because of the decreased effectiveness of glycolysis-dependent phosphorylation; when a critical reduction of glycolysis-dependent phosphorylation is reached, there is a switch in the pathological cortical zone to an 'epileptic' state.

Correlations with Clinical Data

Considering the different clinical profiles in the epileptic patient group, the homogeneity of the GABAergic functional deficit is noteworthy. When the biochemical and functional results are compared with the clinical data, a few interesting observations can be made. There is a significant positive influence of age at surgery on GABAergic function, corroborated by a tendency for improvement of α1-phosphorylation. Other clinical parameters did not significantly interact with the level of deficiency. Concerning the functional GABAergic recovery by the addition G3P or orthovanadate, epilepsy duration had a positive influence only on the effect of G3P. This G3P-specific relationship may be accounted for by a gradual increase in the efficacy of energy metabolic adaptation with epilepsy duration, since a more efficient use of the glycolytic substrate may progressively occur to sustain $GABA_A$ function in long-term epilepsies.

GABAergic Deficiency and Interictal Hypometabolism

^{18}Fluoro-2-deoxyglucose positron emission tomography studies (FDG-PET) have shown that glucose utilization is decreased 10–20% during the interictal periods in the pathological cortical zones in patients with partial epilepsies. It has been suggested that hypometabolism is related to ictal discharge generation and propagation pathways. However, the relationship between this metabolic alteration and the epileptic pathology has never been fully explained. Recent reviews point out the very high energy cost related to the recycling of amino acid neurotransmitters. We propose that, in the epileptogenic tissue from patients with epilepsy, interictal hypometabolism is related to decreased effectiveness of inhibitory transmission, secondary to the deficiency of $GABA_AR$ endogenous phosphorylation. In the majority of our patients, FDG-PET studies showed that the affected zones were indeed in a hypometabolic state. A direct relationship can thus be postulated between the deficiency of glycolysis-dependent α1-phosphorylation shown in vitro and the interictal hypometabolism observed in vivo in patients with partial epilepsy.

It is doubtful that the decrease in $GABA_AR$ α1-phosphorylation per se contributes significantly to the decrease in glucose consumption. However, decreased glucose uptake should be expected as a functional consequence of the observed α1-phosphorylation deficiency on postsynaptic inhibition, if the activity of inhibitory synapses is energy consuming. It has been shown that specific activations of GABAergic inhibitory circuitry result in a marked increase in glucose consumption, as evaluated by ^{14}C-deoxyglucose uptake autoradiography (both in hippocampal slices and in the intact central auditory system). In further support of this hypothesis, FDG-PET studies have revealed that administration of a $GABA_A$ agonist increases cerebral glucose metabolism by >17% in healthy volunteers and even more in epileptic patients (by ~25% at the cortical zones where seizures originate). This GABAergic metabolic effect is of the same order of magnitude as that of the hypometabolism measured in the temporal pole of patients with partial epilepsies (as compared with healthy volunteers). It would be useful to quantify the varying degrees of hypometabolism in these patients, to determine if the degree of hypometabolism correlates with the observed GABAergic deficiencies. Unfortunately, normalization of the data in our study was not suitable for such comparisons, due to FDG-PET acquisition procedures and variability of individual hypometabolic values.

Another metabolic dysfunction, affecting mitochondrial enzymes, has been disclosed in the hippocampus of patients with epilepsy. This dysfunction may also contribute to interictal hypometabolism. An additional primary deficiency in energy metabolism would further affect $GABA_AR$ α1-phosphorylation if the glycolysis-dependent ATP production falls locally below a critical level. However, as already mentioned, the endogenous glycolytic ATP produced at the neuronal plasma membrane from glycolytic substrates is largely preferred to the ambient ATP. We propose that the deficiency of the glycolysis-dependent modulation of GABAergic inhibition can at least partially account for the interictal glucose hypometabolism. Because the α1-phosphorylation level is determined by the opposing actions of a kinase and a phosphatase, equilibrium may be reached at a lower level of α1-phosphorylation when the activity rate of GAPDH (as a kinase) is decreased or when the glycolytic

ATP is less available. The reduction in the effectiveness of endogenous phosphorylation of the $GABA_AR$ in the epileptogenic tissue should reduce the energy costs of inhibition and thus glucose consumption. It is worth noting that this view contrasts with the common opinion that an increase in the effectiveness of GABAergic inhibition produces a glucose hypometabolism by reduction of electrical neuronal activity.

Genetics of $GABA_AR$ α1 Subunit Related to Human Epilepsy

Mutation in the $GABA_A$ receptor subunit genes have been linked to several inherited forms of epilepsies. The α1 subtype is the most widely expressed of the α subunits, with expression increasing throughout development. A single mutation in the $GABA_AR$ α1 subunit was identified in a family with juvenile myoclonic epilepsy (JME), in which an alanine in the third transmembrane domain (TM3) is replaced by an aspartate (A322D). The corresponding mutation in the rat α1 subunit (A294D), expressed in HEK293 cells, reduced GABA sensitivity of the receptor, increased the deactivation rate, and slowed desensitization. However, this mutation does not concern the α1 subunit phosphorylation sites on Ser and Thr residues within the long intracytoplasmic loop (between TM3 and TM4), and therefore the mechanisms underlying this α1-related genetic disorder seem to be different from the that of $GABA_AR$ endogenous phosphorylation involved in intractable partial epilepsy.

Future Goals

Glycolysis-dependent $GABA_AR$ phosphorylation and GABAergic inhibition are deficient in cortical tissue excised from patients suffering from drug-resistant partial epilepsies. These observations have led to our hypothesized direct link between interictal hypometabolism (observed in and around the epileptogenic zones in these patients) and the epileptic pathology. Imaging, NMR spectroscopy, and modeling studies are needed to make clear such metabolic relationships.

In our study, enhancing the $GABA_AR$ endogenous phosphorylation, either by favoring the glycolytic role of GAPDH or by inhibiting receptor dephosphorylation, compensated for the GABAergic functional deficiency. This observation opens up new and promising prospects for the development of antiepileptic molecules active in drug-resistant epilepsies, which affect nearly 25% of patients with epilepsy. Two approaches should certainly be considered: (1) activating the kinase activity of GAPDH; (2) inhibiting the atypical membrane-bound phosphatase. The first approach may certainly give rise to severe side-effects, given the ubiquitous metabolic role of GAPDH. The second approach, however, appears more clinically relevant. We are presently trying to identify the membrane-bound phosphatase(s) that counteracts the $GABA_AR$ glycolysis-dependent phosphorylation. A further important step will be to design and validate specific inhibitors of the involved protein phosphatase(s), as a novel approach to developing new antiepileptic drugs.

See also: **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain; Synaptic Activities and Transmitter Function: Receptor Trafficking; **Non-Synaptic Mechanisms**: Ionic Microenvironment in Living Human Brain and Epileptic Seizures; Role of Gap Junctions in Epileptic Synchronizations; **Transporters**: GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability.

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Role of Metabolic Substrates and Acid-Base Balance in Seizures

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Introduction

As glucose is the main source of energy for neurons in the CNS, disturbances in glucose metabolism are often associated with alterations of brain function. Indeed, seizures are a common complication of diabetes mellitus, associated with either hyperglycemia or hypoglycemia. Mechanisms involved in hypoglycemic seizures are especially intriguing because low glucose levels lead to general neuronal silencing, and EEG recordings show generalized slowing of background activity with prevailing theta rhythm. Similarly in laboratory animals, insulin-induced hypoglycemia leads to generalized seizures. During severe hypoglycemia in these animals, EEG recordings (from cortex or hippocampus) prior to seizures show slowing of activity resulting in an isoelectric state – from which the seizure activity abruptly arises. As seizures start as a result of synchronized neuronal firing, it seems curious that hypoglycemic seizures appear to occur under conditions of general synaptic silencing.

Similarly, changes in acid–base balance have significant effects on neuronal excitability and seizure susceptibility. Hyperventilation leading to respiratory alkalosis is one of the diagnostic tools to evoke seizure in epileptic patients during the EEG exam. In contrast, certain antiepileptic drugs (such as acetazolamide) alter the acid–base balance of the body by inhibiting carbonic anhydrase. Accordingly, the mice deficient in carbonic anhydrase isozyme II have reduced seizure susceptibility compared with their normal littermates. Finally, moderate acidosis induced by ketogenic diet is one of the proposed anticonvulsant mechanisms of the ketogenic diet.

Background

Disturbances in Glucose Metabolism and Neuronal Excitability

Hypoglycemia is often associated with antidiabetic treatment (especially in children and during adolescence) or with other conditions such as insulinoma as well as alcoholism. Fasting or vigorous exercise seem to be predisposing factors for seizure expression during hypoglycemic crisis.

Glucose sensing in the brain

Due to the high demand of neurons for a glucose supply, the brain contains specialized 'glucosensing' neurons to regulate glucose homeostasis. These neurons change their firing rates in response to changes in glucose levels and based on this feature, two distinct populations of glucosensing neurons can be recognized: (1) Neurons that decrease their firing rates during hypoglycemia and increase their firing rates when glucose levels rise. Such neurons are called glucose responsive. (2) Neurons that increase their firing rates during hypoglycemia and decrease their firing rates when glucose levels rise. This type of neurons is called glucose sensitive.

The firing rate of neurons under metabolic stresses is regulated primarily by changes in activity of ATP/ADP-sensitive inward rectifying potassium (K_{ATP}) channels. These channels translate the metabolic state into cellular excitability. They are composed of four regulatory subunits (SUR) and four pore-forming subunits (Kir). The channel inhibition (closing) is mediated by binding of sulfonylureas or ATP to the regulatory subunit. Diazoxide or ADP binding results in the channel opening.

In the glucose responsive neurons, glucose uptake leads to an increase of the ATP/ADP ratio and K_{ATP} channels are inactivated (closed) – resulting in accumulation of intracellular K^+, membrane depolarization, and increased cell firing rate. During hypoglycemia (or other metabolic stresses such as hypoxia), the fall in the ATP/ADP ratio activates (opens) the channel and firing rates decrease due to membrane hyperpolarization. This cell hyperpolarization effect has been proposed as a neuroprotective mechanism during metabolic stress. However, it may raise the risk for global neuronal silencing and lead

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Glucose sensing in the brain

Due to the high demand of neurons for a glucose supply, the brain contains specialized 'glucosensing' neurons to regulate glucose homeostasis. These neurons change their firing rates in response to changes in glucose levels and based on this feature, two distinct populations of glucosensing neurons can be recognized: (1) Neurons that decrease their firing rates during hypoglycemia and increase their firing rates when glucose levels rise. Such neurons are called glucose responsive. (2) Neurons that increase their firing rates during hypoglycemia and decrease their firing rates when glucose levels rise. This type of neurons is called glucose sensitive.

The firing rate of neurons under metabolic stresses is regulated primarily by changes in activity of ATP/ADP-sensitive inward rectifying potassium (K_{ATP}) channels. These channels translate the metabolic state into cellular excitability. They are composed of four regulatory subunits (SUR) and four pore-forming subunits (Kir). The channel inhibition (closing) is mediated by binding of sulfonylureas or ATP to the regulatory subunit. Diazoxide or ADP binding results in the channel opening.

In the glucose responsive neurons, glucose uptake leads to an increase of the ATP/ADP ratio and K_{ATP} channels are inactivated (closed) – resulting in accumulation of intracellular K^+, membrane depolarization, and increased cell firing rate. During hypoglycemia (or other metabolic stresses such as hypoxia), the fall in the ATP/ADP ratio activates (opens) the channel and firing rates decrease due to membrane hyperpolarization. This cell hyperpolarization effect has been proposed as a neuroprotective mechanism during metabolic stress. However, it may raise the risk for global neuronal silencing and lead

to a comatose state. To reduce the rapid decline in general excitability, in some neurons the K_{ATP} channels are downregulated – as an apparent compensatory mechanism – during metabolic stress. The mechanism underlying increased firing of glucose sensitive neurons during a hypoglycemic state is not clear, but selective downregulation of the K_{ATP} channels may partially explain their somewhat paradoxical increase in firing.

Glucosensing neurons are abundant in the hypothalamus, midbrain (especially the substantia nigra), pons, and medulla.

The substantia nigra is a candidate structure involved in the hypoglycemic seizures

Our studies show that the SNR seems to be involved in hypoglycemic seizures. We found increased activity of the SNR neurons during hypoglycemic seizures. That this firing rate change may not be just 'coincidental' is suggested by the facts that: (1) The SNR plays an important role in control of generalized seizures and (2) The SNR GABAergic cells are glucosensing neurons.

1. The SNR plays an important role in seizure control: The SNR has been identified as a critical midbrain structure involved in motor control and modulation of seizures, mediated through regulation of GABAergic neurotransmission. The SNR contains mainly GABAergic neurons with high spontaneous firing rates. These neurons receive inputs from striatum directly, and also indirectly (via globus pallidus and subthalamic nucleus). The direct pathway provides GABA-mediated inhibitory effects on SNR neurons; GABA release at the striatonigral terminals or exogenous application of GABA decreases SNR neuron firing rate. The indirect pathway is excitatory, involving glutamatergic inputs from the subthalamic nucleus.

Seizures can be exacerbated when the activity of the SNR GABAergic neurons is increased. Such increase occurs when inhibitory effects of GABA on SNR neurons are decreased, or when glutamatergic excitatory drive on the SNR neurons is increased. Indeed, localized SNR microinfusions of bicuculline (a GABAA receptor antagonist) or kainic acid (AMPA/kainate receptor agonist) in the SNR have proconvulsant effects. In contrast, treatments that decrease the activity of the SNR GABAergic cells (especially in the SNR anterior) produce anticonvulsant effects. This relationship has been directly demonstrated using localized bilateral microinfusions of GABA agonists such as γ-vinyl-GABA (an irreversible GABA transaminase inhibitor that increases local GABA levels) or muscimol (a $GABA_A$ receptor agonist) into the SNR. Similarly, treatments that decrease glutamatergic neurotransmission in the SNR (infusions of AP7; an NMDA receptor antagonist) have anticonvulsant effects.

Under normal conditions, GABA inhibits the SNR neurons and decreases their high frequency of spontaneous firing, especially via activation of $GABA_A$ receptors. Thus, seizures induced by different chemoconvulsant agents under normoglycemic conditions can be attenuated by intranigral microinfusions of different $GABA_A$ receptor agonists or drugs increasing the GABA availability. However, in the case of hypoglycemic seizures, muscimol is ineffective in reducing SNR discharge rate. This failure of muscimol to prevent glucopenic seizures could be related to $GABA_A$ receptor internalization and thus to the unavailability of these receptors under conditions of metabolic stress. In contrast, intranigral microinfusions of AP7 prevents the occurrence of hypoglycemic seizures (**Figure 1**).

2. The SNR neurons are glucosensing: Studies using extracellular recordings in in vitro slices from mice showed that with decreasing glucose concentration in the artificial cerebrospinal fluid (aCSF), the SNR GABAergic neurons increased their firing rates. The SNR contains a very high density of K_{ATP} channels. The molecular structure of these SNR channels within the GABAergic neurons consists of SUR1 (high affinity receptor for sulfonylurea and ATP binding cassette superfamily) and Kir6.2 (inwardly rectifying channel operating subunit). This subunit composition endows these channels with high sensitivity to metabolic changes. The subcellular distribution of pre- or postsynaptic K_{ATP} channels is not known, but some evidence suggests that K_{ATP} channels within the SNR may be present both pre- and postsynaptically. Under

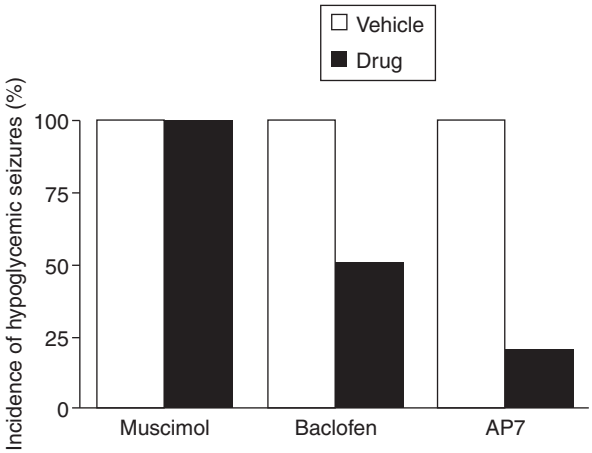


Figure 1 Incidence of hypoglycemia-induced seizures (barrel rotations) following intranigral administration of GABAergic or glutamatergic agents. The drugs or their appropriate vehicle were infused bilaterally into the SNR in a volume of 0.25 µl per side just prior to insulin injection (30 IU kg^{-1} i.p.): Muscimol (0.1 µg), baclofen (0.2 µg), AP7 (1.1 µg). Incidence of barrel rotations has been monitored. Intranigral muscimol did not affect the incidence of hypoglycemic seizures. On the other hand, both baclofen and AP7 significantly decreased the incidence of hypoglycemic seizures. These results suggest that glutamate plays an important role in the initiation of hypoglycemic seizures. The anticonvulsant effect of baclofen can be explained by its action at the presynaptic GABAB heteroreceptors located at the glutamatergic terminals originating from the subthalamic nucleus and thus suppresses glutamate release in the SNR.

metabolic stress, the SNR GABAergic neurons increase their firing rate (unlike the majority of neurons in the CNS which decrease their firing). However, the mechanism of this paradoxical effect is not well established.

Disturbances in pH Control and Neuronal Excitability

Systemic pH control

In the blood, there are four buffering systems for pH: bicarbonate, hemoglobin, protein, and phosphate. However, the buffering capacity of the latter two is small. The most important contribution to pH control in the extracellular environment belongs to the bicarbonate system, consisting of carbon dioxide (CO_2), carbonic acid (H_2CO_3), and bicarbonate (HCO_3^-). Intracellularly, proteins and phosphates are the most important buffers. The intracellular and extracellular compartments communicate with each other, using protons, bicarbonate, and oxygen modulate pH across the membrane. The capacity of the CO_2–H_2CO_3–HCO_3^- buffering system is very large because CO_2 is easily released to the atmosphere via lungs. Additional exchange with the environment is achieved via kidney acidification (H^+ excretion) of urine and, in rare cases, via gastric secretion of H^+.

Role of carbonic anhydrase

In many tissues, the interchange of pH constituents and buffers is accelerated enzymatically by carbonic anhydrase, which hydrates CO_2 to H_2CO_3. H_2CO_3 is in balance (via free dissociation) with a bicarbonate ion (H^+ and HCO_3^-). The carbonic anhydrase family consists of 12 active isozymes in humans and 13 in most other mammals. Carbonic anhydrase plays a significant role in the bicarbonate buffer system, in the proton excretion in urine, in acidification of the stomach content, and in pancreatic bicarbonate secretion, as well as in deposition of calcium in bones. There is also carbonic anhydrase in the nervous system. The principal carbonic anhydrase isozyme found intracellularly (in astroglia, oligodendrocytes, and choroids plexus is the isozyme II). In addition, CNS contains membrane-bound isozymes IV and XIV, and neuronal isozyme VII. In the CNS, carbonic anhydrase isozymes participate in fast buffering of pH transients that occur during neuronal activity.

Carbonic anhydrase inhibitors as anticonvulsants

Acetazolamide, an inhibitor of carbonic anhydrase, has anticonvulsant properties and has been approved for the treatment of epilepsy since 1953. Among newer antiepileptic drugs, anticonvulsant effects of zonisamide may be linked to its inhibitory effects on carbonic anhydrase, and topiramate has weak inhibitory effects on carbonic anhydrase activity (which, however, seems to be unrelated to its anticonvulsant effects).

Methods

Hypoglycemia-Related Experiments

P21 Sprague Dawley rats from Taconic farms have been used. Rats were sacrificed under CO_2 anesthesia, brain quickly removed from the skull, and sagittal slices (400 μm thick) containing the middle part of the SNR were cut on a vibratome in ice-cold aCSF. The aCSF composition was (in mM): 210 sucrose, 3.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO, and 10 D-glucose. Slices were allowed to recover for at least 1 h at room temperature in aCSF containing (in mM): 124 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26 $NaHCO_3$, 2 $CaCl_2$, 1.3 $MgSO_4$, and 20 glucose. During experiments, we manipulated glucose concentrations from 20 to 2 mM and added mannitol to maintain osmolarity when glucose was lowered. All solutions were saturated with 95% O_2/5% CO_2 gas mixture. Slices were transferred to the recording chamber and perfused with oxygenated aCSF at a rate of 4 ml min^{-1}. Recordings were performed at room temperature (22 °C). The pipette filling solution contained (in mM): 140 KCl, 6 NaCl, 2 $MgCl_2$, 10 HEPES, 2 Mg-ATP and the pH was adjusted to 7.3 with KOH. Whole-cell patch clamp recordings were made from electrophysiologically identified GABAergic neurons (lack of sag after negative current injection, biphasic afterhyperpolarization, and fast spiking). After recording firing frequency during a 5-min baseline period in 20 mM glucose aCSF, we switched to aCSF containing 2 mM glucose. Slices were bathed for 10 min in this glucose concentration, and firing frequency was measured in the last 10 s of each minute.

pH-Related Experiments

Carbonic anhydrase II-deficient mice

We used adult mice, in which a null mutation of the Car^{2n} locus was produced using nitrosourea. The homozygotes Car^{2n}/Car^{2n} have an absolute deficit of carbonic anhydrase II (CAII) in all tissues, including brain. These mice suffer from systemic acidosis and decreased bicarbonate levels. The mice were studied in three experiments:

1. *Susceptibility to flurothyl-induced seizures*: Flurothyl is a liquid convulsant which easily evaporates. When the gaseous form is inhaled, it produces clonic and tonic–clonic seizures. For seizure threshold testing, it is delivered to air-tight chamber using a constant rate pump (20 μl min^{-1}). The amount of flurothyl required to induce a particular seizure type can be calculated from the latency to onset of seizures.
2. Susceptibility to seizures induced by a single dose of systemically injected pentylenetetrazole. Pentylenetrazole is a convulsant drug acting on the TBPS site of the GABA receptor. After systemic injection (100 mg kg^{-1}, s.c.), it produces clonic and tonic–clonic convulsions,

sometimes with lethal ending. In this test, some of CAII-deficient mice and controls were pretreated with acetazolamide (100 mg kg^{-1}, i.p.) 30 min prior to pentylenetetrazole injection.

3. Susceptibility to audiogenic seizures, in response to a loud sound. Immature mice were 'primed' with a 5 min exposure to a 120 dB bell during a critical stage of auditory system development (postnatal day 19). They were retested – exposure to 1 min of 120 dB bell sound – at P45. In sensitized animals, this stimulus elicits tonic–clonic seizures, usually consisting of wild running, falling, a tonic phase, and then clonus of all four limbs.

pH changes in vitro

To further illustrate the effects of pH changes on seizure susceptibility, we modified pH in the extracellular environment of hippocampus–entorhinal cortex slices in vitro. Combined transversal hippocampus–entorhinal cortex slices were prepared from normal adult male rats. Epileptiform activity was induced by decreasing extracellular Mg^{2+}. This manipulation unblocks NMDA receptors and the seizure-like activity ensues. pH was changed in the extracellular environment by changing the composition of saturation gas (1.0–20.6% of CO_2, balance of oxygen, with resulting aCSF pH 7.8–6.7). For a control, we directly acidified the aCSF to pH = 6.7 using 10–12 mmol HCl. We investigated the interval between low Mg^{2+}-induced seizure-like events and their amplitudes in control aCSF, in altered pH, and after washout.

Results

Changes in Extracellular Glucose Levels Increase SNR GABAergic Neuron Firing

We evaluated the contribution of presynaptic transmitter release to hypoglycemia-induced changes in firing rate of the SNR GABAergic neurons. A decrease in extracellular glucose concentration resulted in an increase of action potential firing rate of the SNR GABAergic neurons (**Figure 2**). The increase in firing rate occurred with a delay of several minutes after the change in glucose concentration. The pipette we used to record from SNR neurons contained ATP, so that ATP presumably diffused into the impaled SNR GABAergic neurons. Thus, the K_{ATP} channels in these cells were inactivated (closed). We therefore interpreted the change in the firing rate of the SNR GABAergic neurons, under these hypoglycemic conditions, as a function of alterations in presynaptic transmitter release – i.e., as a result of an active role of the presynaptic K_{ATP} channels. Further studies, however, are needed to establish whether this effect is directly related to opening of the K_{ATP} channels at the presynaptic GABAergic terminals – leading to complete lack of GABAergic inhibition (including the tonic inhibition) on the spontaneously firing SNR GABAergic neurons, or due to an involvement of glutamate. Unlike GABAergic presynaptic terminals in the SNR, presynaptic glutamatergic terminals lack K_{ATP} channels, and thus glutamate release is not regulated by K_{ATP} channels. The possible involvement of these glutamatergic inputs in increasing SNR GABAergic neuron firing seems to be supported by in vivo data showing that bilateral intranigral microinjections of NMDA receptor antagonist AP7 significantly decreased the incidence of hypoglycemic seizures (**Figure 1**).

Seizure Susceptibility of CAII-Deficient Mice

In the flurothyl seizure model, seizures will eventually occur in all subjects. The latency to seizure is a measure of the subject's susceptibility – i.e., seizure threshold. CAII-deficient mice had an increased latency to both clonic and tonic–clonic flurothyl-induced seizures, by ~20%, compared to wild-type mice. Additionally, after tonic–clonic seizures, ~46% of wild-type mice died,

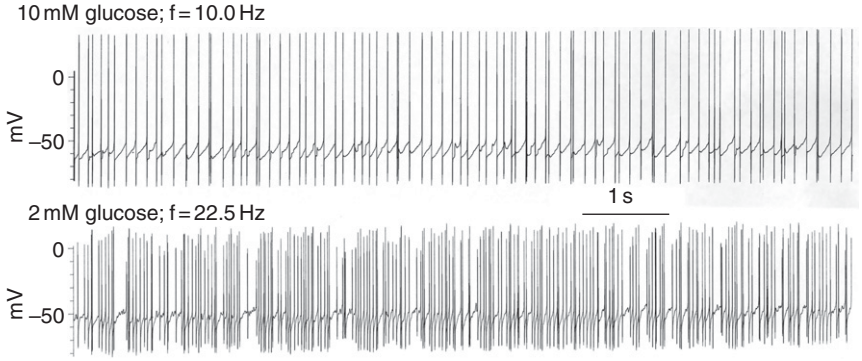


Figure 2 Whole-cell recording in a current clamp mode from an SNR GABAergic neuron. The basal average firing rate of this neuron in 10 mM glucose solution was ~10 Hz. Lowering glucose to 2 mM resulted in increase in average spontaneous firing of this neuron to 22.5 Hz.

but none of the CAII-deficient mice died. Clonic seizures induced by pentylenetetrazole developed significantly less frequently in CAII-deficient mice compared to wild-type mice (Figure 3). Additionally, pretreatment with acetazolamide in wild-type mice decreased incidence of seizures to the values found in CAII-deficient mice; pretreatment with acetazolamide did not further decrease occurrence of pentylenetetrazole seizures in CAII-deficient mice. Finally, when all mice were primed by loud sound during the critical developmental period, CAII-deficient mice experienced fewer loud sound-induced seizures in the adulthood compared to wild-type mice (Figure 3). Thus, in all seizure models, CAII-deficient mice displayed increased seizure resistance (compared to wild-type mice); this resistance was similar to the anticonvulsant effects of pretreatment with the carbonic anhydrase inhibitor acetazolamide. As the CAII-deficient mice suffer from systemic acidosis, the data suggest that systemic acidosis is associated with anticonvulsant effects.

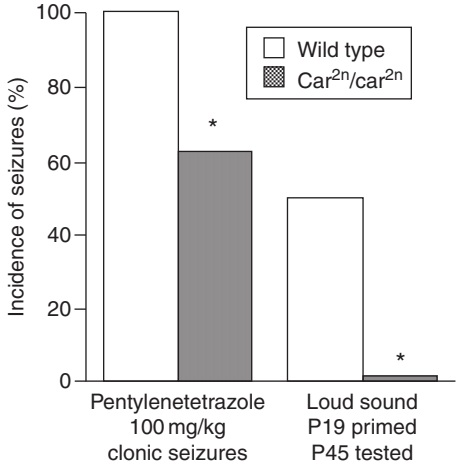


Figure 3 Incidence of clonic seizures induced by pentylenetetrazole (100 mg kg^{-1}, s.c.) and seizures evoked by loud sound after critical period priming in wild-type and Car^{2n}/Car^{2n} mice. Car^{2n}/Car^{2n} mutants demonstrated significant decrease in seizure incidence consistent with their carbonic anhydrase isozyme II deficiency. *Y*-axis: incidence in per cent. *Indicates significant difference ($P < 0.05$) compared to the wild type.

Effects of pH Changes on Seizure Susceptibility In Vitro

To test the effect of extracellular acidotic pH on epileptiform activity and seizures, we used an in vitro slice model. We induced epileptiform activity in the entorhinal cortex of combined hippocampus-entorhinal cortex slices using low Mg^{2+} and then applied aCSFs with different pH levels. When the aCSF was acidified to pH 6.7 (by either changing the oxygenation gases or by direct acidification), the interval between seizure-like events increased and the amplitude of events decreased. These effects were reversible (Table 1) and consistent with the anticonvulsant effects of extracellular acidosis, possibly working through NMDA receptor blockade.

Future Goals

The model of hypoglycemic seizures may not be relevant only to patients with metabolic disorders (diabetes mellitus or hyperinsulinism). Although it is known that a short epileptic seizure leads to increased glucose levels, a recent study showed that (in cerebral cortex) extracellular glucose levels significantly decrease in later stages of prolonged seizures. This study did not investigate the changes in extracellular glucose levels in other brain regions. The possibility exists that seizures can be maintained, and status epilepticus develops, if glucose levels decrease in structures normally responsible for seizure control or termination. This concept may have direct application to the SNR. Studies using the [^{14}C]-2-deoxyglucose (2DG) technique show that 2DG uptake is significantly increased in the SNR just prior to and during seizures – perhaps because blood

Table 1 Effects of extracellular acidification on the interval between and amplitude of low Mg^{2+}-induced seizure-like events in the medial entorhinal cortex in vitro

	pH = 6.7 achieved by pCO_2 = 20.6% in the ACSF			*pH = 6.7 achieved by direct acidification of the ACSF*		
Event parameter	Baseline pH = 7.3	pCO_2 = 20.6%, pH = 6.7	Wash pH = 7.3	Baseline pH = 7.3	pH = 6.7	Wash pH = 7.3
Interval (min)	2.59 ± 0.90	8.36 ± 2.35[a,b]	1.85 ± 0.62	5.20 ± 1.48	8.58 ± 1.28[a,b]	3.60 ± 1.07
Peak amplitude (mV)	1.92 ± 0.29	1.32 ± 0.19[a,b]	1.68 ± 0.24	1.40 ± 0.07	0.90 ± 0.04[a,b]	1.38 ± 0.14

[a]Significant difference compared to baseline ($P < 0.01$).
[b]Significant difference compared to wash ($P < 0.01$).
Interval denotes interval between individual seizure-like events. The longer the interval, the fewer seizure-like events occurred consistent with anticonvulsant effects. Decrease in the peak amplitude of the seizure-like event is also consistent with anticonvulsant effects.

flow is increased specifically in the SNR and is reflected by increased 2DG uptake. However, another explanation for the increased 2DG uptake is that there is decreased glucose availability under these conditions, which then favor 2DG uptake. Future studies should determine the details of glucose metabolism, its maintenance, and glucose transport during seizure activity.

Studies have demonstrated that extracellular pH may have multiple effects on neurons. First, proton excess inhibits NMDA receptors – perhaps the most important link between the acid–base balance and neuronal excitability. Thus, hyperventilation leads to respiratory alkalosis, which unblocks NMDA receptors and thus provokes seizures. However, there are many additional potentially pH-sensitive phenomena in the brain microenvironment, which need to be further studied. Brief acid–base transients developing during transmitter release from axonal terminals may affect the 'tone' of neurons and set the background excitability level for basic synaptic transmission. Glial cells, and especially astrocytes, significantly participate in the control of pH with their potent carbonic anhydrase and proton exchanger machinery, and therefore, pH changes may represent one means of glia-neuron communication. Extracellular pH is tightly coupled with pH maintained intracellularly; the effects of pH changes in either compartment will affect pH in the other compartment. These interactions, and their consequences for neuronal excitability, require clarification.

Acknowledgements

The authors thank Dr. Ondřej Chudomel for performing the whole cell patch clamp recording experiment. The work has been supported in part by grants NS 056093, NS059504, and NS-20253 from NINDS and Heffer family research grant.

See also: **Antiepileptic Drugs**: Antiepileptic Drug Mechanisms; **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Sex Differences in Seizure Sensitivity; **Imaging**: Physiologic Correlates of Metabolic Imaging in Human Epilepsy; **Metabolic Changes**: Glucose Metabolism and $GABA_A$ Receptor Dysfunction in Human Partial Epilepsy; Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain; **Networks**: The Substantia Nigra in the Control of Seizures.

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Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain

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Introduction

The use of neuroimaging is rapidly increasing, both in animal models and human neuropathology. Imaging techniques that allow for the mapping of metabolic and hemodynamic changes, and particularly, autoradiographic techniques, have been widely applied in the assessment and treatment of neurologic diseases. Measurements of ictal blood flow and interictal metabolic changes are used in most epilepsy centers as presurgical tools to locate the epileptic focus and to delineate the seizure circuit, mainly in pharmacoresistant temporal lobe epilepsy.

Application of autoradiographic techniques to animal models allows investigators to measure local cerebral metabolic rates for glucose (LCMRglcs) by the [^{14}C]2-deoxyglucose (2DG) technique, and local cerebral blood flow (LCBF) rates using the [^{14}C]iodoantipyrine (IAP) technique. These techniques map LCMRglcs or LCBF in as many regions of interest as needed, in conscious freely moving animals in experiments that manipulate physiological, pharmacological, behavioral, or pathological conditions. The spatial resolution of these techniques is sufficient to provide precise identification of the areas in which epileptic seizure leads to changes in functional activity. However, the resolution of these techniques remains regional, and most attempts to reach the cellular level with these methods have failed. Thus, there is a lack of information about the nature of the cell type (e.g., neuronal or glial) whose function/activity is affected during or after the epileptic event. A recently developed approach, combining micro-autoradiography and immunohistochemistry, has now provided that level of discrimination, so that studies can determine whether it is in neurons or astrocytes that uptake of [^{14}C]2DG is taking place. However, this technique has not yet been applied to epilepsy models. Finally, nuclear magnetic resonance spectroscopy (NMRS) with ^{13}C-labeled glucose and acetate as precursors for the neuronal and astrocytic tricarboxylic acid cycle, respectively, allows investigators to study not only metabolic activity but also the interaction between neurons and astrocytes. Again, this type of analysis can only be performed at the regional (not single cell) level. Clearly, imaging involves constant compromises between information that can be obtained at the level of the circuit, brain region, or cell type.

Background

The autoradiographic techniques for the measurement of cerebral blood flow and metabolism in the rat were first published in the late 1970s. After their initial development, they were applied to numerous paradigms, including the consequences of seizures on cerebral activity. In the immature brain, the autoradiographic techniques were used to map seizure circuits in a nonquantitative way, until methods were developed to provide in vivo quantitative data in immature rats. Indeed, quantification of brain metabolism and blood flow requires the insertion of catheters into blood vessels so as to allow the simultaneous i.v. injection of the radioactive tracer and withdrawal of arterial samples. The application of these techniques in small immature rodents was initially limited by the size and fragility of blood vessels, and by the necessity to miniaturize methods of measurements that would provide accurate data based on very limited volumes of blood (i.e., so that blood withdrawal would not compromise the physiology of the animal). More details on these techniques and their adaptation to the immature brain can be found in the Methods section.

The first studies performed in normal adult rodents showed that, in normal conditions, rates of LCBF and LCMRglcs are quite heterogeneous throughout the brain, with low rates in white matter and levels varying by as much as a factor of 4 in gray matter regions. LCBF and LCMRglcs are coupled in all brain regions; that is, the regions with the highest glucose needs have the highest blood flow in order to adjust the delivery of substrates, oxygen, and glucose to the metabolic demand. Finally, in the adult brain, glucose can be considered the sole energy substrate, and hence LCMRglcs can also be seen to provide rates of energy metabolism while both LCMRglcs and LCBF reflect cerebral functional activity.

Using these techniques (in the 1970s and 1980s), several well-known groups showed that during severe sustained seizures in the adult brain, LCMRglcs increase to very high levels, and the regions most vulnerable to the insult (i.e., showing neuronal loss) are those in which glucose use is increased to the greatest extent. Investigators showed that neuronal loss is independent of systemic factors, by carrying out these studies also in anesthetized, artificially ventilated, and well-oxygenated animals. Interestingly, during sustained seizures, LCBF and LCMRglcs

do not follow a parallel temporal evolution. While the metabolic demand and hence LCMRglcs remain elevated during long seizure periods, LCBF rates first increase greatly to match metabolic demand and then start to decrease, decreasing first to normal levels and then dropping to very low levels. Thus, the late phase of severe sustained seizures is characterized in the adult brain by a pronounced mismatch between LCBF and LCMRglcs, leading to marked relative hypoperfusion. The regions in which this mismatch takes place are vulnerable to neuronal loss; these regions are usually those with very high seizure-induced metabolic increases.

In our group, we adapted these quantitative autoradiographic techniques to the immature rat, so that we could study age-related changes in seizure circuits. We also tried to revisit, in immature brain, the issue of coupling between blood flow and metabolism in the genesis of neuronal loss. Finally, we have used ^{13}C NMRS and in situ hybridization to map the brain nutrient transporters during seizures, in order to clarify the age-specific nature of the response of the immature brain to seizures.

Methods

Autoradiography: Techniques and Limitations

LCMRglcs are measured by means of the 2DG technique, and LCBF rates are assessed using the IAP technique. Both techniques have been adapted to the adult mouse and the immature rat. The spatial resolution of these techniques is excellent, and is based on the revelation of silver grains (on autoradiographic films), which are excited by the electrons emitted from the ^{14}C-labeled molecule that has accumulated in different brain regions. The optical density of the regions of interest can be measured (e.g., on serial coronal brain sections) and compared with that of calibrated [^{14}C]-methylmethacrylate plastic standards. The final step is the calculation, by means of specific operational equations, of the relation of ^{14}C concentration to rates of cerebral glucose utilization or blood flow, in each specific brain region of interest, using an image processing system. Many software packages are now available for these procedures (including free software offered on a NIH website).

A major technical difficulty in both techniques relates to the fact that the experiments require the insertion of polyethylene catheters, usually into the femoral artery and vein, for quantification of results. The venous catheter allows for the i.v. injection (2DG) or infusion of the tracer (IAP), while the arterial catheter is needed to withdraw timed blood samples for the quantification of the temporal evolution of the specific activity of the tracer in blood. In the adult rat, the insertion of catheters does not present any technical difficulty and the volume of blood is sufficient to allow for repetitive sampling, with no significant consequences on the health status of the animals. However, in immature rats (or in mice), it was more difficult to insert the catheters; this difficulty is compounded in immature animals, where the small size of the vessels is combined with the immaturity of the vessel (especially the vein) wall. Moreover, because of the small body blood volume, it is necessary to reduce the volume and number of the samples.

The choice of the technique of interest is also limited by temporal resolution. While LCBF can be measured over very short durations ranging from 20 to 60 s, the quantification of LCMRglcs requires a 45-min period of stable metabolism, which is impossible to achieve with events like short seizures. This limitation has led some authors to use the 2DG technique to map brain metabolism during seizures in a nonquantitative manner. In this latter case, data are expressed as ratios – of the optical density of the structure of interest over that of a structure that is supposed to remain unchanged (most often the corpus callosum or the cerebellum). However, this semiquantitative approach should not be used over too short durations; longer durations are needed to minimize potential errors because of a large proportion of free 2DG present in blood and brain tissue at the time of sacrifice. When studying short events, it is preferable to choose alternate methods like the autoradiographic measurement of LCBF, which can be quantified over very short durations.

^{13}C Nuclear Magnetic Resonance Spectroscopy

The study of neuronal/astrocytic metabolism and the interactions between cell types can be studied using ^{13}C NMRS. Acetate is selectively taken up by astrocytes, while glucose is considered to be mainly metabolized by the neuronal tricarboxylic acid cycle. Thus, the simultaneous injection of ^{13}C-labeled acetate and glucose, and NMRS analysis of brain extracts, gives information on the neuronal and astrocytic metabolism in the same animal. It allows experimenters, also, to study the fluxes and exchanges of amino acid transmitters between neuronal and glial compartments. Indeed, as a result of neurotransmission, the neuronal compartment constantly loses intermediates such as glutamate and GABA that need to be rapidly cleared from the synaptic cleft. A large proportion of the two neurotransmitters is taken up by glial cells. In astrocytes, GABA and glutamate are converted to glutamine by glutamine synthetase, a glia-specific enzyme. Glutamine is shuttled back to neurons, where it is broken down to glutamate and GABA. This pathway is known as the GABA–glutamate–glutamine cycle. In this context, ^{13}C NMRS is a very useful technique for studying metabolic compartmentation, and changes in glutamate and GABA metabolism, in animal models of epilepsy as well as in human epileptic patients.

Results

In our laboratory, we have mainly focused on seizure- and epilepsy-induced changes in brain metabolism and blood flow. We have used these changes as a means of delineating the seizure circuits, and as a method of determining the vulnerability of the different brain structures, especially within the immature brain. Our interest has been focused on three types of seizures or epilepsy models: (1) primarily generalized seizures, as produced in the pentylenetetrazol (PTZ) model; (2) idiopathic generalized absence epilepsy, as seen in Genetic Absence Epilepsy Rats from Strasbourg (GAERS); and (3) temporal lobe epilepsy induced by the systemic injection of lithium and pilocarpine. In all our studies, we have focused on age-related changes in the response of the brain to, and long-term consequences of, the seizures or the epilepsy.

The Pentylenetetrazol Model of Seizures and Status Epilepticus

In the pentylenetetrazol (PTZ) model of status epilepticus (SE), induced by repetitive injections of low doses of the convulsant, the pattern of metabolic activation varies with age of the animal. At very young ages (postnatal day (PN) 10 and PN14), continuous seizures induced widespread, general activation of all brain structures. The largest metabolic increases occurred in brainstem regions, involved in seizure circuits and the control of autonomic functions essential for survival. In PN17 and PN21 rats, LCMRglcs also showed the greatest increase in most brainstem regions, but were either similar to or lower than control values in most anterior regions (Figure 1). These low values could reflect a redistribution of high levels of activity to the posterior brain regions that are critical for survival, as also reported by another group in the newborn marmoset monkey subjected to status epilepticus. Conversely, the younger (PN10 and PN14) rat brain can sustain prolonged activation without this adaptation for redistribution. Prolonged and sustained PTZ-induced seizures did not lead to brain damage in any of the 4 ages studied, but long-term decreases in LCMRglc were recorded, which reflect plasticity-induced functional changes, as also recorded by other groups in the absence of neuronal loss. Long-term functional consequences were more widespread when SE was induced at PN21 than at PN10. This difference is consistent with the widely reported higher vulnerability of the brain of the weanling compared with the suckling rat.

Metabolic Activation in a Genetic Model of Idiopathic Absence Epilepsy: The GAERS

The quantitative 2DG technique was applied to the measurement of LCMRglc in GAERS. These rats are characterized by the spontaneous occurrence of absence seizures accompanied by spike-and-wave discharges (SWD) on the EEG, occurring within a thalamocortical loop. When measured in adult rats in the basal situation (a period in which there is a mixture of ictal and interictal events), LCMRglc showed widespread increases in all brain regions – as also seen in patients with childhood absence epilepsy – although the seizure circuit is limited to a thalamocortical pathway. The metabolic increases seem to occur during the interictal phases rather than during seizures, as confirmed by continuous recording of LCBF by Laserflow Doppler. In the GAERS strain, absence seizures appear at 30–40 days postnatal. Their frequency and duration increases, up to 3–4 months of

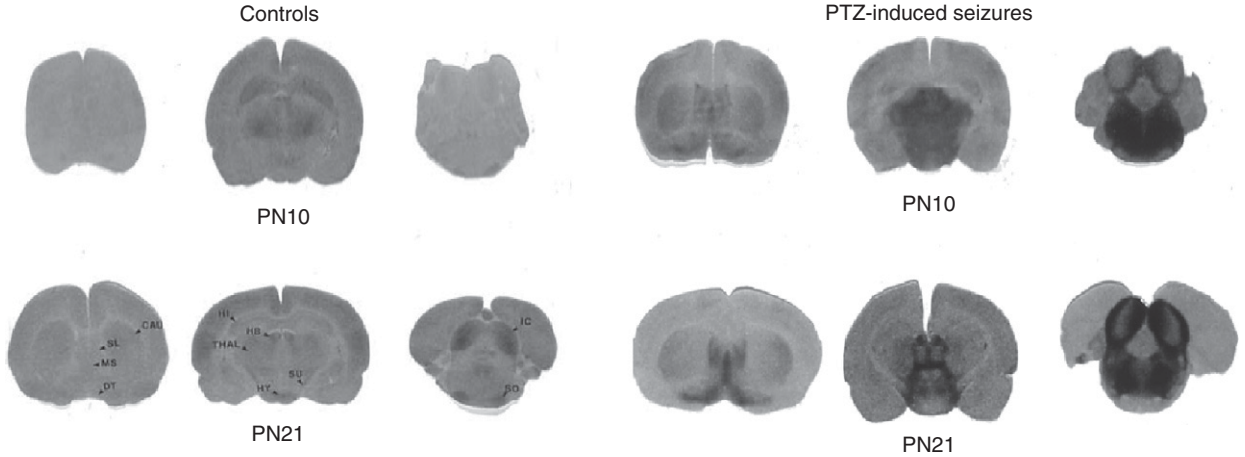


Figure 1 Age-related increases in LCMRglcs induced by prolonged pentylenetetrazol (PTZ) seizures. The response to seizures is quite homogeneous in the very immature PN10 rat brain involving mostly subcortical regions at all anteroposterior brain levels. In PN21 rats, LCMRglcs are no longer increased by seizures, even decreased compared to control levels in some forebrain regions, or regionally very heterogeneous as in thalamus. Conversely, at the brainstem level, marked metabolic increases are indicative of a redistribution of LCMRglcs in favor of vital regions mediating cardiorespiratory functions.

age, and then remains stable until the animal's death. In PN21 rats, i.e., before the occurrence of SWDs, LCMRglcs are only increased in a limited number of structures, mainly limbic regions in which SWDs are never recorded and structures belonging to the nigral system of seizure control. This finding points to the high sensitivity of limbic regions to seizure events and to cellular events preceding the occurrence of the seizure – which presumably already activate the nigral system of seizure control in these young animals.

Likewise, before the onset of SWDs, the interactions between glutamatergic neurons and astrocytes, assessed by NMRS using [1–^{13}C]glucose and [1,2–^{13}C]acetate, appear normal. In contrast, increased astrocytic metabolism takes place in adult GAERS, suggesting that astrocytic alterations could possibly be the cause of seizures. Consequently, we have hypothesized that in this rat model, seizures appear with maturation when cortical metabolism is sufficiently enhanced – and accompanied by a slight reduction in inhibitory neurotransmission, as reflected by reduced glutamine supply to GABAergic neurons.

Temporal Lobe Epilepsy: Insights from the Lithium-Pilocarpine Model

The incomplete maturation of brain structures and connections leads to age-dependent clinical seizure expression. This age-dependency is of particular relevance, since in human temporal lobe epilepsy, an initial injury occurs most often before the age of 4 years. In the lithium-pilocarpine model of SE, the initial SE induced by the chemoconvulsant leads to age-dependent neuronal loss and spontaneous seizures (or no epilepsy) after a latent phase. During SE, LCMRglcs vary with postnatal age. In PN10 rats, pilocarpine-induced SE leads to functional activation in many forebrain regions – but not in hypothalamic and thalamic nuclei. In PN21 and adult rats, increases in LCMRglc are generalized and quite dramatic in the cerebral cortex (mainly entorhinal and piriform), the hippocampus, amygdala, septum, and thalamus, while the brainstem is less activated. The largest increases occur in cortical and hippocampal regions (**Figure 2**).

Other groups have mapped LCMRglcs in immature rats subjected to kainate SE, and have emphasized the age-related maturation of the structures involved in seizure circuits. However, none of these studies was performed in a quantitative manner. In rats less than 3 weeks old, the pattern of brain activation is limited to the hippocampus and septum, reflecting the distribution of paroxysmal discharges. By the end of the third week, when kainate leads to limbic motor seizures, other regions are activated, and metabolic maps become similar to adult ones. This evolution reflects mainly the maturation of limbic circuits, which allows the propagation of seizures to extrahippocampal and subcortical sites.

The active and sustained metabolic increase associated with SE usually leads to neuronal death. In adult rats, pilocarpine-induced (and also kainate-induced) seizures lead to widespread brain damage, mainly in hippocampus, amygdala, septum, entorhinal and piriform cortex – where the largest metabolic increases are recorded. When SE is induced in rats between PN10 and PN18, the activation of a limited number of brain regions is associated with no or quite limited brain damage. In PN21 rats, neuronal loss is limited to entorhinal cortex, lateral thalamus, amygdala, and the hilus of the dentate gyrus.

There are distinct age-related differences in the long-term metabolic consequences of lithium-pilocarpine SE. In PN10 rats, in which no overt damage and no epilepsy develop, SE has no long-term metabolic consequences. Conversely, some PN21 rats and all adult rats will become epileptic after a latent phase. At these two ages, LCMRglcs are decreased during the latent and chronic phases in the hippocampus, amygdala, thalamus and

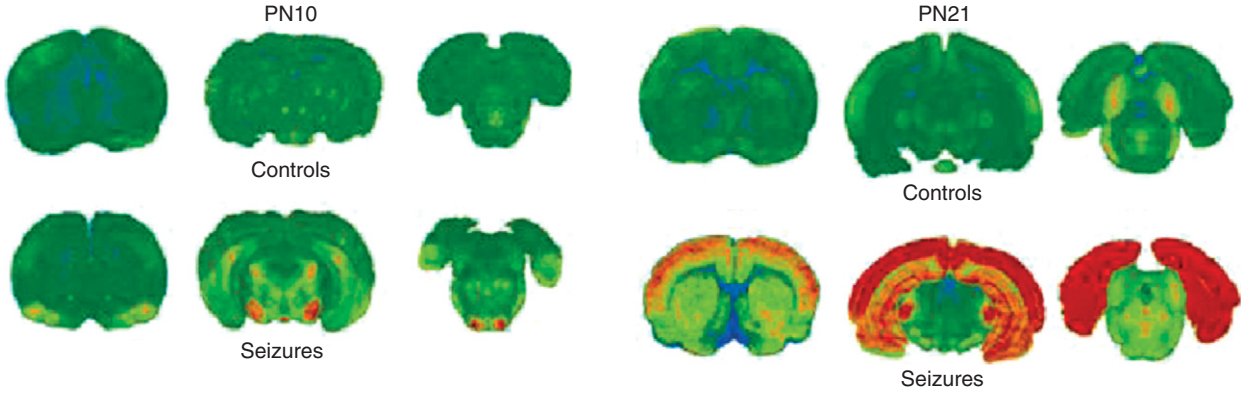


Figure 2 Age-related increases in LCMRglcs induced by prolonged lithium-pilocarpine seizures. In PN10 rats, seizures induce moderate increases in LCMRglcs compared with the response in PN21 rats. Increases are quite marked in cortical and hippocampal regions and less marked in subcortical areas. They are also more pronounced in anterior regions than in posterior regions and absent in thalamus of PN10 rats.

entorhinal cortex – even though the SE-related neuronal loss is limited to the lateral thalamus and hilus in PN21 rats. However, the most striking feature is the mismatch between the number of neurons surviving in the hilus – which is very low at both ages – and the level of metabolic activity – which is close to normal at both ages. This mismatch suggests a major role of the hilus in the initiation and maintenance of epilepsy.

Coupling Between Cerebral Blood Flow and Metabolism During Seizures and Its Relation with Neuronal Damage

In adult and immature animals and humans, LCMRglcs and LCBF are tightly coupled in most situations. In adult animals, LCBF rates and LCMRglcs usually increase to a similar degree during the early phase of seizures. In vulnerable structures undergoing neuronal damage (such as the hippocampus, cerebral cortex and thalamus), this early increase is followed by a pronounced mismatch between LCBF and LCMRglcs, the latter remaining elevated while the former is lower than the control levels (Figure 3). This mismatch can be found in the whole forebrain of both immature and adult rats, whether neuronal loss occurs or not. Hence, despite an extended flow-metabolism mismatch, neuronal loss in PN21 rats is only found in a few regions; and in PN10 rats, SE does not lead to neuronal loss. In posterior regions of the brain (i.e., brainstem), there is no mismatch between LCBF and LCMRglcs and no SE-related neuronal death at any age. Thus, in the lithium-pilocarpine model, marked hypoperfusion during SE correlates well with neuronal damage in adult rats, correlates only partly in PN21 rats, and does not correlate at all in PN10 rats. Although the mechanisms underlying the resistance of the immature brain to brain damage are unknown, the age-related differences in the consequences of the flow-metabolism mismatch calls into question the role of this mismatch in neuronal loss. These changes in cerebral functional activity are rather the consequence of SE-related cellular activation. The cellular and molecular events occurring during SE differ in nature and severity according to maturation, and these differences clearly underlie the variability in extent of neuronal death.

Transport of Nutrients Between Blood and Brain

Nutrient delivery to the brain is mediated by transporter proteins, GLUT1 and GLUT3 for glucose and mainly MCT1 and MCT2 for lactate and other monocarboxylic acids. GLUT1 and GLUT3 density is low in the immature brain and progressively increases concurrently with LCMRglcs. Conversely, the vascular expression of MCT1 peaks during suckling and declines with maturation, while nonvascular MCT1 and MCT2 stay at a constant level. Sustained seizures largely increase LCMRglcs, and a limited rate of glucose transport has been considered as a possible cause of neuronal death, especially in the immature brain. We have shown that during lithium-pilocarpine SE,

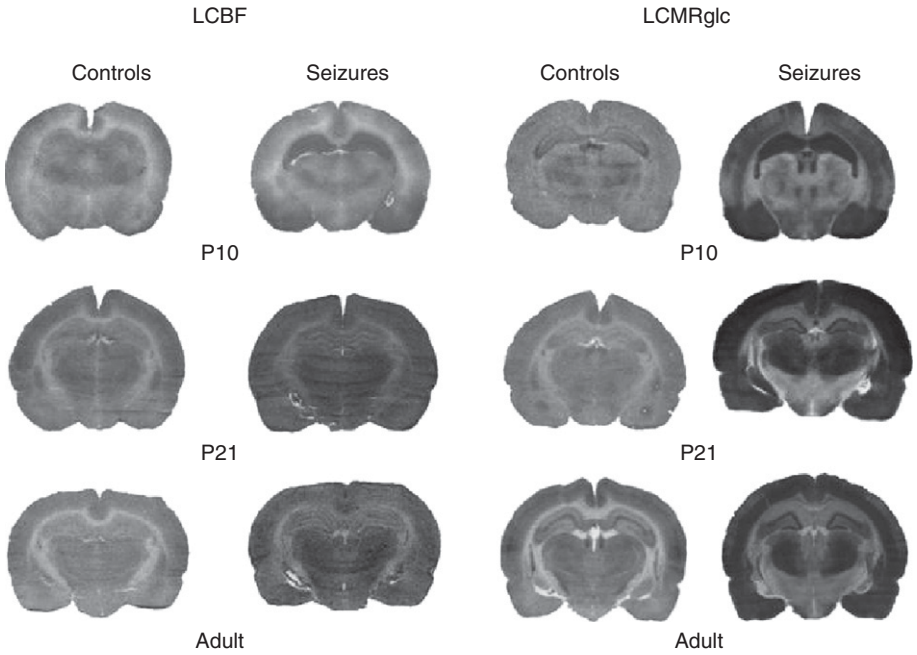


Figure 3 Age-related changes in metabolism-flow coupling induced by prolonged lithium-pilocarpine seizures. As illustrated in Figure 2, LCMRglcs increase with seizures at all ages, mainly in PN21 and adult rats while changes in LCBF rates do not match at any age the striking increases in metabolic demand leading to a mismatch between blood flow and metabolism. This seizure-induced hypoperfusion is regionally coupled with neuronal loss in adult but not immature rats.

the blood–brain barrier GLUT1 transporter undergoes widespread increases particularly in adult rats, and the neuronal GLUT3 transporter is upregulated mainly in PN10 rats. The vascular MCT1 transporter is upregulated at all ages, but 5–10 times more so in adult than in immature rats; MCT2 expression increases mainly in adult rats. Altogether, the upregulation of nutrient transporters appears to be quite well regulated to respond to the large energetic demand occurring during SE, and to the potentially large accumulation of lactate as a result of glucose metabolism that can be extruded from the brain. During the latent and chronic phases, changes in nutrient transporter expression generally reflect neuronal loss.

Future Directions

Most of the data obtained to date in the measurement of metabolic and hemodynamic changes in the immature and mature brain during seizures originate in studies using autoradiography. These techniques have proven to be very useful and to achieve a very good resolution. However, these techniques require that the experimental animal be sacrificed at each time-point studied. Therefore, the number of animals necessary to run these studies is very large, and no temporal follow-up can be conducted on the same animal. Alternative methods that would allow longitudinal tracking of pathological changes in a given animal include positron emission tomography (PET) and single photon computed emission tomography (SPECT) imaging, and functional magnetic resonance imaging (fMRI).

PET detects positron emission from selected radionucleotides. The miniaturization of PET devices has led to the development of a commercially available 'micro-PET' instrument. For the measurement of brain metabolism, [^{18}F]fluorodeoxyglucose (FDG) – which has a relatively long half-life (110 min) – is usually used. The micro-PET imaging technology has thus far had limited application to animal models of epilepsy. Micro-SPECT has been used in preclinical studies, but not yet for following blood flow changes in epileptic animals. The major limitation in the use of micro-PET and micro-SPECT in rats are their relatively poor spatial and temporal resolution. Hopefully, the resolution of both technologies will improve, and hence provide additional methods for the temporal follow-up of the evolution of seizure circuits in single animals, providing data that can be combined with the precise information obtained with autoradiography.

The fMRI technique reflects altered metabolic neuronal activity, and the method has recently been extended to the study of epilepsy in animal models. In general, fMRI measures increased blood flow as a reporter of increased neuronal activity. Blood-oxygen-level-dependent (BOLD) fMRI uses the oxygenation status of hemoglobin as the 'contrast' reporter. Altered BOLD signal changes have been reported in the thalamus and cortex after administration of γ-hydroxybutyric acid (a chemical model of absence seizures) and in a genetic strain of absence epilepsy.

See also: **Glutamate**: Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain; **Imaging**: Imaging Approaches to Seizure Analysis: PET Analysis; SPECT in Epilepsy Research; **Pediatric Epilepsy**: Developmental Aspects of Seizures; Epileptogenesis in Childhood.

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Acutely Dissociated Neurons

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Introduction

Epilepsy is a term used for several chronic neurological syndromes characterized by abnormal and inappropriate neuronal activity and synchrony. Often the neuronal synchrony is so widespread in the brain that consciousness is impaired. The particular neurons that are affected, the particular neuronal abnormalities, the particular connections that are affected, and the particular synaptic (or non-synaptic) abnormalities appear to be at least as varied as the number of named epilepsy syndromes.

The study of the basic mechanisms of epilepsy has benefited from the use of many different in vitro experimental preparations, including the intact whole-brain model and progressively more 'reduced' preparations such as brain slices, acutely dissociated neurons, neuron fragments (e.g., dendritic fragments), and single channel patch preparations. The cells in the acutely dissociated neuron preparation remain sufficiently intact for studies of many kinds of cellular activity, including action potentials, voltage-gated currents, and currents associated with activation of specific ionotropic or metabotropic receptor/transmitter systems. The acutely dissociated neuron preparation is particularly well-suited for some types of investigations that focus on the intrinsic properties of neurons or their responsiveness to activation of specific receptors. There are, in addition, several advantages to recordings from dissociated cells as compared with recordings from single neurons embedded within intact brains or brain slices or single neurons in culture. Those advantages will be discussed and exemplified in this article.

Background - The Pros and the Cons

Most studies using the acutely dissociated cell are attempts to understand the roles of ion channels in action potential generation and patterning. Such studies of ionic

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The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy

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Introduction

To date only a minority of epilepsy research has been focused on mitochondria. However, recent advances provide compelling evidence of mitochondrial involvement in the epilepsies. Direct mitochondrial dysfunction is associated with a small percentage of inherited epilepsies – e.g., mitochondrial encephalopathies. A role of mitochondria in acquired epilepsies, which account for ~60% of all epilepsy cases, remains to be fully explored. Temporal lobe epilepsy (TLE) is the most notable example of acquired epilepsy usually preceded by injury such as episode(s) of prolonged seizures or status epilepticus (SE), childhood febrile seizures, hypoxia, or trauma. These initial insults are thought to set in motion complex molecular, biochemical, and structural changes that culminate in the development of spontaneous recurring seizures, or 'epileptogenesis.' Since injury per se can set in motion molecular events that trigger the epileptic condition, it is important to understand the biochemical causes and consequences of epileptic seizures. Identifying these biochemical mechanisms may lead to therapies that interrupt the process of epileptogenesis.

Background

Several major features of the epileptic phenotype suggest that mitochondrial involvement may not be a mere epiphenomenon, but rather a key event contributing to the initiation and progression of TLE. Mitochondria are intimately involved in important cellular events that are leading candidate mechanisms of hyperexcitability and/or its consequences. These potential mechanisms include metabolic and bioenergetic changes associated with seizure activity, the involvement of mitochondria in the processes underlying seizure-induced cell loss (i.e., excitotoxicity and apoptosis), mitochondrial regulation of amino-acid cycling, neurotransmitter biosynthesis, and ionic homeostasis, particularly calcium buffering. Another important reason to consider mitochondrial mechanisms, particularly the involvement of oxidative stress in epilepsy, is the knowledge that the incidence of epilepsy abruptly rises with advancing age; mitochondrial oxidative stress is a leading mechanism of aging and age-related degenerative disease.

The most compelling evidence for mitochondrial (dys) function in epilepsy is the recognition that dramatic metabolic and bioenergetic changes occur following acute seizures and during different phases of chronic epilepsy (**Figure 1**). The acute consequence of seizures associated with SE is an increase in cellular glucose uptake and metabolism that is unparalleled by most conditions. Cerebral blood flow is increased to match this hypermetabolism; since the increased rate of glycolysis exceeds pyruvate utilization by pyruvate dehydrogenase, there is an increased lactate buildup. While *hyper*metabolism occurs in human epileptic foci during ictal phases (seizure episodes), *hypo*metabolism is prevalent during interictal phases (between seizure episodes). A bioenergetic defect is also evident in the measurement of ^{31}P and the loss of mitochondrial *N*-acetyl aspartate in human epileptic tissue, suggesting the involvement of mitochondria in altered neurotransmitter metabolism. Severe metabolic dysfunction characterized by biphasic abnormal NAD(P)H fluorescence transients and changes in mitochondrial membrane potential (dsm) have been observed in ex vivo preparations from both chronically epileptic rats and human subjects. The mechanisms of both hypometabolism and hypermetabolism in seizure-induced brain damage, and in subsequent development of epilepsy, need to be determined.

Methodological Approaches and Recent Results

Oxidative Stress and Mitochondrial Dysfunction: Acute Consequences of Epileptic Seizures

ROS and reactive nitrogen species (RNS) are thought to play important roles in diverse nervous system disorders

such as stroke, spinal cord injury, Parkinson's disease, Alzheimer's disease, Huntington's disease, Freidrich's ataxia, and amyotrophic lateral sclerosis (ALS). A role for ROS in seizure-induced neuronal death is supported in part by the observations that prolonged seizures result in increased oxidation of cellular macromolecules and compounds with antioxidant properties (superoxide dismutase (SOD) mimetics, vitamin C, spin traps, and melatonin) prevent seizure-induced pathology. Moreover, oxidative stress is thought to be an important consequence of excitotoxicity, which plays a critical role in epileptic brain damage. Finally, seizure-induced neuronal death involves overlapping processes of necrosis and apoptosis, which are strongly influenced by mitochondrial function and oxidative stress. Seizure activity initiates the mitochondrial pathway of apoptosis via involvement of proapoptotic factors, cytochrome c translocation, and caspase-3/9 activation. The following sections review the studies demonstrating oxidative stress following acute SE.

Mitochondrial ROS Production

Since reactive species are transient, unstable, and localized to cellular compartments, methodological assessment of ROS in biological systems in vivo is challenging. One approach has been the use of surrogate markers in vivo that are easily modified by ROS and are stable enough to be analyzed by reliable quantitative methods. Seizure-induced oxidative damage to susceptible targets (proteins, lipids, and DNA) has been assessed following chemoconvulsant-induced SE. The presence of a labile iron motif in the iron–sulfur (Fe–S) center of cytosolic and mitochondrial aconitase(s) renders them sensitive to O_2^- and related species, allowing the measurement of aconitase activity to serve as an index of steady-state O_2^- levels. Measurement of O_2^- production using endogenous aconitase inactivation as a surrogate marker has several advantages in that it is highly sensitive to inactivation by O_2^-, relatively specific for O_2^- and allows estimation of compartmentalized (mitochondrial or cytosolic) O_2^- production. The usefulness of aconitase inactivation is evident by the demonstration that brain and heart mitochondrial aconitase activity is drastically diminished in Sod2 homozygous knockout (Sod2−/−) mice. One disadvantage of using an endogenous marker such as aconitase is that its synthesis may be regulated, particularly during chronic studies. Since oxidative inactivation is a posttranslational event, this problem may be overcome by measurement of apoprotein levels in addition to enzyme activity. Another disadvantage is the sensitivity of aconitase to multiple reactive species; therefore, aconitase inactivation necessitates verification of the inactivating species by multiple approaches (e.g., examining mutant mice, use of specific scavengers).

In rats, kainate-induced seizures inactivate O_2^- susceptible mitochondrial aconitase but not oxidatively stable fumarase. This inactivation is specific for the mitochondrial aconitase, and not its cytosolic counterpart that is also sensitive to O_2^-, implicating mitochondria as the major site of seizure-induced O_2^- production. Maximal inactivation of mitochondrial aconitase occurs several hours after the cessation of SE (16 h post kainate injection) and at times preceding the death of susceptible hippocampal neurons. Kainate-induced mitochondrial aconitase inactivation and hippocampal neuronal loss are attenuated in transgenic mice overexpressing Sod2 and exacerbated in mice partially deficient in Sod2. Manganese (III) tetrakis (4-benzoic acid) porphyrin (MnTBAP), a broad spectrum antioxidant, protects rats against seizure-induced mitochondrial aconitase inactivation and hippocampal damage without decreasing behavioral seizure intensity or frequency. These findings support a role for mitochondrial O_2^- in hippocampal pathology produced by kainate-induced seizures. Furthermore, seizure-induced O_2^- production has been demonstrated by hydroethidium staining in the rat model of lithium/pilocarpine-induced SE. Other mitochondrial enzymes with high susceptibility to ROS include α-ketoglutarate dehydrogenase, the activity of which is also decreased following pilocarpine-induced seizures.

Lipid Peroxidation

Polyunsaturated lipids are highly susceptible to oxidation and their diverse products have been used as markers to indicate oxidative stress in vitro and in vivo. Seizure-induced lipid peroxidation has been shown to occur by the measurement of end-products such as thiobarbituric acid reactive substances and F_2-isoprostanes (F_2-IsoPs). F_2-IsoPs are a novel class of prostaglandin F_2 (PGF_2)-like compounds, produced in vivo by a noncyclooxygenase and free-radical-catalyzed mechanism involving the peroxidation of arachidonic acid. F_2-IsoPs are a validated sensitive, stable, and reliable marker of free radical-induced lipid peroxidation in vivo. Prolonged seizures produce a large increase in prostaglandin derivatives, including prostaglandin-$F_2\alpha$, a precursor of F_2-IsoP. In adult rats, kainate-induced SE produces a large subregion-specific increase in F_2-IsoP levels in the highly vulnerable CA3 region prior to cell damage. Interestingly, the dentate gyrus, a region that contains granule neurons that are resistant to kainate-induced neuronal death also shows marked increases in F_2-IsoP levels. Recently, novel products of free radical-mediated peroxidation of arachidonic acid were discovered that have a substituted tetrahydrofuran ring, which have been termed 'isofurans' (IsoFs). Interestingly, it was shown that the formation of IsoFs and F_2-IsoPs are differentially modulated by oxygen

tension; high oxygen tension favors the formation of IsoFs whereas the formation of F_2-IsoPs is favored at low oxygen tension. SE-induced IsoF formation occurs with overlapping but distinct time courses as F_2-IsoP formation in hippocampal subregions. IsoF, but not F_2-IsoP, formation coincides with other indices of mitochondrial oxidative stress. SE resulted in a transient decrease in hippocampal pO_2 measured by in vivo electron paramagnetic resonance oximetry, suggesting an early phase of seizure-induced hypoxia. Whereas SE-induced F_2-IsoP formation coincided with the peak hypoxia phase, IsoF formation coincided with the 're-oxygenation' phase. It remains to be determined whether these potentially bioactive lipid derivatives exert a cytotoxic or signaling role in response to seizure activity and if mitochondrial oxidative stress contributes to their formation.

Oxidative DNA Lesions

Seizure-induced oxidative DNA damage has been demonstrated by using the measurement of 8-hydroxy-2-deoxyguanosine (8-OHdG), an oxidatively modified guanine adduct, as an index of oxidative DNA damage. The ratio of steady-state levels of 8-OHdG to 2-deoxyguanine (2-dG) reflects oxidative DNA damage. Kainate administration in adult rats produces a large increase in the ratio of oxidized:nonoxidized bases (8-OHdG:2dG) at times preceding overt cell death, suggesting a potential role for oxidative DNA damage in the sequence of events leading to seizure-induced neuronal death. More recent studies confirm that mitochondrial, not nuclear DNA is the source of this seizure-induced change in 8-OHdG levels, as would be expected by the close proximity of mitochondrial DNA to the major source of O_2^- production, i.e., the electron transport chain (ETC), which accounts for the several fold higher oxidized DNA bases in mitochondrial DNA in comparison to nuclear DNA. Direct damage to the mitochondrial genome and base excision repair of the lesion are major consequences of increasing the steady-state levels of 8-OHdG. Recent studies in our laboratory suggest that both mtDNA damage and repair is activated by SE.

Redox Status

In addition to the measurement of oxidatively modified cellular targets as indicators of oxidative stress, another approach has been to directly determine the subcellular redox status by measuring a redox couple such as glutathione (GSH) and its disulfide (GSSG). Decreased levels of GSH have been observed in the parietooccipital region of the brain and in the plasma of epilepsy patients. Although some studies have shown decreased GSH levels in hippocampal tissue early following SE in animal models, others have reported no changes. Studies in our laboratory demonstrated a time-dependent decrease in GSH/GSSG ratios predominantly in the mitochondrial fractions compared with whole hippocampal homogenates. This decrease in GSH/GSSG ratio was accompanied by a mild increase in GSH peroxidase activity and a decrease in GSH reductase activity in hippocampal homogenates and mitochondria, respectively. Since coenzyme A (CoASH) and its disulfide with GSH (CoASSG) are primarily compartmentalized within mitochondria, their measurement in whole tissue homogenates was undertaken to overcome problems associated with GSH/GSSG measurement following subcellular fractionation. Hippocampal tissue CoASH/CoASSG ratios were decreased following kainate-induced SE; the time-course and magnitude of change paralleled mitochondrial GSH/GSSG levels. Cysteine, a rate-limiting precursor of GSH, was also decreased following SE in both hippocampal tissue homogenates and mitochondrial fractions. These changes in altered redox status provide further evidence for seizure-induced mitochondrial oxidative stress. The increased mitochondrial GSSG that was formed following SE in situ under conditions of oxidative stress may become available to exert local injury. Additionally, decreased ratios of GSH/GSSG could induce structural damage to mitochondrial membranes, changes in mitochondrial enzyme activities, and mitochondrial dysfunction.

Relationship to Neuronal Vulnerability and Age

In general, oxidative damage occurs to a greater extent in brain areas that are vulnerable to kainate-induced brain damage (hippocampal area CA3, CA1, and piriform cortex) than in resistant areas (dentate gyrus and cerebellum). Further, seizure-induced mitochondrial oxidative stress shows a strong developmental regulation, which may explain the age-related vulnerability of the brain to seizure-induced brain damage. The higher expression of uncoupling protein-2 (UCP-2) in the neonatal brain than in the adult brain was shown to be an underlying factor that renders the neonatal brain more resistant to seizure-induced free-radical production and neuronal injury. Advancing age, which renders animals more susceptible to seizure-induced mortality, also modulates the response to oxidative stress. For example, an otherwise subtoxic dose of kainate in young adult rats resulted in significant oxidative damage to aged rats.

Verification in SOD Transgenic/Knockout Mice

An additional approach for assessing the role of oxidative stress in seizure-induced brain damage is the use of SOD mutant mice. Although oxidized proteins, lipids, and DNA are useful surrogate markers for detecting the presence of oxidative stress, it is difficult to identify precisely

the reactive species and its cellular source. Hippocampal damage produced by local application of kainate in transgenic and knockout mice of the three endogenous SODs – in the cytoplasm (CuZnSOD or Sod1), mitochondria (MnSOD or Sod2), and extracellular compartment (EC-SOD or Sod3) – showed that overexpression of Sod2 (and to a lesser extent Sod3, but not Sod1) was protective. Moreover, mice overexpressing Sod2 were protected against kainate-induced mitochondrial aconitase inactivation and hippocampal cell death, whereas mice with partial Sod2 deficiency (Sod2−/+) showed exacerbation of these effects. A caveat associated with the use of mutant mice to study mechanisms of seizure-induced cell loss is the sensitivity of the genetic background strain to the injury. To circumvent this strain-sensitivity necessitates the use of intracerebral rather than systemic administration of kainic acid. Together with the demonstration that seizures inactivate mitochondrial, not cytosolic aconitase, these studies have suggested that mitochondria play a disproportionately greater role in seizure-induced O_2^- production (compared with cytosolic aconitase).

Mitochondrial Dysfunction Following Acute and Chronic SE

These studies demonstrate that prolonged seizures acutely result in oxidative damage to lipids, DNA, and susceptible proteins. However, whether oxidative stress and/or mitochondrial dysfunction occur several weeks after SE is unknown. This issue has been addressed by assessing mitochondrial dysfunction several weeks following pilocarpine-induced SE. They showed that the activities of complexes I and IV of the ETC decreased, and complex II increased, one month after piloparpine SE. These changes were accompanied by lowered mitochondrial membrane potential measured by rhodamine123 fluorescence in the CA1 and CA3 areas. The authors attributed these changes to decreased mitochondrial DNA copy number that results in down-regulation of oxidative phosphorylation (OXPHOS) enzymes encoded by mtDNA. These studies suggest that the acute effects of SE – i.e., increased mitochondrial oxidative stress – may result over time in oxidative damage to mtDNA (as suggested by increased levels of 8-OHdG) and decreased expression of mitochondrially encoded proteins required for the functioning of the ETC. Seizure-induced accumulation of oxidative mitochondrial DNA lesions and resultant somatic mtDNA mutations could, over a period of time, render the brain more susceptible to subsequent epileptic seizures, particularly in the context of advancing age. A link between mitochondrial dysfunction and epilepsy is strengthened by the finding that some patients with TLE show mitochondrial complex I deficiency in the seizure foci, which is the leading cause of increased O_2^- production and mitochondrial dysfunction in patients with Parkinson's disease. In addition to pilocarpine-induced seizures, inhibition of complex I has also been observed in homocysteic acid-induced seizures. Furthermore, ultrastructural damage to the mitochondria has been observed in the hippocampus of both kainic acid- and pilocarpine-treated epileptic rats.

Mechanistic studies assessing the role of mitochondrial functions on neuronal excitability have been attempted by measuring seizure-like events (SLEs) in hippocampal explant cultures. The SLEs, usually generated by a combination of electrical stimulation of the axons of the granule cells (i.e., the mossy fibers) and lowering the Mg^{2+} concentration in the bathing media (0 Mg^{2+}) to relieve the voltage-dependent Mg^{2+} blockade of the NMDA receptors, are associated with increased mitochondrial calcium accumulation, mitochondrial depolarization, decreased nicotinamide adenine dinucloetide [NAD(P)H] autofluoresence, and O_2^- generation. O_2^- production measured by hydroethidium progressively increases during, and with each consecutive SLE, along with increases of both cytosolic and mitochondrial calcium, thus providing a link between mitochondrial calcium, free-radical production, and neuronal death.

Mitochondrial Dysfunction and Neuronal Excitability

Although mitochondrial dysfunction is an acute consequence of epileptic seizures, whether mitochondrial dysfunction can be a causative factor in acquired epilepsies is unknown. The precedence for mitochondrial dysfunction producing epilepsy has already been established from our knowledge regarding the molecular basis of certain inherited epilepsies – i.e., the mitochondrial encephalopathies. The most prominent example of mitochondrial dysfunction causing epilepsy is the occurrence of epilepsy in mitochondrial disorders due to mutations in mitochondrial DNA (mtDNA) or nuclear DNA. For example, myoclonic epilepsy with ragged red fibers (MERRFs) is a syndrome in which a single mutation of the mitochondrially encoded $tRNA^{lys}$ results in a disorder consisting of myoclonic epilepsy and a characteristic myopathy with ragged red fibers. Defects in complex I and complex IV of mitochondrial OXPHOS are the leading mechanisms by which this mitochondrial gene mutation produces MERRF. Several normal functions of mitochondria, ranging from its bioenergetic functions to metabolic functions, can impact neuronal excitability. These include cellular ATP production, ROS formation, synthesis and metabolism of neurotransmitters, fatty-acid oxidation, calcium homeostasis, and control of apoptosis/necrotic cell death. These vital functions are closely interrelated, and which of these factors contributes to the seizures associated with mitochondrial dysfunction remains unclear.

Several common neurological insults such as hypoxia, trauma, and aging, as well as several neuronal diseases such as stroke and Alzheimer's disease render the brain susceptible to epileptic seizures. Further, although epilepsy occurs in all age groups, the incidence of epilepsy is markedly increased in the elderly. Oxidative stress and mitochondrial dysfunction is common to each of these conditions. Seizure activity can be induced by paradigms (e.g., increased oxygen tension (hyperbaric hyperoxia) and local infusion of redox-active iron salts or mitochondrial toxins) that are capable of producing mitochondrial oxidative stress and dysfunction. Thus, mitochondrial dysfunction initiated by free-radical production could contribute to lesion-induced hyperexcitabililty. In studying this relationship, mice partially deficient in Sod2 (Sod2−/+) are particularly useful because they provide a model of sublethal chronic elevation of mitochondrial O_2^- to test the hypothesis. Work from our laboratory provides experimental evidence linking mitochondrial oxidative stress with increased seizure susceptibility induced by aging, environmental stimulation, or kainate administration. Whereas Sod2−/− mice show extensive mitochondrial dysfunction and behavioral phenotypes such as ataxia and seizures before neonatal death, Sod2−/+ mice appear both biochemically and phenotypically normal at birth but develop age-related deficits consistent with chronic mitochondrial oxidative stress. A subset of Sod2−/+ mice developed spontaneous and handling-induced seizures as a function of advancing age. The age-related onset of seizures in Sod2−/+ mice correlated with increased mitochondrial oxidative stress (mitochondrial aconitase inactivation and mitochondrial, but not nuclear 8OHdG formation) and mitochondrial dysfunction as measured by O_2 utilization. Prior to the age at which spontaneous and handling-induced seizures occurred, Sod2−/+ mice showed increased susceptibility to kainate-induced seizures and hippocampal cell loss.

Although several factors associated with mitochondrial dysfunction have the ability to enhance seizure susceptibility in Sod2−/+ mice, the most likely suspects include targets sensitive to oxidative insults that raise plasma membrane excitability. We therefore examined the high affinity astroglial and neuronal glutamate transporters, based on their known sensitivity to oxidative damage and their crucial role in maintaining low levels of synaptic glutamate from reaching neurotoxic levels. Sod2−/+ mice show an age-dependent decrease in the hippocampal expression of glial glutamate transporters (GLT-1 and GLAST), which may explain the increased vulnerability of these mice to epileptic seizures. Chronic O_2^- production in the mitochondrial matrix can be rapidly converted to H_2O_2 by dismutation and Fe–S inactivation. Unlike O_2^-, H_2O_2 is freely permeable across cellular membranes and can be generated on an equimolar amount as O_2^- by oxidative inactivation of mitochondrial aconitase, which also showed parallel age-related decline in Sod2−/+ mice. H_2O_2 generated in this manner could oxidize astroglial glutamate transporters and lead to their decreased expression. These results suggest that mitochondrial oxidative stress and resultant dysfunction is sufficient to increase seizure susceptibility. Given the dependence of neurotransmitter and ion transport systems on ATP, depletion of ATP may be another important independent factor contributing to increased seizure susceptibility associated with mitochondrial dysfunction.

Therapeutic Implications and Future Challenges

Therapies for the epilepsies are largely aimed at decreasing neuronal excitability and thereby controlling the occurrence of epileptic seizures. A recent therapeutic approach is to search for antiepileptogenic, rather than antiepileptic, drugs that would target underlying processes that lead to the development of epilepsy. Testing antiepileptogenic therapies aimed at mitochondrial bioenergetics and oxidative stress pathways has, to date, been largely limited to animal studies. Clinical trials of vitamin E as an add-on therapy for refractory epilepsy have been controversial, with largely failed attempts to influence the occurrence of epileptic seizures in pediatric patients. Creatine, an endogenous guanidine, functions with phosphocreatine and a mitochondrial form of creatine kinase as a spatial energy buffer between the cytosolic and mitochondrial compartments. Administration of creatine has been shown to be protective in animal models of CNS injury, including trauma, ischemia, 3-nitropropionic acid, MPTP-induced parkinsonism, and ALS. Creatine supplementation has been effective in reducing hypoxia-induced seizures in both rats and rabbits. The clinical management of intractable epilepsies with strategies such as caloric restriction or modification – e.g., ketogenic diet – suggests the role of mitochondrial bioenergetics in the protective effects. The ketogenic diet has been shown to produce mitochondrial biogenesis, bioenergetically efficient mitochondria, and lower levels of ROS. The latter has been show to occur because of up-regulation of UCP2 in mice fed a ketogenic diet. In these experimental studies, seizure-induced brain damage and subsequent epilepsy could be inhibited by caloric restriction or a ketogenic diet. Diet modification by ketogenic diet or caloric restriction represents a nonpharmacologic strategy that decreases seizure frequency in the epileptic EL mouse. Interestingly these paradigms are also known to limit free-radical formation. Consistent with this effect, recent studies in our laboratory have shown an up-regulation of GSH biosynthesis in animals fed the ketogenic diet. Several newer antiepileptic

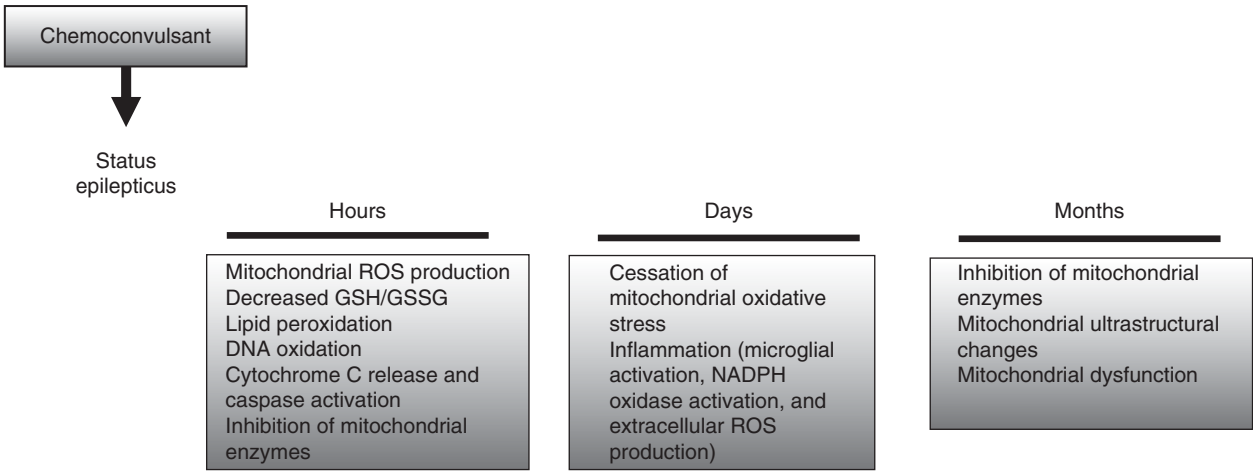


Figure 1 Documented and proposed cellular events initiated hours, days, and months following an acute episode of chemoconvulsant-induced status epilepticus. Acute and chronic biochemical events known to occur following chemoconvulsant-induced SE.

drugs such as zonisamide, topiramate, and levetiracetam inhibit mitochondrial dysfunction and/or possess antioxidant properties, raising the possibility that inhibition of mitochondrial dysfunction may in part underlie their actions.

Oxidative stress and neuronal damage induced by kainate can be ameliorated by at least two types of SOD mimetics: the manganese porphyrin, MnTBAP, and the salen compound, EUK134. However, acute administration of catalytic antioxidants does not alter chemoconvulsant-induced behavioral seizure severity. Previous generations of manganese porphyrins (e.g., MnTBAP) showed limited ability to cross the blood–brain barrier (BBB), which necessitated intracerebral administration, and it remains to be determined if newer classes that are BBB permeable, orally active, and validated in animal models of neuronal injury modulate seizure-induced injury and/or epileptogenesis. Other compounds with antioxidant properties that inhibit seizure-induced brain injury include the hormone melatonin, a nitrone spin trap, and vitamin C. It should be noted that since ROS play an important physiological role in cell signaling, their removal with antioxidants may have deleterious consequences on cellular functions during chronic administration. Although iron chelators are poorly tolerated in vivo, chelation of the mitochondrial pool may be another avenue for therapeutic intervention. One promising example is the use of HBED, a lipophilic iron chelator that penetrates mitochondria following in vivo administration and inhibits seizure-induced hippocampal injury.

Recent advances have unequivocally shown that mitochondrial dysfunction and oxidative stress are an acute consequence of seizure activity. Another exciting yet unexplored area of research is the role of RNS in neuronal excitability and epileptogenesis. The precise mechanisms by which these processes contribute to the progression of epilepsy and whether chronic improvement of mitochondrial bioenergetics has an effect on epileptogenesis remain to be determined. To develop antiepileptic (and antiepileptogenic) therapies influencing mitochondrial bioenergetics and oxidative stress, it will be important to unravel the role of mitochondrial mechanisms in the disease process (i.e., how mitochondrial defects modify neuronal excitability).

See also: **Cell Damage/Excitotoxicity**: Molecular Mechanisms of Cell Death After Seizures; **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; Dietary Therapies for Epilepsy; **Metabolic Changes**: Role of Metabolic Substrates and Acid-Base Balance in Seizures.

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Glucose Metabolism and GABA$_A$ Receptor Dysfunction in Human Partial Epilepsy

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Introduction

Phosphorylation of synaptic receptors modulates several neurotransmission systems in the vertebrate central nervous system. It has been shown, in particular, that glutamate and γ-aminobutyric acid (GABA) ionotropic receptors can be phosphorylated on the intracellular loops of several of their subunits by known kinases. These kinases primarily phosphorylate the β or γ subunits – but not the α subunits – of the type A GABA receptor (GABA$_A$R).

A different phosphorylation mechanism was recently described for this receptor, and termed 'endogenous,' since it does not require exogenous kinase or kinase activators. The endogenous kinase must then be closely related to the receptor at the neuronal membrane. This phosphorylation mechanism prevents rundown of the GABAergic response of acutely dissociated hippocampal or cortical neurons. In the paragraphs below, we will briefly describe the characteristics of this mechanism and then show that this mechanism is deficient in the cortical zones epileptic seizures originate in patients with partial epilepsy.

Background

A New Mechanism: Glycolysis-Dependent Modulation of GABAergic Inhibition

The first step was to identify the GABA$_A$R endogenous kinase. The protein that was isolated after purification and phosphorylation tests of the receptor was not a known kinase, but rather a key glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A kinase activity of GAPDH, independent of its role as a dehydrogenase in glycolysis, has been described in rabbit muscles, where it was shown that GAPDH autophosphorylates using glycolytic ATP and then transfers the phosphate group to other muscle proteins – thus playing the role of a kinase. We showed that GAPDH is physically associated with the receptor and is partially colocalized with GABA$_A$R α1 subunits at the plasma membrane of neurons in culture.

We also showed that local synthesis of ATP occurred in washed cerebral cortex membranes when glyceraldehyde-3P (G3P), the specific glycolytic substrate of GAPDH, was supplied. These results indicate that the enzyme that follows GAPDH in the chain of glycolytic reactions, the phosphoglycerate kinase, is also present at the neuronal membrane and that it uses the product of the reaction catalyzed by the GAPDH to produce glycolytic ATP, very likely without diffusion. The ATP is then used for GAPDH autophosphorylation, probably also without diffusion. Next, the phospho-GAPDH phosphorylates the associated GABA$_A$R on the I2 intracellular loop of its α1 subunit, at two identified serine and threonine residues. The endogenous phosphorylation of the GABA$_A$R is thus a glycolysis-dependent mechanism. These results establish a direct link between glucose metabolism and GABAergic neurotransmission (**Figure 1**).

Does GABA$_A$R endogenous phosphorylation have a functional role? We showed, on acutely dissociated pyramidal neurons from rat cortex, that any factor supporting GABA$_A$R endogenous phosphorylation retards rundown of GABA$_A$ currents in whole-cell patch-clamp recordings, and that any factor preventing GABA$_A$R endogenous phosphorylation reinforces rundown. The addition of G3P to the

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MODELS

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Acutely Dissociated Neurons

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Introduction

Epilepsy is a term used for several chronic neurological syndromes characterized by abnormal and inappropriate neuronal activity and synchrony. Often the neuronal synchrony is so widespread in the brain that consciousness is impaired. The particular neurons that are affected, the particular neuronal abnormalities, the particular connections that are affected, and the particular synaptic (or non-synaptic) abnormalities appear to be at least as varied as the number of named epilepsy syndromes.

The study of the basic mechanisms of epilepsy has benefited from the use of many different in vitro experimental preparations, including the intact whole-brain model and progressively more 'reduced' preparations such as brain slices, acutely dissociated neurons, neuron fragments (e.g., dendritic fragments), and single channel patch preparations. The cells in the acutely dissociated neuron preparation remain sufficiently intact for studies of many kinds of cellular activity, including action potentials, voltage-gated currents, and currents associated with activation of specific ionotropic or metabotropic receptor/transmitter systems. The acutely dissociated neuron preparation is particularly well-suited for some types of investigations that focus on the intrinsic properties of neurons or their responsiveness to activation of specific receptors. There are, in addition, several advantages to recordings from dissociated cells as compared with recordings from single neurons embedded within intact brains or brain slices or single neurons in culture. Those advantages will be discussed and exemplified in this article.

Background - The Pros and the Cons

Most studies using the acutely dissociated cell are attempts to understand the roles of ion channels in action potential generation and patterning. Such studies of ionic

currents in acutely dissociated cells have been useful in defining the firing patterns characteristic of principal cells and interneurons of many brain areas, to compare developmental changes in these firing properties, and to contrast 'normal' and 'abnormal' cells from the same brain region. Acutely dissociated neurons have been obtained from various animal models of interest, as well as from tissues obtained during human surgery (e.g., resections for medically-refractory epilepsy, even when tissue is removed by aspiration).

There are important technical advantages of the preparation that make possible some approaches to studying electrogenesis that are difficult or impossible to accomplish in other preparations. First, acutely dissociated cells have reduced dendritic appendages (**Figure 1**), and as such are more suitable for voltage clamp studies because space clamping is considerably improved. Second, ionic currents are more amenable to pharmacological isolation. Thus, it is possible to study the currents underlying action potential and firing pattern generation, as contributed by both the soma and proximal dendrites. Finally, the isolated cell preparation offers the cleanest access to cell contents, for studies of specific mRNA by PCR.

There are obvious disadvantages to studying the acutely dissociated cell. Cells are literally torn from the brain during the isolation process. Perhaps surprisingly, the main disadvantage is not poor cell condition. Indeed, acutely dissociated cells routinely remain viable for hours during recordings. Rather, the main disadvantage is that the partial preservation of the cell produces a distorted view of the intact cell's properties. For example, complex firing properties that depend on distributed conductances along the somato-dendritic axis are no longer available for analysis. Another important disadvantage is the absence of synaptic (or non-synaptic) neuronal connectivity. Studies of cell-to-cell interactions are limited to an analysis of transmitter action – where the dissociated cell is one of the 'cleanest' preparations.

Although it is not possible for an isolated, acutely dissociated cell to generate seizure activity, the advantages of the preparation for addressing cellular questions makes it an important complement to other methods in the study of epilepsy.

Methods

The Preparation

There are many variations in the isolation protocol. We have varied our own protocol several times. In our current protocol, we used Sprague-Dawley rats; animals of essentially any age can be used (we currently use animals that are 21–35 days old). Preparation begins by anesthetizing the animal with 2-bromo-2-chloro-1,1,-trifluoethane (halothane) and decapitating it. The brain is quickly removed from the skull by first cutting the scalp along the dorsal surface of the head, then splitting the skull and carefully removing bone and dura mater from over the dorsal surface of the brain. A small spatula is used to lift the brain from the skull as it cuts through the cranial nerves. The whole brain is placed in ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 $NaHCO_3$, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 glucose, which is bubbled with 95% O_2/5% CO_2 to maintain a pH of 7.4. Blocks of tissue, several millimeters thick, and containing the region of interest (e.g., ventral hippocampal regions cut in the horizontal plane) are cut from each hemisphere. These blocks are sectioned at 400 to 500 μm (slightly thicker than slices used for electrophysiological studies on acute slices) using a Vibratome sectioning system, and transferred to ACSF solution at room temperature. Each slice is itself then dissected to isolate a particular brain region – e.g., CA3 or subiculum. The resulting pieces of tissue are approximately 1 mm square (and the thickness of the slice). Microdissection of brain slices is a useful way to refine the isolation of a specific brain region and cell type. Microdissection of slices is relatively straightforward in hippocampal/limbic system slices, since the boundaries for many subregions are very clearly visible with a dissecting microscope.

At least one enzyme treatment (sometimes more) is used to facilitate cell dissociation. The specific enzyme, its concentration, and the duration of exposure vary considerable among the investigators using these techniques. Our current enzyme protocol for acutely dissociating cells starts with the transfer of tissue pieces (<1 cubic mm) to a small chamber containing a PIPES dissociation solution (in mM): 120 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 PIPES, 25 Glucose, and 1 mg/ml Protease type XIV (pH 7.0 with NaOH) at temperature 31°C, where the tissue remains for 30–40 min. After enzymatic treatment, the tissue is washed three times with enzyme-free PIPES solution, and pieces remain in this solution until they are ready for dissociation. For the final step, a single piece of the enzyme-treated tissue is triturated by passing through progressively smaller tip sizes of fire-polished pipettes (approximately, 0.8, 0.4, and 0.2 mm tip diameters). The dissociated neurons are placed in an uncoated 35 mm Petri dish (Corning) containing ACSF solution bubbled with 95% O_2/5% CO_2. Cells are allowed to settle for 3–5 min by stopping the gas supply to the dish. Cellular debris can be reduced by gently replacing ACSF and allowing the cells to resettle. The petri dish is moved to the microscope stage for cell selection and recording.

Characterization of Intrinsic Neuronal Behavior

The acutely dissociated cell is an 'intermediate' preparation – more 'reduced' than a preparation involving neurons

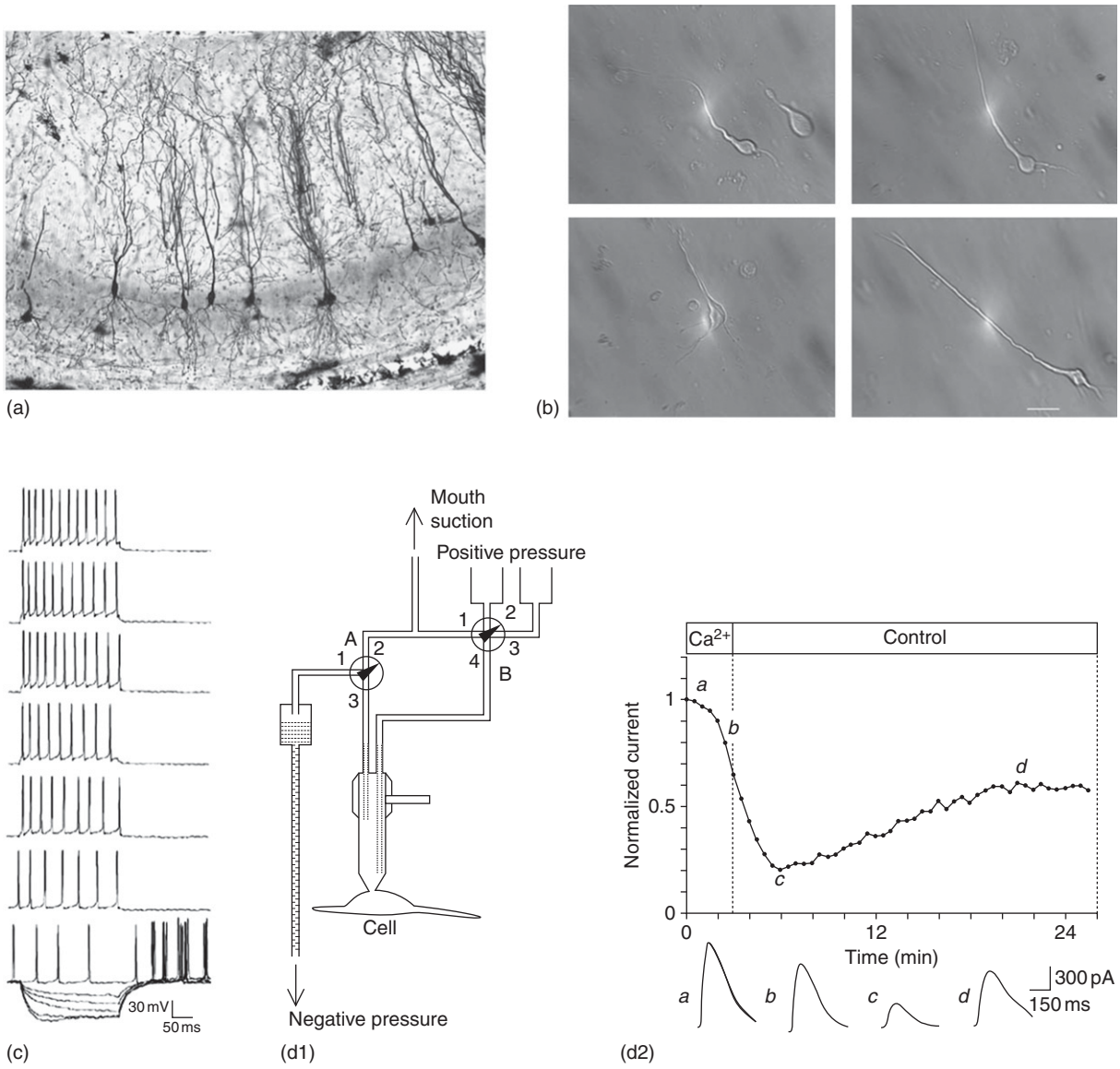


Figure 1 Examples of acutely dissociated neurons and recordings of voltage and ligand-gated currents in current-clamp and voltage-clamp configurations. (a) Golgi-stained pyramidal neurons in rat area CA1. Contrast the dendritic arbors of the intact cells (a) with the examples of acutely dissociated rat CA1 pyramidal neurons (b). (c) Current clamp recordings from area CA1 pyramidal neurons show action potential generation in response to depolarizing current steps and and voltage responses to hyperpolarizing current injection. Cell was held at −70 mV and stimualted with currents that were incremented in 50 pA steps. In part d, we illustrate the intracellular perfusion setup (d1) developed by Qiang Xiao Chen *et al.*, and an example of GABA-mediated whole cell currents (d2). The intracellular perfusion setup permits the switching of solutions inside the recording pipette and therefore inside the neuron while it is being recorded. The valve labeled B switches the perfusion solution; the valve labeled A is used to switch between mouth suction to establish the recording and steady 'pull' for perfusion. A full description of the intracellular perfusion setup is given in the publication. (d2) Effects of intracellular calcium ion concentration on GABA-A receptor-mediated whole cell currents. Top is a plot of normalized peak currents elicited by pressure ejection of GABA. Below are example current records at the labeled times. A high intracellular concentration of calcium (5×10–6 M) induces a rapid rundown of GABA-mediated currents. Switching to low intracellular calcium (1×10–8 M) reverses the rundown. Cell is voltage-clamped at −10 mV. We thank Bolek Srebro for the Golgi-stained tissue (a), and Wei-Ping Chen and Mark Stewart for the data shown in parts b and c. Both portions of part d were adapted from Chen, Stelzer, Kay, and Wong (1990) $GABA_A$ receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurons. *J. Physiol.* (*Lond.*) 420: 207–221.

in situ (e.g., brain slices, whole brain, or even tissue culture), but more 'complete' than subcellular preparations (preparations used for all forms of frank patch recording – e.g., inside-out, outside-out, etc.). Action potential discharge properties are typically studied with current clamp techniques. Voltage-clamping methods can be used to study currents, and take advantage of the dissociated cell's relatively compact electrical structure (for effective space clamping).

Pharmacological isolation of currents is particularly effective in this preparation, since the cell's membrane is entirely exposed to the extracellular environment.

The most commonly used recording methods (we will not discuss any of these in detail here) employ low resistance pipettes for whole cell recording techniques (various configurations – whole cell mode, perforated patch mode, and cell-attached patch mode). The selection of recording configuration and pipette solution will depend on the specific goals of the study. It has been shown that the ionic composition of the low resistance pipettes can itself be a significant variable in such studies, since ingredients of the pipette solution can block some channels or substantially alter the kinetic properties of these channels. In general, to assess firing properties of neurons, the pipette solution should be 'physiological.' Alternatively, the investigator may choose a recording configuration (such as perforated patch) that will minimally alter the intracellular contents of a cell. To study particular currents, one can also use pipette solutions that will be helpful in isolating currents of interest – e.g., QX-314 to block sodium channels, or cesium ions to block potassium channels). Any study of how a particular cellular 'pathology' (such as pacemaker bursting) or a genetic mutation affecting an ion channel (channelopathy) contributes to epileptogenic mechanisms requires an understanding of the baseline characteristics of intrinsic currents of the neuron. Such measurements and comparisons can be made on cells from 'normal' and 'experimental' tissue.

A general plan for characterizing the acutely dissociated cell includes the following:

1. Determine the basic properties of a cell type by recording the cell's behavior in current-clamp mode, to define the action potential firing capabilities and patterns of the neurons. This behavior can be compared to the firing patterns of comparable cells in slices or in 'intact' preparations. Injected current (steady direct current, or brief pulses) can be used as the stimulus to evoke action potential firing.
2. Make a qualitative assessment of currents that are activated in response to hyperpolarizing or depolarizing stimuli. Determine the channels that are involved in suprathreshold activity (e.g., burst firing vs. regular spiking; various firing rates) and subthreshold changes (e.g., membrane oscillations), by testing the effects of specific channel blockers (one at a time).
3. Make a quantitative assessment of currents, using voltage-clamp techniques in the presence of drugs that will block all channels except the one you wish to study.

From a practical standpoint, experimental emphasis on a small subset of these guidelines (e.g., studies of a single current) can be used to compare cells from animals of different ages, or to compare cells from normal animals with cells from a disease model (e.g., following kainic acid treatment or kindling). In studies of neurons removed from human patients (tissue resection as part of a surgical treatment), it is difficult to establish the supra and subthreshold parameters for 'normal' cells. As such, conclusions about disease-related features of human cells are risky.

Characterization of Receptor and Channel Properties

The acutely dissociated cell cannot, by definition, be used as a model to study seizures per se. However, the preparation can be used to study abnormalities in cellular properties that contribute to seizure generation. For example, transmitter receptor agonists and antagonists, as well as ion channel blockers, can be applied to a recorded cell using any of a variety of methods: pressure ejection, iontophoresis, and bath application are all appropriate methods. The special advantage of the dissociated cell preparation is that a drug delivery device can be placed anywhere in relation to the recorded cell, and can be moved within the recording chamber without substantially disturbing the recording. This feature permits the rapid application not only of single drugs, but also of multiple drugs, to a given cell.

Results – Examples of the Preparation's Utility

Endogenous Bursting and Pacemaker Cells

One definition for a 'seizure generator' is a brain region containing 'pacemaker' neurons to drive the activity, and connectivity to the surrounding tissue to cause synchrony. While the acutely dissociated cell preparation cannot evaluate connectivity, it is ideal for the study of pacemaker properties – those intrinsic neuronal properties that lead to a burst firing pattern. These properties may be observed as properties of normal cells (physiological pacemakers), such as hippocampal CA3 pyramidal cells and subicular pyramidal cells, which have endogenous burst firing properties. We have compared properties of normal, acutely dissociated CA1 pyramidal cells in hippocampus with subicular pyramidal cells; the acutely dissociated subicular cells (by not CA1 cells) are characterized by a pattern of inward currents (when the cells are stimulated with depolarizing current) that is consistent with action potential burst generation. Burst electrogenesis in a single neuron is a complex phenomenon that appears to vary across cell types (even among closely related areas such as CA1, CA3, and subiculum) and is a key determinant of a pacemaker region.

Action potential generation, and more specifically burst electrogenesis, depend on voltage-gated sodium and calcium currents. Accordingly, these currents have been studied in various animal models of epilepsy and in

cells from human patients with epilepsy. Calcium and potassium currents have been shown to be altered in acutely dissociated neurons from rat dentate gyrus and hippocampal CA1 after kindling. Changes in calcium currents from neurons of human dentate gyrus, potassium currents from human neocortical neurons, and sodium currents from human entorhinal and subicular neurons have all been hypothesized as possibly contributing to seizure generation (or at least characterizing an epileptic brain region).

T-current and absence seizures. The acutely dissociated cell, with its advantages for voltage clamp studies of ionic currents, has been useful for evaluating the role of the transient, low-threshold (T-type) voltage-gated calcium current in absence seizures. 'Rebound' bursting in thalamic relay neurons was found to be suppressed by ethosuximide (a drug commonly used to treat absence seizures), and the acutely dissociated cell preparation was used to demonstrate that ethosuximide selectively blocks the T-current in these thalamic cells. A similar study of the T-type calcium current showed that this current is prominent in hippocampal pyramidal cells prior to postnatal day 30, but disappears after this age. Interestingly, ethosuximide did not appear to affect the T-currents in immature hippocampal CA1 pyramidal cells, suggesting that there are different subtypes of T-channels present in thalamic vs. hippocampal neurons.

Ionotropic receptor properties. The hypersynchronized activity that defines a seizure is largely dependent on glutamate receptor activation, and is prevented (at least to some extent) by synaptic inhibition. Many dissociated cell studies have been aimed at defining the properties of ionotropic receptor types, such as GABA and NMDA, as well as defining the pharmacological modulation of receptor-ion channel properties. An important aspect of these studies has been the comparison of the receptor properties of normal vs. abnormal neurons. For example, larger amplitude NMDA receptor-mediated currents were found in dentate granule cells isolated from kindled rat brains, as compared to currents in normal control granule cells. The sedative and anticonvulsant properties of drugs such as pentobarbital and phenobarbital were examined in acutely dissociated CA1 neurons in studies that contrasted the differential drug actions on calcium currents and GABA receptor-mediated currents.

Metabotropic receptor properties. The preservation of an intact cell soma in acutely dissociated cells suggests that many intracellular signaling pathways – e.g., involving g-protein coupled receptors ('metabotropic' receptors) – remain intact. Previously, it was thought that such structurally complex receptors would not survive the enzymatic action used for the cell dissociation. However, through careful monitoring of enzymatic concentration and duration of treatment, robust g-protein coupled receptor activity can be preserved. This development, together with the accessibility of the intracellular space in the acutely dissociated cell, has made it possible to characterize intracellular signaling cascades activated by these g-protein coupled receptors. Interestingly, one prominent metabotropic receptor-mediated current, the M-current, is not seen in acutely dissociated cells. This absence may be explained by the recent finding that these channels are localized near the axon hillock. The cholinergic receptors coupled to the M-current may be located on the membrane of acutely dissociated cells, but the coupled potassium channels (KCNQ) required for the M-current appear to be eliminated by the dissociation process.

Currents recorded as a consequence of receptor activation in the acutely dissociated cell preparation (as well as other preparations that employ 'bulk' drug application) will necessarily include an unknown combination of synaptic and extra-synaptic receptor-mediated channel activation. To specifically study synaptically-mediated channel activation, it is possible to preserve attached vesicle-containing boutons with the isolation of neuronal somata during the dissociation process. The dissociation process is similar to the one described above, with the critical differences being in the concentrations of calcium (0–0.2 mM) and magnesium (5 mM) salts used during the isolation and incubation steps. The procedure that maintains the attached boutons permits the investigator to observe miniature synaptic currents in a preparation that is well-suited for high resolution voltage clamp recordings.

Studies of genetic models. The genetically-altered animal is an increasingly useful tool for the neuroscientist. Channels, pumps, or signaling cascades that determine channel behavior can be studied in acutely dissociated cells in order to determine the consequences of genetic manipulations at the level of simple cell properties. The acutely dissociated cell preparation can be useful for testing the physiological consequences of altered channels in genetically engineered animals, or for evaluating altered channel behavior as a possible basis for the seizures in a diseased animal or human. This approach may be particularly useful in studying specific channelopathies that have been associated with some seizure disorders (such as Severe Myoclonic Epilepsy of Infancy).

As an adjunct to physiological studies on tissue with potential genetic abnormalities, a potentially useful manipulation is to employ single cell PCR techniques, to obtain, amplify, and identify specific genetic material (or its absence or modification, in the case of knock-out or transgenic animals) from a single cell. With the dissociated cell preparation, this process is considerably simplified since one is spared the task of aspirating the contents of a cell (the required procedure when single cell PCR is applied to neurons, e.g., in slices) because the whole cell can be processed directly, with a small risk of contamination by cellular material from other cells.

Future Directions and Conclusions

The set of tools for manipulating ion channels and other proteins in cells continues to grow. These manipulations have led to numerous insights into cellular physiology for many cell types, including neurons. Critical to our understanding specific channel or protein function is experimental access for measurements of complex cellular processes. The acutely dissociated neuron preparation offers superior access for studies of coordinated ion channel function that is required for action potential generation and changes in firing properties. It will likely continue to find utility in exploring the ever-widening spectrum of specific cellular mutations that can be produced by genetic manipulation, and as one of the most versatile tools for functional studies of human biopsy material.

We have offered some examples of how the acutely dissociated cell preparation can be useful for studying the properties of neurons (or, in theory, even glia) that may contribute to epilepsy (e.g., burst electrogenesis), for studying the changes in cells that may provide the basis for seizure activity, and for testing the effects of drugs that may act to interfere with channels and receptors that participate in epileptiform activity. As the genetic bases for cell physiology are defined, we predict that the acutely dissociated cell soma – with its intact nucleus – will be further developed into a preparation that allows the direct correlation of genetic and physiological studies. Thus, the preparation has great utility in elucidating basic properties that may be involved in seizure-related cellular behavior. Since dissociated cells cannot, in themselves, generate seizure-like 'models,' the dissociated cell approach is most powerful when applied in combination with studies of animal models or human epileptic tissue.

see also: **Absence Seizures**: Thalamic Neurons and Networks Related to Absence Epilepsy; **Glutamate**: Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; **Ion Channels**: Channelopathies in Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; Seizure Models in Acute and Organotypic Slices; The use of Cell Culture Models to Study Mechanisms Related to Epilepsy and Antiepileptic Drugs; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization.

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Characteristics and Mechanisms of Kindling

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Introduction

A consistent theme emerging from the first century of research in neuroscience is that the brain does not respond passively to its environment. The active brain generates patterns of neural firing and produces behavior spontaneously. Likewise, the adaptive brain does not respond passively to interventions such as lesions or electrical stimulation; it reacts to the intervention and is modified in a lasting way by the experience. Not surprisingly, epilepsy is characterized by active and adaptive changes in brain function, as suggested in several preparations of experimental epilepsy. However, the progressive and adaptive nature of neural changes is not easy to track in most preparations, due to the unpredictability of, and lack of, experimental control over seizure development. A more predictable pattern of seizure development, with excellent experimental control, is found in the kindling preparation, and kindling has become the dominant preparation for studying this kind of active and lasting plasticity in experimental epilepsy. In this article we review the characteristics and potential mechanisms of kindling, with an emphasis on both enduring findings and recent developments. We start with a brief description of the discovery of kindling.

Background

Discovery of Kindling

Early investigators of the phenomenon of intracranial self-stimulation (ICSS) described the occurrence of the development of either electrographic seizures or behavioral convulsions during ICSS of sites in the forebrain. A number of early researchers interested in epilepsy also stumbled over the progressive development of seizures after repeated application of either drugs such as cocaine or focal electrical stimulation. Perhaps the clearest example of this is found in a series of studies from Jose Delgado's laboratory at Yale in the late 1950s and early 1960s, in which it was observed that behavioral seizures developed during repetitive ictal electroencephalographic (EEG) discharge. However, none of the researchers observing seizure development followed up on the observation systematically.

Graham Goddard's doctoral research in Psychology at McGill University in the early 1960s was concerned with the effects of electrical stimulation of the amygdala on conditioning in rats. Because of an equipment malfunction, repeated short trains of fairly high-intensity stimulation were accidentally delivered to the amygdala of some rats, one of which developed seizures after a few repetitions. The rat continued to display seizures even after the equipment had been repaired, and similar stimulation given to naive rats did not cause seizures. Goddard realized that it was the repeated application of stimulation that led to seizure, not the stimulation itself; after observing the phenomenon in other rats, Goddard surmised that the brain was changing in response to the repeated application of stimulation. Because stimulation initially produced no behavioral effects and the convulsions developed gradually, Goddard and his students called his phenomenon 'kindling.' They went on to show that kindling results in a state of increased susceptibility to convulsions that is long-lasting if not permanent, and that kindling can be observed in other species, including mice, cats, and monkeys. Subsequent research by the eminent neurologist Juhn Wada confirmed the occurrence of kindling in cats and primates, and first documented the development of spontaneous seizures during kindling.

Inspired by Goddard's preliminary observations, Ronald Racine carefully monitored EEG activity during kindling and convincingly demonstrated that stimulation has to elicit afterdischarge (AD) in order to kindle seizures. He found in addition that repeated stimulation significantly lowers threshold for AD in amygdala and hippocampus, and that threshold reduction persists even after a 6-week period without stimulation. The changes in threshold are localized to the area stimulated, in that there is generally no effect on thresholds measured in other limbic sites. Racine also conceived the idea of testing for transfer of both electrographic and behavioral kindling between sites in the forebrain. He found that kindling with stimulation of a limbic or cortical site results in a facilitation of subsequent kindling from other limbic or cortical sites, respectively, and that this transfer effect persists even after lesioning of the initial site of kindling. Thus transfer is not due merely to increased epileptiform output from the site of initial stimulation, but instead involves sensitization of a substrate for seizure that can be activated by multiple cortical and subcortical areas. Finally, Racine demonstrated that kindling is associated with potentiation of excitatory synaptic transmission, as discussed later.

Methods

The seminal experiments of Goddard's and Racine's groups have defined the paradigm for most subsequent research on kindling. In typical kindling studies, regardless of species, experimenters apply short trains of electrical stimulation, typically 1 or at most 2 s in duration. The waveform is usually balanced biphasic square pulses, although some experimenters apply monophasic (cathodal) pulses or sine waves. The intensity of stimulation is set at, or a fixed amount above, the threshold for triggering AD. EEG is recorded from the stimulated site and one or more additional sites, usually for 30 or more seconds before and 2 or more minutes after each stimulation.

Results

Characteristics of Kindling

Behavioral characteristics

There are at least two distinct patterns of kindling, determined by locus of stimulation: a limbic forebrain pattern and a neocortical (nonlimbic) pattern. The pattern of seizure development evoked by stimulation of limbic sites best exemplifies kindling, because it involves gradual emergence of partial seizures, hemiconvulsions, and then generalized convulsive seizures from an initially negative behavioral response. That is, early limbic ADs are associated with nonconvulsive responses, often simple behavioral arrest, and these are gradually replaced by frank convulsive movements as the AD develops and spreads, resulting in generalized seizures that become more intense with repeated stimulations. In contrast, stimulation of neocortex and several other sites (e.g., claustrum, perirhinal cortex, brainstem) typically evokes a brief forced motor response followed by a clonic or short clonic–tonic seizure. With repeated stimulations, the convulsive response often evolves to include a late-phase limbic-type generalized clonic–tonic seizure.

Electrographic characteristics

Stimulation of limbic and paleocortical sites initially triggers relatively simple ADs of short duration, low amplitude, and minimal propagation. With repeated stimulations, limbic AD dramatically increases in duration and amplitude, and it propagates widely to distant sites, many of which eventually develop patterns of AD that are independent of the stimulated site. In contrast to the striking changes in limbic or paleocortical AD during kindling, initial stimulations of the anterior or posterior neocortex or some other sites (e.g., claustrum) fail to result in any appreciable changes in the duration or amplitude of primary-site ADs. When repeated stimulation of the anterior neocortex results in the late development of generalized limbic-type seizures, however, there is an abrupt increase in the duration and amplitude of neocortical ADs associated with development of independent AD in the amygdala.

An important feature of the electrographic response during kindling is its sensitivity to the interstimulation interval. Goddard and colleagues found that seizures can reliably be kindled when stimulations are sufficiently spaced. Racine's work showed that the reliability of kindling is related to the ability of stimulation to trigger AD, with AD occurring most reliably at interstimulation intervals of 2 h or greater; stimulation at shorter intervals produces short-lasting inhibitory effects that can suppress AD or the behavioral seizures triggered by AD. These inhibitory effects of 'massed' stimulation must be taken into account in studies attempting to identify physiological, biochemical, or morphological correlates of kindling.

Neural circuitry

Numerous studies have been concerned with identifying the neural circuitry underlying kindling. A common strategy is to map the susceptibility of different sites on the assumption that sites kindling more rapidly are closer to the substrates of convulsive seizure generalization. The early research pointed to the amygdala as a highly susceptible site, and more recent work has identified high susceptibility to kindling in the piriform cortex, perirhinal cortex, claustrum, thalamic nuclei (e.g., dorsomedial nucleus, nucleus submedius), and particular nuclei in the brainstem (e.g., mesencephalic reticular formation, central gray, reticularis pontis oralis), suggesting that the limbic generalization network may be quite widespread. A second strategy is to track the patterns of seizure discharge that occur during establishment of seizures (epileptogenesis) or during the triggering of an established seizure. Another strategy is to destroy a structure implicated by its susceptibility to kindling or electrographic criteria. This approach has resulted in evidence strongly suggesting that the perirhinal cortex, piriform cortex, claustrum, and thalamus play important roles in generalization. For technical reasons, the effects of brainstem lesions have not been examined.

Interictal behavioral consequences

The behavioral consequences of kindling that are specific to the site being kindled have been examined for several decades. The results suggest, overall, that kindling produces modification of function in the structure or circuits subjected to kindling, as would be expected if kindling involves a pathological distortion of the neuronal mechanisms involved in normal plasticity. Discussion of these results are dealt elsewhere.

Mechanisms

Potential mechanisms of kindling have been investigated at the electrophysiological, morphological, and molecular levels. We provide a brief introduction to what is known to date.

Electrophysiology

Three dominant themes have emerged in this research: (1) the hypothesis that kindling-induced potentiation (KIP) of excitatory synaptic drive is the cellular mechanism of kindling; (2) the hypothesis that erosion of the synaptic inhibition mediated by GABA leads to the elaboration and spread of AD underlying kindling; and (3) the hypothesis that kindling is due to the development of the burst response, due to alterations in either network properties or cellular membranes that lead neurons to become 'epileptic.' These hypotheses are of course not mutually exclusive, and the events they postulate could either be correlated or causally related. For example, kindling could involve a shift in the balance between excitatory drive and inhibitory control, and repeated episodes of enhanced excitation and diminished inhibition could in turn lead to membrane or network changes that result in bursting in vulnerable neurons.

Kindling-induced potentiation

Prior to the first report of long-term potentiation (LTP), Racine and colleagues in the 1970s showed that kindling stimulation produces potentiation of potentials evoked in secondary sites, particularly late (presumably polysynaptic) components of the potentials. Even nonepileptogenic tetanic stimulation produces long-lasting effects that can result in facilitation of subsequent kindling, although something more than KIP seems to be required for kindling. Subsequently, Goddard and his collaborators established the paradigm of seeking correlations between kindling and changes in extracellular potentials evoked in an area connected monosynaptically to the site of kindling stimulation; the typical site of stimulation was the perforant path, and evoked potentials were monitored in the dentate gyrus. Their results, and those of others employing the same paradigm, are unfortunately inconsistent, with some studies reporting enduring KIP of both the excitatory postsynaptic potential (EPSP) and population spike (PS), others of the EPSP alone, others of the PS alone, and others of neither the EPSP nor the PS. Given the inconsistency of the results, it is clear that kindling is not reliably correlated with potentiation of monosynaptic responses evoked in the dentate gyrus.

Other studies have employed kindling stimulation at sites other than the hippocampal formation and have confirmed that KIP is not restricted to hippocampal kindling. A form of KIP occurs in the piriform cortex during olfactory bulb kindling, the persistence of which exceeds that previously described with kindling of other sites. This observation raises the possibility that KIP in the piriform lobe could be a candidate mechanism of kindling. It is not likely that these forms of extrahippocampal KIP can account for kindling, however, both because they reach maximum before kindling of behavioral seizures and because not all subjects that kindle show KIP. A plausible explanation for these findings is that KIP at the first synapse could result in sequential potentiation of successive synapses in a multisynaptic neuronal network, a process that would not necessarily be evident in the field potentials recorded at the first synapse in the system.

Disinhibition

A straightforward hypothesis for kindling, and the development of epilepsy in general, is that a physiological process results in disinhibition. Although there is considerable evidence for an inhibitory effect of noradrenaline (NA) on epileptogenesis and for an association between kindling and a decrease in NAs effects, most of the research has focused on a putative collapse of inhibition mediated by GABA. This hypothesis has been tested in numerous studies employing the paired-pulse technique to measure the strength of inhibition, where it has been found that limbic kindling produces KIP of feedback inhibition in the dentate gyrus, at both short and long interpulse intervals. Kindling also produces potentiation of feed-forward inhibition in the dentate gyrus. It is obvious that KIP of inhibition is contrary to the collapse of inhibition predicted by the GABA hypothesis. The picture is different when inhibition is measured in other sites, however. In field CA1, in particular, kindling is associated with decreased paired-pulse inhibition, and in some studies this decrease has been related to a decrease in the number of GABA-immunoreactive neurons in the hippocampus. Furthermore, research by Schinnick-Gallagher and by Leung have shown that kindling results in decreased sensitivity of a presynaptic $GABA_B$ receptor, in amygdala and hippocampus. These results suggest that disinhibition in the hippocampus, amygdala, or elsewhere might contribute in two ways to limbic kindling. First, loss of the specific subpopulation of GABAergic interneurons producing feed-forward inhibition would lead to a decrease in inhibitory postsynaptic potentials (IPSPs), thereby increasing the effects of excitatory input. Second, a long-lasting decrease in the sensitivity of presynaptic $GABA_B$ receptors would lead to increased release of glutamate or other excitatory neurotransmitters, and this action could in turn alter the systems properties of affected neural circuits such that runaway excitation would result. According to this hypothesis, disinhibition and increased excitatory drive interact profoundly, with the outcome being the development of epileptiform bursting in amygdaloid neurons.

Bursting

Bursting involves a pattern of high-frequency discharge of action potentials, usually riding on a sudden and large

(20–50 mV) depolarization of the membrane – the paroxysmal depolarizing shift (PDS) that can last for 100 ms. Interictal discharge is the EEG correlate of bursting. PDSs synchronize over a group of neurons during the transition from normal to epileptiform activity. Finally, bursting (i.e., the PDS) is the cellular behavior observed during AD triggered by a variety of treatments. Bursting is a widespread correlate of kindling that has been observed in field potential recordings and single-cell recordings, both extracellular and intracellular. Burst discharge is most prominent in the amygdala and piriform cortex, but has also been observed in the dentate gyrus, hippocampus, substantia nigra, locus coeruleus, and elsewhere. Intracellular recordings have shown that multiple action potentials typically riding on a PDS are the correlates of the bursting recorded extracellularly, similar to that observed in other forms of experimental epilepsy. Thus bursting is likely to play an important causal role in kindling, perhaps in conjunction with diminished inhibition.

The question then becomes whether the development of epileptiform bursting is a consequence of alterations in endogenous passive membrane properties of the neuron, or whether it is network driven, a distinction made by Johnston and Brown. The evidence is that kindling fails to affect passive or intrinsic membrane properties of neurons in most of the areas that have been tested, including the amygdala, piriform cortex, and hippocampus proper. In contrast, granule cells of the dentate gyrus display long-lasting changes after kindling, including increased input resistance, alterations in Ca^{2+} currents and Ca^{2+} inactivation, and evidence of enhanced participation of NMDA receptors in baseline synaptic transmission. For most sites, however, available evidence indicates that the bursting associated with kindling generally reflects epileptiform activity in a network of cells driven by synaptic transmission. This conclusion is based, in part, on the lack of evidence for alterations in intrinsic membrane properties in areas other than the dentate gyrus. If endogenous bursting is not implicated, network-driven bursting is the only likely alternative. Stronger evidence in favor of network-driven bursting is, however, available from studies investigating the effects of intracellular current injection on bursting, which have shown that the occurrence of bursting (or PDSs) after kindling is unaffected by depolarization of the membrane, suggesting that the kindling-associated bursting it is not due to endogenous bursting, which is voltage dependent. Other evidence suggests that kindled bursting in several sites, including amygdala and hippocampus, involves persistent activation of NMDA receptors. Collectively, these results support the hypothesis that kindling involves a long-lasting increase in the release of glutamate or a closely related excitatory amino acid, a mechanism that is both a cause and an effect of changes in inhibitory neurotransmission.

Morphological changes

Most investigators have found that inducing a limited number of seizures fails to produce evidence of widespread damage. Thus potential morphological substrates of kindling must be subtler than overt damage. At the light microscopic level, there has been evidence of an association of kindling with sprouting of the mossy fibers of the dentate gyrus, as seen in other preparations of experimental epilepsy and in resected tissue from patients with epilepsy. Because the mossy fibers are normally excitatory, this sprouting has been hypothesized to create aberrant excitatory loops that contribute to epileptogenesis. Contrary to the hypothesis, however, numerous studies have described dissociations between mossy fiber sprouting and kindling in several species and different kindling paradigms. Thus, for example, the time course of rapid kindling can be dissociated from mossy fiber sprouting, with generalized seizures developing well before any sprouting is evident. One interpretation of these results is that sprouting is not causally related to kindling but may be a consequence of kindling that contributes to both the intensity of kindled seizures and the behavioral comorbidities measured after kindling.

Even if mossy fiber sprouting was reliably associated with kindling, its interpretation would be clouded by recent research from the laboratory of Rafael Gutierrez. He has found that seizures are associated with an alteration of the phenotype of dentate granule cells, which give rise to the mossy fibers, such that normally excitatory glutamatergic neurons become capable of synthesizing and releasing GABA. Thus, sprouted mossy fibers may produce inhibitory as well as excitatory effects after seizures, which could explain the increased inhibitory drive seen in the dentate gyrus after kindling.

Yuri Geinisman first reported an increase in the proportion of specific, highly efficacious perforated synapses within the kindled hippocampus, at the ultrastructural level. Teskey has found that this observation generalizes to other structures, and recently reported an increase in perforated synapses in the neocortex following kindling in that area. Geinisman has published three-dimensional reconstructions that suggest that perforated synapses are, in fact, formed from nonperforated synapses, either as an endpoint of greater synaptic efficacy or as an intermediate before splitting into two separate synapses. He has also suggested that increases in the proportion of perforated synapses represent selective maintenance of a more efficacious subtype of synapse that is important for sustaining KIP. Perforated synapses may be more efficacious because of the narrower synaptic cleft between presynaptic and postsynaptic membranes at the site of the perforation and because of the additional 'edges' at the perforation sites. Perforated synapses also have ~6.5 times greater AMPA receptor density and 80% higher NMDA receptor density, and are significantly larger, than nonperforated synapses.

Molecular changes

Much of the research on possible molecular mechanisms of kindling has focused on changes in markers for neurotransmitters, and a vast literature has been generated over the past four decades. Fairly consistent results have suggested that GABAergic and noradrenergic function is diminished in areas other than the dentate gyrus, supportive of the hypothesis of disinhibition. In parallel, other molecular markers suggest that the excitatory effects of glutamate are increased, for example at NMDA receptors. Particularly striking is work by John Slevin's group showing a long-lasting increase in release of glutamate after kindling. This finding suggests that excitatory drive is increased, which might result in increased network-maintained bursting. James McNamara and colleagues have reported that conditional deletion of the trkB receptor for BDNF in mice results in the complete abolition of kindling, suggesting that particular neurotrophins are critical for epileptogenesis. The group of mice showing the effect comprised only four animals, however, and acceptance of the results awaits replication in another laboratory.

There are large species differences in susceptibility to kindling, suggestive of an important role for genetic mechanisms. For example, rats develop generalized seizures fairly quickly, whereas guinea-pigs are extremely resistant to seizure generalization. Similarly, during kindling, baboons develop bisynchronous and bisymmetrical primary generalized seizures, both triggered and spontaneous, whereas macaques develop only asynchronous and asymmetrical secondary generalized seizures and fail to show spontaneous seizures. A very promising approach to genetic determinants of susceptibility to kindling is being conducted by Dan McIntyre and colleagues. They have been examining molecular differences between the fast and slow kindling strains of rat, originally developed by Racine through inbreeding based on their rate of amygdaloid kindling. The fast strain requires only half as many stimulations and the slow strain requires up to three times more stimulations as 'normal' Long-Evans rats to kindle. Fast rats are more susceptible to the convulsant effects of GABA antagonists, and slow rats are more susceptible to the depressant effects of GABA agonists, suggesting that GABAergic transmission may be altered in the strains. In confirmation, fast rats show underexpression of the adult form of the $\alpha 1$ subunit of the $GABA_A$ receptor, and overexpression of neonatal forms of α subunits; and the reverse pattern is found in slow rats. Thus, enhanced susceptibility to kindling is, in this preparation, associated with developmental abnormalities in inhibitory mechanisms and this immaturity is, in turn, reflected functionally in differences in miniature IPSCs in the perirhinal cortex. McIntyre's ongoing research is mapping out differences in gene expression in the fast and slow strains, and should provide additional insight into key mechanisms.

In other recent research concerned with changes in gene expression associated with kindling, Corcoran and his colleagues have been using microarray analysis to compare expression of genes after hippocampal kindling and after amygdaloid kindling, with the view to identifying the gene changes possibly related to the behavioral consequences specific to kindling of that site. Ontological analysis of amygdaloid kindled tissue reveals significant enrichment in expression of categories of genes related to cytoskeletal reorganization and biogenesis, cation transport, glutamatergic neurotransmission, and neurogenesis. Hippocampal kindled tissue also displays enrichment in expression of genes related to cytoskeleton reorganization and biogenesis and cation transport, and unique enrichment in transcription factor activity and GTPase-mediated signal transduction. Slevin's group has been using proteomics to examine changes in proteins, the product of gene expression, after kindling. Kindling is associated with significant increases in the catalytic subunits and decreases in the regulatory subunit of the mitochondrial ATP synthase complex, and with large increases in the subunits of the synaptic vesicle H^+-ATPase involved in transmitter uptake. Thus, at the molecular level, both energy metabolism and transmitter uptake show a long-lasting increase in kindled tissue.

Conclusions and Future Goals

Kindling is one of the most dramatic and enduring changes in brain activity produced by focal electrical stimulation. It is a function of the repeated induction of AD, which grows, propagates, and triggers the development of behavioral seizures. The permanence of the kindled increase in seizure susceptibility suggests that dramatic reorganization of neural structure and function is involved, and research into mechanism has been pursued at the molecular, electrophysiological, and morphological levels.

Although much has been learned about the characteristics and mechanisms of kindling, much more remains to be learned. For example, kindling results in several morphological changes, but none can yet be viewed as convincingly accounting for the permanent change in seizure susceptibility underlying kindling. Furthermore, many laboratories are working to identify possible molecular substrates of kindling. Analysis of the epigenetic changes associated with kindling is particularly promising, and may provide important insights into the fundamental mechanisms of epileptogenesis. Additional characterization of the behavioral consequences of kindling should help us to understand the comorbidities of the clinical epilepsies, and future research should address the intriguing possibility that molecular or other interventions will be able to dissociate the behavioral changes consequent to kindling from the induced epileptic state. Finally, notwithstanding the provocative results of

preliminary studies and anecdotal reports, a key question remains as yet unanswered: Do kindling-like events underlie the occurrence of some kinds of epilepsy in humans?

The 'engram' of kindling (in Goddard's terminology) has not yet been identified conclusively, but there is every reason to expect that investigation of kindling will continue to generate insight into fundamental mechanisms of the brain's active responses to change in its environment, as well as into epilepsy and epileptogenesis.

See also: **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Model Characterization in Relationship to Human Disorders; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Peptides**: Cannabinoids and Epileptiform Activity; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Seizure-Induced Alterations in Motor and Sensory Maps; Synaptic Potentiation and Inhibition after Partial Seizures.

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Computer Models of Seizures and Epilepsy: Understanding Epileptogenesis using Complex Large-Scale Models

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Introduction

Scientific understanding of the complex circuits involved in epileptogenesis and seizures has traditionally been obtained through the use of animal models, culture systems, and numerous other experimental approaches. In the past several decades, however, computational modeling has become an increasingly useful tool for studying and understanding neuronal network structure and function. In epilepsy research, such models have been employed to investigate the role of specific pathologies in epileptogenesis, in conjunction with experimental work or as stand-alone modeling studies. These models vary greatly in both scope (e.g., a given model can range from simulations of the effect of mutations on specific ion channels to representations of entire brain areas and their associated connectivity)

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Computer Models of Seizures and Epilepsy: Understanding Epileptogenesis using Complex Large-Scale Models

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Introduction

Scientific understanding of the complex circuits involved in epileptogenesis and seizures has traditionally been obtained through the use of animal models, culture systems, and numerous other experimental approaches. In the past several decades, however, computational modeling has become an increasingly useful tool for studying and understanding neuronal network structure and function. In epilepsy research, such models have been employed to investigate the role of specific pathologies in epileptogenesis, in conjunction with experimental work or as stand-alone modeling studies. These models vary greatly in both scope (e.g., a given model can range from simulations of the effect of mutations on specific ion channels to representations of entire brain areas and their associated connectivity)

and complexity (e.g., models can consist of simple integrate-and-fire neurons, or they can incorporate realistic channels and conductances, combined with detailed three-dimensional reconstructions of their anatomical features). While each specific model is uniquely suited to answering a particular question, understanding the intricacies of complex pathological states such as epileptogenesis requires approaches that combine complex single cells into large networks with a broad scope. Fortunately, the extraordinary increases in computational power over the last 10 years have made this type of approach quite feasible. This article will illustrate how complex single-cell models, derived from and replicating experimental data, can be combined into large-scale networks to yield substantial increases in our understanding of the relationships between structure and function of large, pathologically relevant brain areas such as the dentate gyrus in the hippocampus.

Background

The dentate gyrus in the mammalian brain is composed of a complex neuronal circuit that is thought to serve as a gate for activity propagation throughout the limbic system. Following repetitive seizures, head trauma, and ischemia, the dentate gyrus undergoes marked structural reorganization, primarily due to loss of hilar cells and the occurrence of mossy fiber (granule cell axon) sprouting, the hallmarks of seizure-induced end-folium sclerosis in temporal lobe epilepsy. Within the resulting, fundamentally transformed dentate gyrus microcircuit, there are simultaneously ongoing alterations in intrinsic cellular and synaptic properties, prompting the question of how to study the impact of network structural changes in isolation.

Computational modeling offers a unique tool to help isolate and demonstrate the effects of individual pathologies, allowing for the implementation of only structural changes in the network while leaving intrinsic neuronal and synaptic properties unaltered. Several studies (see Further Readings) have examined potential epileptogenic roles for enhanced synchronization and activity propagation in small-world network architectures using idealized model networks. However, to model processes involved in epileptogenesis, and to make confident experimental predictions, model networks must be strongly data-driven and include detailed structural and functional properties.

In this article, we discuss results from research employing a complete 1:1 scale structural model of the rat dentate gyrus (over 1 million nodes with full connectivity), as well as a 1:20 scale functional model composed of more than 50 000 multi-compartmental neurons, representing four different excitatory and inhibitory cell types, each reliably reproducing firing properties from electrophysiological experiments. The latter model is made possible by the incorporation of numerous realistic ion channel mechanisms, complete with biologically accurate kinetics, densities, and distributions. The structures of these dentate networks can be characterized using tools from graph theory. These tools have recently been employed in studies of real world networks such as the electric power grid, the internet, and the *C. elegans* worm nervous system. Initially, we determine for the first time the topological characteristics of the healthy dentate gyrus structural model as well as the subsequent topological changes during epileptogenesis. Subsequently, we determine the functional relevance of these topological changes, leading to an intriguing demonstration of hyperexcitability arising exclusively from structural alterations in a realistic neuronal network.

Methods – Implementation of Dentate Network Models

Vast amounts of information about the dentate gyrus, detailing cell types and numbers, connectivity, and intrinsic single cell and synaptic properties, have been collected in the past decade, allowing for the construction of the realistic models discussed in this article. While a summary of the model components and their implementation is provided in this section, the interested reader should refer to the papers listed under 'Further Reading' for a detailed discussion of the experimental justification of model components based on the published literature. The models themselves are available from the ModelDB database, located online at http://senselab.med.yale.edu/senselab/modeldb/.

A Purely Structural Model

The structural model of the dentate gyrus is a complete 1:1 scale representation containing eight distinct and well-described cell types: granule cells, mossy cells, basket cells, axo-axonic cells, MOPP cells (molecular layer cells projecting to the perforant path termination zone), HICAP cells (hilar cells projecting to the commissural-associational pathway termination zone), HIPP cells (hilar cells projecting to the perforant path termination zone), and IS (interneuron-specific) cells (see connectivity diagram in **Figure 1(a)**). The cell numbers and connectivity of each neuron type were determined from published data and are indicated in **Table 1**. The connectivity is displayed as the numbers of postsynaptic targets within a population that a single presynaptic neuron of a given type innervates (e.g., looking at the third row, second column of **Table 1**, a single basket cell innervates approximately 1250 granule cells; mean and ranges are indicated).

Not only the number of connections made by a presynaptic cell, but also the distributions of these connections along the dentate septo-temporal axis, are very

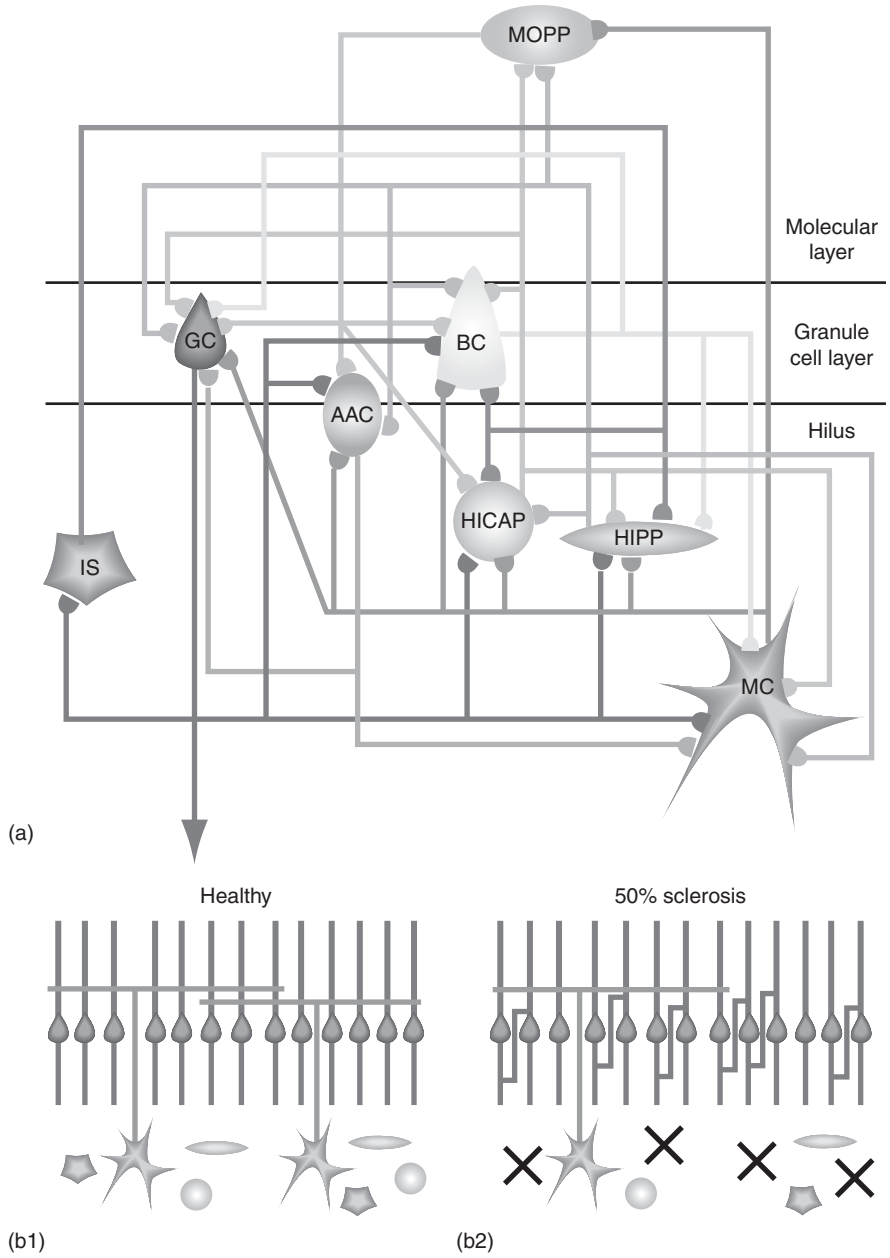


Figure 1 Basic circuitry of the dentate gyrus and the network alterations during sclerosis. (*a*) Schematic representation of connectivity of the eight major cell types in the healthy dentate gyrus (GC: granule cell; BC: basket cell; MC: mossy cell; AAC; axo-axonic cells; MOPP: molecular layer interneurons projecting to the perforant path termination zone; HIPP: hilar interneurons projecting to the perforant path termination zone; HICAP: hilar interneurons projecting to the commissural-associational pathway termination zone and IS: interneuron specific cells). The diagram shows the location of different cell types within the three layers of the dentate gyrus, but does not indicate the actual axonal connection topography (implemented in structural and functional dentate models) or the specific location of the synapses on the post-synaptic cell (implemented in the functional network models). (b1) Diagram of the excitatory connections in the healthy dentate gyrus (only granule cells and hilar neurons are shown). Note that the mossy fibers (granule cell axons) do not make connections with other granule cells in the healthy network. (b2) Diagram of the dentate gyrus at 50% sclerosis indicating the loss (represented as large X-s) of half the of hilar neuron populations, and the 50% sprouting of mossy fiber connections to other granule cells (note that this is a simplified representation: all granule cell axons sprouted new contacts in both structural and functional models of sclerosis). Used with permission from *The Journal of Neurophysiology*.

important for an accurate representation of network structure. In the model, single or double Gaussian distributions were fitted to average axonal distributions determined from published in vivo single cell fills from the literature (**Figure 2**). All cell types were distributed evenly in 100 bins along a simulated 6 mm strip of dentate gyrus, and the cell-to-cell connectivity was determined probabilistically based on the fitted axonal distributions.

Table 1 Connectivity matrix for the healthy dentate gyrus

	Granule cells	*Mossy Cells*	*Basket cells*	*Axo-axonic cells*	*MOPP cells*	*HIPP cells*	*HICAP cells*	*IS cells*
Granule cells	*X*	*9.5*	*15*	*3*	*X*	*110*	*40*	*20*
(1 000 000)	X	7–12	10–20	1–5	X	100–120	30–50	10–30
Mossy cells	*32 500*	*350*	*7.5*	*7.5*	*5*	*600*	*200*	*X*
(30 000)	30 000–35 000	200–500	5–10	5–10	5	600	200	X
Basket cells	*1250*	*75*	*35*	*X*	*X*	*0.5*	*X*	*X*
(10 000)	1000–1500	50–100	20–50	X	X	0–1	X	X
Axo-axonic cells	*3000*	*150*	*X*	*X*	*X*	*X*	*X*	*X*
(2000)	2000–4000	100–200	X	X	X	X	X	X
MOPP cells	*7500*	*X*	*40*	*1.5*	*7.5*	*X*	*7.5*	*X*
(4000)	5000–10 000	X	30–50	1–2	5–10	X	5–10	X
HIPP cells	*1550*	*35*	*450*	*30*	*15*	*X*	*15*	*X*
(12 000)	1500–1600	20–50	400–500	20–40	10–20	X	10–20	X
HICAP cells	*700*	*35*	*175*	*X*	*15*	*50*	*50*	*X*
(3000)	700	30–40	150–200	X	10–20	50	50	X
IS cells	*X*	*X*	*7.5*	*X*	*X*	*7.5*	*7.5*	*450*
(3000)	X	X	5–10	X	X	5–10	5–10	100–800

Numbers and connectivity values for granule cells, mossy cells, basket cells, axo-axonic cells, molecular layer interneurons projecting to the perforant path termination zone (MOPP), hilar interneurons projecting to the perforant path termination zone (HIPP), hilar interneurons projecting to the commissural-associational pathway termination zone (HICAP), and interneuron specific cells (IS) were estimated from published data. Connectivity is listed as the number of connections from a single presynaptic neuron (column 1) to a postsynaptic population (corresponding rows). The mean number of connections implemented in the graph theoretical calculations is indicated in italic. However, small world structure was preserved even if the extreme low or the extreme high estimates (ranges given below mean values) were used for the calculation of L and C. Used with permission from *The Journal of Neurophysiology*.

The structural model was represented as a 'graph' in the computer, detailing 'nodes' (representing individual cells) and the 'links' (representing synapses) connecting them. Each node represented a unique single cell in the biological dentate gyrus and its complete connectivity. This representation resulted in a graph with more than 1 billion directed synaptic links connecting over 1 million nodes.

Adding Functionality

The approach for implementing the functional model network was similar to that described for the structural model above; however, some important differences stand out: The functional model only represented four cell types (granule cells, mossy cells, basket cells, and HIPP cells) with sufficient electrophysiological data available to implement faithful representations of their firing characteristics in single cell models. These four cell types comprised the granule and mossy cells (the only excitatory cell types in the dentate gyrus) and two major (i.e., numerically dominant and functionally important) types of interneurons – the parvalbumin and somatostatin-positive interneurons. Furthermore, the functional model is a 1:20 scale representation of the dentate, rather than 1:1 representation implemented in the structural model. This reduced model is due to constraints imposed by available computational resources. Even with downscaling, the number of represented cells remains extremely large (with over 50 000 multicompartmental model neurons), allowing the network to maintain the topological characteristics of the full scale model (see below). Finally, the functional model network includes active, biophysically realistic model neurons connected with weighted synapses, each accurately reproducing a number of input-output characteristics determined experimentally.

Implementation of Sclerosis

'End-folium' sclerosis (subsequently termed 'sclerosis') comprises several pathological changes occurring in the dentate gyrus, including hilar cell loss, mossy fiber sprouting, and gliosis. For investigating alterations of the neuronal network architecture, hilar cell loss and mossy fiber sprouting are the primary determinants, and thus these two factors were implemented in the model and will be referred to as sclerosis in this article.

To allow for the implementation of progressive sclerosis in the network, maximal sclerosis was defined as (1) complete hilar cell loss (i.e., loss of all hilar interneurons and all mossy cells) and (2) simultaneous maximal mossy fiber sprouting (as reported in the literature, an average of 275 new contacts per granule cell – in this model represented exclusively as the addition of previously nonexistent granule cell-to-granule cell connections). Subsequently, any intermediate level of sclerosis could be represented as a simple percentage of this maximum. For example, at 50% sclerosis, 50% of mossy cells and hilar interneurons were lost, and 50% of the maximum number of mossy fiber contacts was added (schematically shown in **Figure 1(b)**).

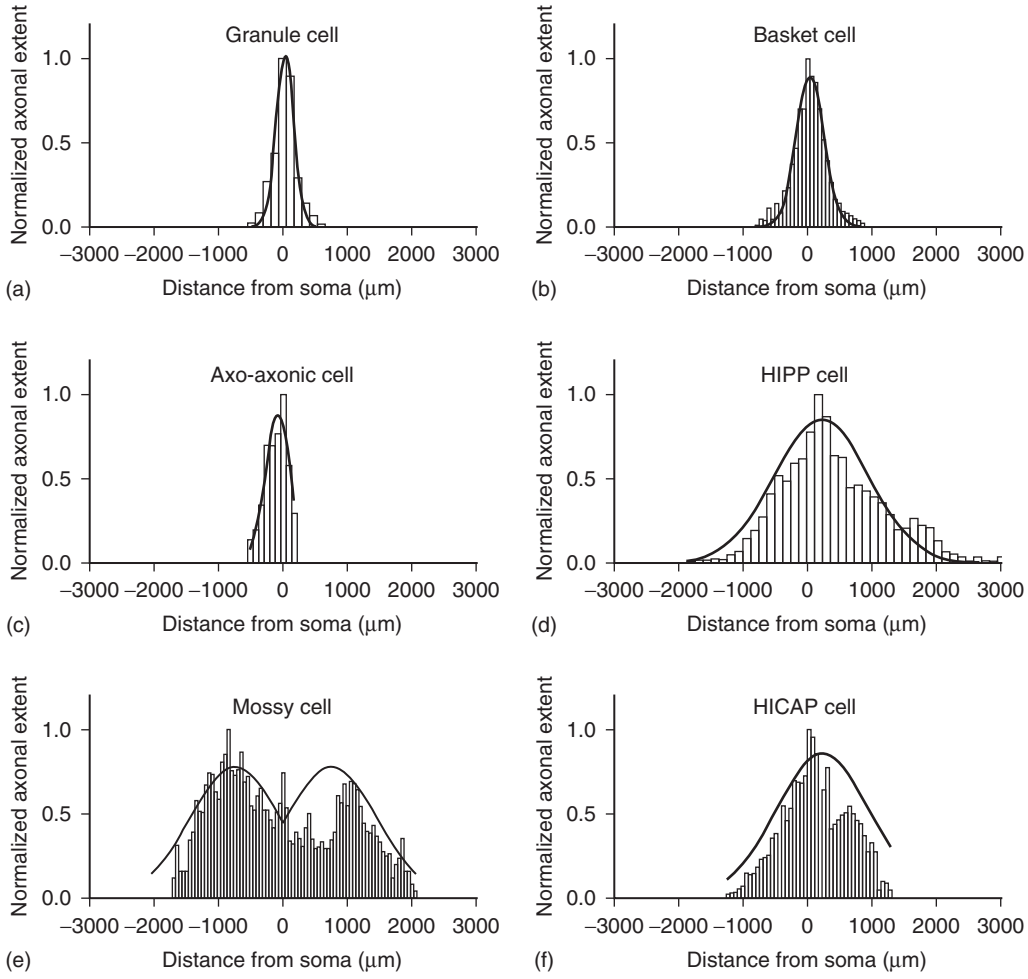


Figure 2 Gaussian fits to distributions of axonal branch lengths from experimental in vivo fills used in model connectivity. (a) Graph of the averaged axonal distribution from 13 granule cells and the fitted Gaussian distribution. (b) Fitted septo-temporal distribution of axonal length of a reconstructed basket cell. (c) Fitted axonal distribution of a CA1 axo-axonic cell. (d) Averaged axonal distributions of 3 HIPP cells from gerbil and the corresponding Gaussian fit. (e) Fitted averaged axonal distributions of 3 mossy cells with a characteristic bimodal pattern of distribution. (f) Septo-temporal distribution of the axonal lengths of a HICAP neuron and the fitted Gaussian. In all graphs, the septal pole of the dentate gyrus is to the left (negative coordinates) with the soma positioned at zero. Used with permission from *The Journal of Neurophysiology*.

Structural and functional calculations were performed at increments of 10% of maximal sclerosis in both the structural and functional models.

Results – Structural and Functional Characterization of the Networks

Topological Assessment of the Structural Network

Two measures originally employed to assess the structure of the nervous system of the worm *C. elegans* were used to characterize the network topology of the healthy and sclerotic dentate: The average path length, L, and the average clustering coefficient, C. The average path length is defined as the average number of steps required to move from any node to any other node in the network, and thus measures the global connectivity of a network. The average clustering coefficient is a measure of local connectivity, defined as the average fraction of all possible connections between 'postsynaptic' nodes, of each node, that are actually present. These topological measures, C and L, can perhaps be best understood in the context of a social network: C measures the probability that any two friends of a given person also know each other. L measures how many steps in a chain of friends would be necessary to connect two randomly selected people in the network.

Based on these two topological measures, networks can be divided into three classes: (1) Regular, with a long path length and large clustering coefficient (**Figure 3(a)**; high local connectivity, low global connectivity); (2) Random, with a short path length and small clustering coefficient

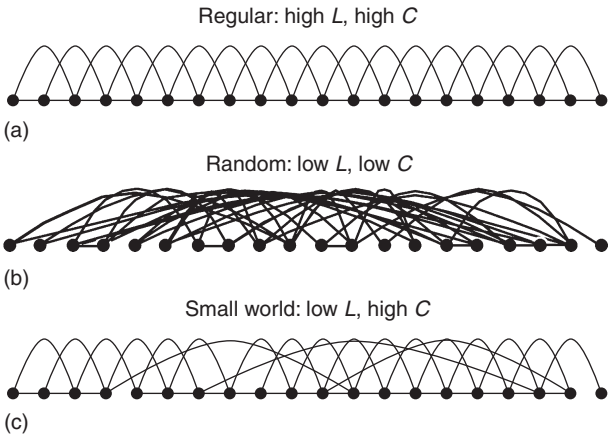


Figure 3 Diagrams of three basic network types. (a) A network with regular topology. All nodes in a regular network form nearest neighbor connections, leading to strong local interconnectedness (high clustering coefficient *C*), but also demanding a large number of steps to move from a given starting point to any other node in the network (high average path length *L*). (b) A network with random topology. A random network has no spatial constraints on the connectivity distribution of individual nodes, leading to a low average *L* but at the same time a low *C*. (c) A network with 'small world' topology. Randomly reconnecting even a few local projections in a regular network topology to distal nodes results in the emergence of a network with 'small world' topology, displaying a high *C* along with a low average *L*. Used with permission from *The Journal of Neurophysiology*.

(**Figure 3(b)**; low local connectivity, high global connectivity); and (3) Small World, with a short path length and large clustering coefficient (**Figure 3(c)**).

The small world network (**Figure 3(c)**) is a unique intermediate between the random and regular graphs, combining a high degree of local connectivity with a number of long range connections, resulting in a both locally and globally well-connected network.

While L and C values are clearly important for network classification, it is difficult to understand topological alterations during sclerosis in terms of their absolute values, as the size of the network and number of connections change with progressive sclerosis. Rather, it is important to use a random network of equivalent size, with an equivalent number of connections as a reference. Using the ratios of C/C_{random} and L/L_{random} normalizes each network to the reference and allows a direct characterization of the randomness (or lack thereof) of its topological features. Both absolute values and these normalized ratios will be illustrated in the present article; however the ratios are most meaningful in terms of comparing networks with different degrees of sclerosis.

The Dentate Gyrus is a Small World Network

Initially, the structure of the healthy or control (also referred to as the model at 0% sclerosis) structural network was established. Remarkably, considering the more than 1 million nodes represented, the average path length was very low ($L = 2.68$, marked ○ in **Figure 4(a)**), meaning that any two neurons on average are separated by fewer than 3 synapses in the dentate gyrus. This is remarkably close to the average path length of the *C. elegans* nervous system (2.65), which has only 282 nodes, a full four orders of magnitude smaller than the dentate gyrus!

When comparing the average path length of the control dentate network to an equivalent random graph, the L/L_{random} ratio obtained was very close to one (1.19; horizontal dotted line in **Figure 4(c)**). This value indicates a globally extremely well-connected control dentate network, with the average path length only slightly increased over the case of a random network- and could perhaps indicate that the neural network of the dentate gyrus simply is a random graph. However, when comparing the calculated clustering coefficient to the clustering of an equivalent random graph, this possible 'equivalence' was clearly not the case. Specifically, the calculated high ratio of C/C_{random} demonstrated a high degree of local connectivity ($C/C_{\text{random}} = 0.026751/0.001135 = 24.7$; horizontal dotted line in **Figure 4(d)**; the absolute value for C in the control network is marked with a ○ in **Figure 4(b)**). Together with the high degree of global connectivity, this ($L \sim L_{\text{random}}$ and $C \gg C_{\text{random}}$) indicates that the dentate gyrus has a small world network topology.

Small World Characteristics are Initially Enhanced, then Transformed during Sclerosis

Similar to the characterization of the control dentate graph, calculations were performed for networks during progressive sclerosis. Maximal sclerosis resulted in a dramatic loss of connections in the network (953 198 800 links are removed out of a total of 1 287 363 500, 74% reduction), while the number of nodes changed minimally (48 000 hilar cells are lost out of 1 064 000 total cells, only 4.5% reduction). Despite the massive loss of connections (due to the loss of richly connected hilar mossy cells and interneurons, which is only slightly compensated by relatively sparse mossy fiber sprouting), the structural graph calculations revealed that L did not significantly increase until beyond 90% sclerosis (solid black line in **Figure 4(a)**). Furthermore, the ratio L/L_{random} surprisingly decreased below the control value during submaximal sclerosis (solid black line in **Figure 4(c)**; control value indicated by horizontal, dotted line in **Figure 4(c)**), suggesting a more globally well-connected graph. Calculation of the clustering coefficient demonstrated similar results, with the ratio C/C_{random} increasing and remaining above the control value until approximately 90% sclerosis (solid black line in **Figure 4(d)**; control marked with

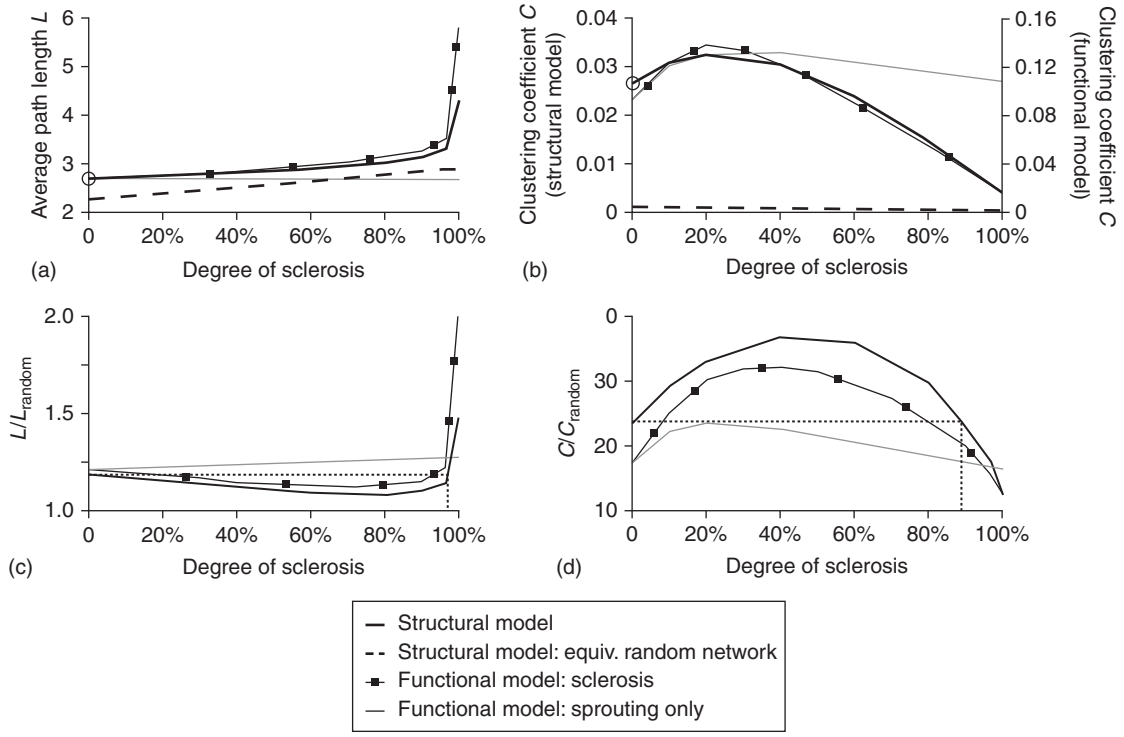


Figure 4 *L* and *C* changes during sclerosis for the different models of the dentate gyrus. (a,c) Alterations of the average path length *L* with sclerosis; (b,d) Alterations of the clustering coefficient *C* with sclerosis. Legend in a also applies to (b,d). Legend in e also applies to f. Solid black lines in a–d: 1:1 structural models; Solid black lines in e–f: graphs of the isolated excitatory/inhibitory networks. Black lines with squares: Structural graph of the 1:20 functional model during sclerosis; Gray lines: Structural graph of the 1:20 functional model network during sprouting only. Dashed black lines in a,b: Equivalent random graphs of the 1:1 structural models. (a,b) Changes in *L* and *C* of the different dentate graphs. The values of *L* and *C* for the healthy (i.e., at 0% sclerosis) full scale structural model dentate gyrus are indicated by '○' on the *y*-axis. (c,d) The relative *L* ($=L/L_{random}$, from panel a) and relative *C* ($=C/C_{random}$, from panel b) changes during sclerosis. In panels c and d, the relative *L* and *C* for the 1:1 structural model of the healthy dentate graph are indicated by dotted horizontal lines. The vertical dotted lines indicate the degree of sclerosis at which the relative *L* exceeds, and the relative *C* decreases below the values for the healthy control graph. Note that the changes of the relative *L* and *C* during sclerosis in the structural model of the 1:20 functional model network (50 000+ nodes; black lines with squares) closely resemble those in the 1:1 full-scale structural model (over 1 million nodes; solid black lines). Note that panel b has two *y* axes. Used with permission from *The Journal of Neurophysiology*.

dotted horizontal line in Figure 4(d)). Taken together, these changes demonstrate a surprising enhancement of small world characteristics at sub-maximal sclerosis, with improved global and local connectivity.

Beyond 90% sclerosis, the absolute value of *L* increased rapidly (solid black line in Figure 4(a)), with the ratio L/L_{random} surpassing the control value at 96.6% sclerosis (vertical dotted line in Figure 4(c)). Furthermore, at approximately 90% sclerosis, the C/C_{random} ratio decreased below the control value (vertical dotted line in Figure 4(d)). Since both a short path length and a large clustering coefficient are required to confer a network with small world structure, the greatly increased path length and decreased clustering coefficient (albeit still high compared to an equivalent random network) indicated a transformation of network topology from a small world into a more regular network structure (high *L*, high *C*) at maximal sclerosis.

Mechanisms of the Biphasic Structural Alterations

In order to understand the factors underlying the described biphasic transformation of dentate network topology during progressive sclerosis (i.e., the initial enhancement of small world properties followed by a transition to a more regular network structure), the excitatory and inhibitory parts of the dentate gyrus graph were studied in isolation.

The behavior of the isolated excitatory graph (granule and mossy cells alone; solid black lines in Figure 5) closely resembled the findings from the complete dentate graph (solid black lines in Figure 4). As described for the complete graph, the average path length remained low during submaximal sclerosis and increased rapidly above 90% sclerosis, while the changes in clustering coefficient displayed the earlier described biphasic pattern

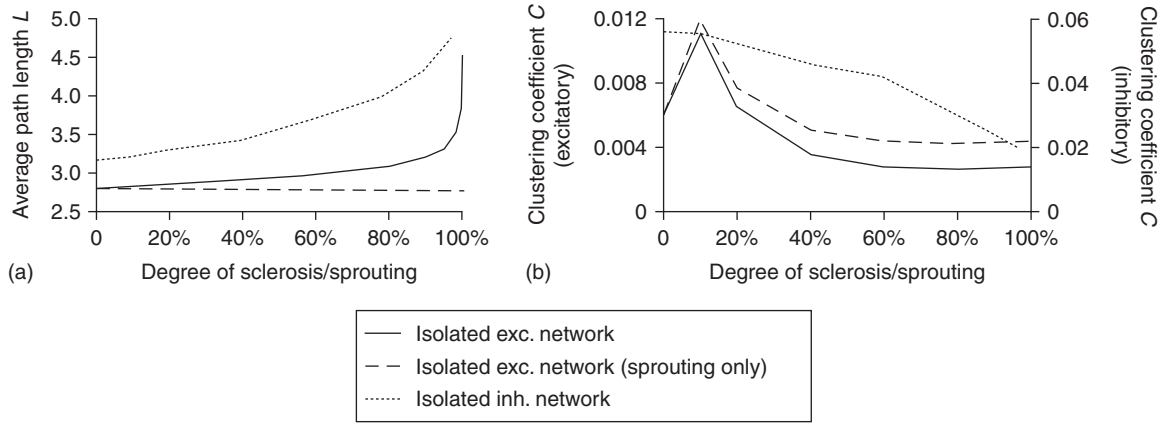


Figure 5 Changes in *L* and *C* during sclerosis for the isolated excitatory and inhibitory graphs. (a,b) Alterations in *L* and *C* for graphs of the isolated excitatory and inhibitory networks during sclerosis, and graphs of the isolated excitatory networks with sprouting alone (no mossy cell loss). Dotted lines: Alterations in *L* and *C* for inhibitory graphs following hilar interneuron loss; solid lines: excitatory graphs (granule and mossy cells); dashed lines: excitatory graphs with mossy fiber sprouting in the absence of mossy cell loss. Note that panel b has two y axes. Used with permission from *The Journal of Neurophysiology*.

as sclerosis progressed. This pattern of change suggests that loss of long range connections resulting from mossy cell loss, and addition of new recurrent granule cell connections through mossy fiber sprouting, govern the biphasic changes described in the complete graph. Further analysis incorporating mossy fiber sprouting in the absence of hilar cell loss showed no significant effect on path length in the isolated excitatory graph (dashed line in **Figure 5(a)**), emphasizing the fact that surviving long range connections provided by mossy cells are the main determinant of *L*. Conversely, the clustering coefficient still displayed a biphasic pattern when hilar cell loss was omitted (dashed line in **Figure 5(b)**), pointing to mossy fiber sprouting as the determining factor for the alterations in C. In the isolated inhibitory network, sclerosis simply resulted in loss of interneurons and an increasingly sparse network, with monotonically increasing L and monotonically decreasing *C* (dotted lines in **Figure 5**).

In summary, two primary factors govern the biphasic topological alterations of the dentate network during sclerosis: During sub-maximal sclerosis, mossy fiber sprouting maintains a high degree of local connectivity and indirectly connects granule cells to surviving mossy cells, conserving the relatively low *L*. With severe sclerosis, the main factor switches to the loss of long-range projecting hilar neurons, particularly the mossy cells, when the increased local connectivity created by mossy fiber sprouting no longer adequately compensates for the massive loss of long distance connections. This latter condition leads to the dramatic increase in average path, and transition of network structure from a small world to a more regular, lattice-like structure.

Characterization of the Functional Network

To understand the functional impact in temporal lobe epilepsy of the described topological changes during sclerosis, the activity in the described network architectures was analyzed in the 1:20 scale functional model of the dentate gyrus previously introduced. The use of a computational model here allows for a dissociation of structural alterations from underlying cellular or synaptic modifications confounding such investigations in experimental preparations.

At first, a structural graph of the 1:20 scale functional model was constructed, and *L* and *C* calculations during sclerosis were performed as in the full scale model. These calculations verified that the structural alterations in the 1:20 scale graph closely resembled those of the full scale graph, with biphasic changes retained for both *L* and *C* (lines with squares in **Figure 4**; compare with solid black lines).

Activity in the functional model network was stimulated by a single, simulated perforant path input to 5000 granule cells, 10 mossy cells, and 50 basket cells in the centrally located bins of the network, 5 ms after simulation start. The resulting activity of the functional network was quantified using four different measures: (1) Duration of granule cell firing (the time between the first and the last granule cell action potential in the network); (2) Average number of action potentials per granule cell; (3) Time until action potentials were fired in granule cells at the edges of the network, furthest from the stimulation point (i.e., latency to full network activity); and (4) Synchrony of granule cell action potential firing assessed by an established coherence measure.

Network Activity Mirrors the Biphasic Structural Alterations

Built from detailed, biophysically realistic single cell models reliably reproducing electrophysiologically determined firing properties, the healthy functional model network showed limited firing in response to a single perforant

path input (**Figure 6(a)**), in accordance with experimental data, and it became increasingly hyperexcitable up to approximately 80% sclerosis (**Figure 6(b–e)**). However, surprisingly, hyperexcitability in the functional model network actually decreased at levels of sclerosis exceeding 80% (**Figure 6(f)**). This pattern of hyperexcitability in the functional network followed the same biphasic pattern seen in the structural network, and the functional effects were solely due to structural network alterations. During submaximal sclerosis and small world structural enhancement, hyperexcitability increased, while hyperexcitability decreased when the network transitioned to a more regular structure.

As previously described, network activity was quantified by four different measures (**Figure 7**); the biphasic structural changes in the network during sclerosis were mirrored by both the maximal activity duration and mean number of action potentials per granule cell, as well as the latency to full network activation (solid black lines in **Figure 7(a–c)**). Synchrony of granule cell firing was assessed both locally (within 0.12 mm along the virtual dentate strip; dashed black lines in **Figure 7(d)**) and globally (up to 2.5 mm separated sampled granule cells; solid black lines in **Figure 7(d)**). While local and global synchrony measures were similar at moderate levels of sclerosis (40–60%), global synchrony subsequently decreased with severe (>60%) sclerosis, while local synchrony increased substantially. These data demonstrate that sclerosis leads to a fundamentally transformed microcircuit where biphasic patterns of activity changes follow biphasic structural changes in network architecture.

To ensure that network activity levels did not simply follow changes in levels of granule cell synaptic input, the net excitatory and inhibitory conductances were calculated at each level of sclerosis (**Figure 8**). However, as both net excitatory and inhibitory peak conductance declined in a monotonic fashion with progressive sclerosis (black lines,

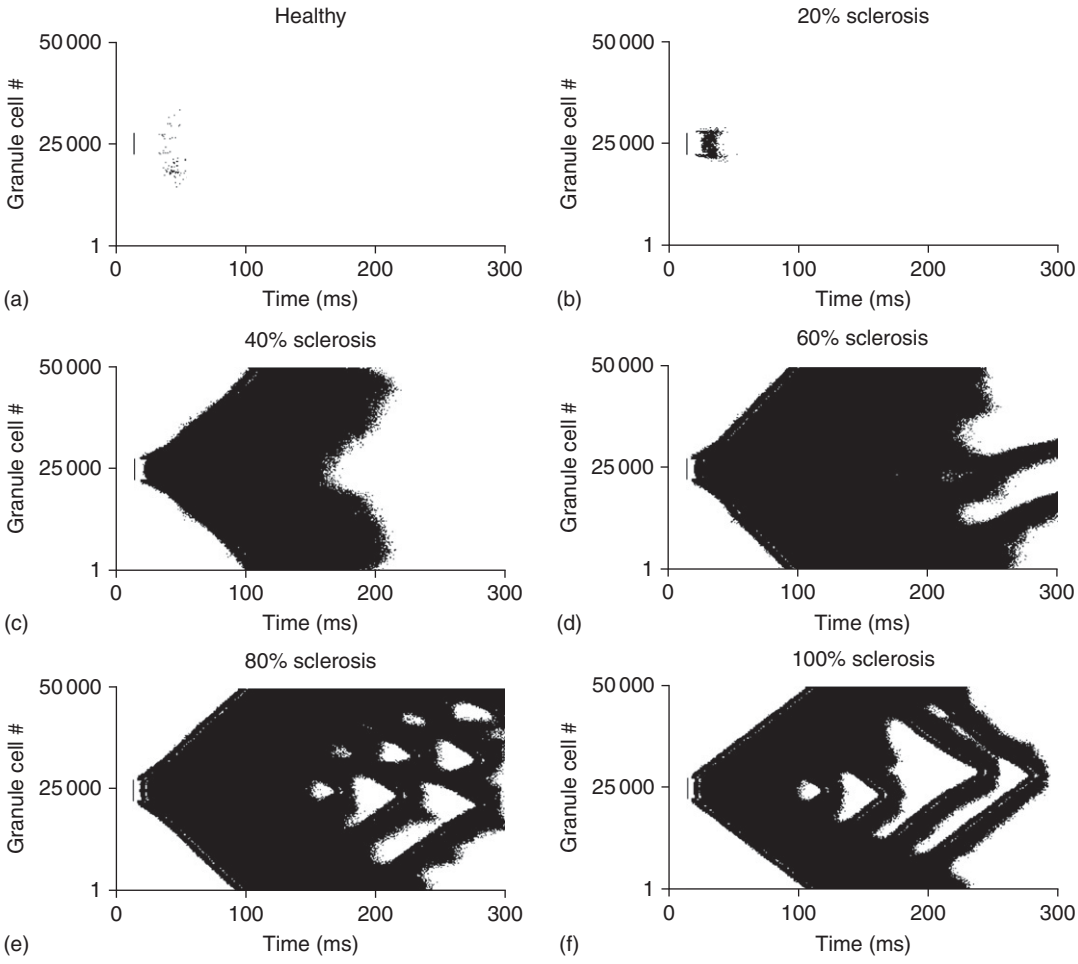


Figure 6 Impact of sclerosis-related topological changes on granule cell firing in functional model networks. (a–f) Raster plots show the first 300 ms of granule cell action potential firing in the functional model network (Granule cells #1–#50 000, plotted on the *y*-axis) for increasing degrees of sclerosis. Activity was initiated by a simulated, single shock stimulation of the perforant path input to granule cells #22 500–#27 499 along with 10 mossy cells and 50 basket cells (co-localized with the stimulated granule cells) at $t = 5$. Note that peak hyperactivity was observed at less than maximal (80%) sclerosis (see quantification in **Figure 7a–d**). Used with permission from *The Journal of Neurophysiology*.

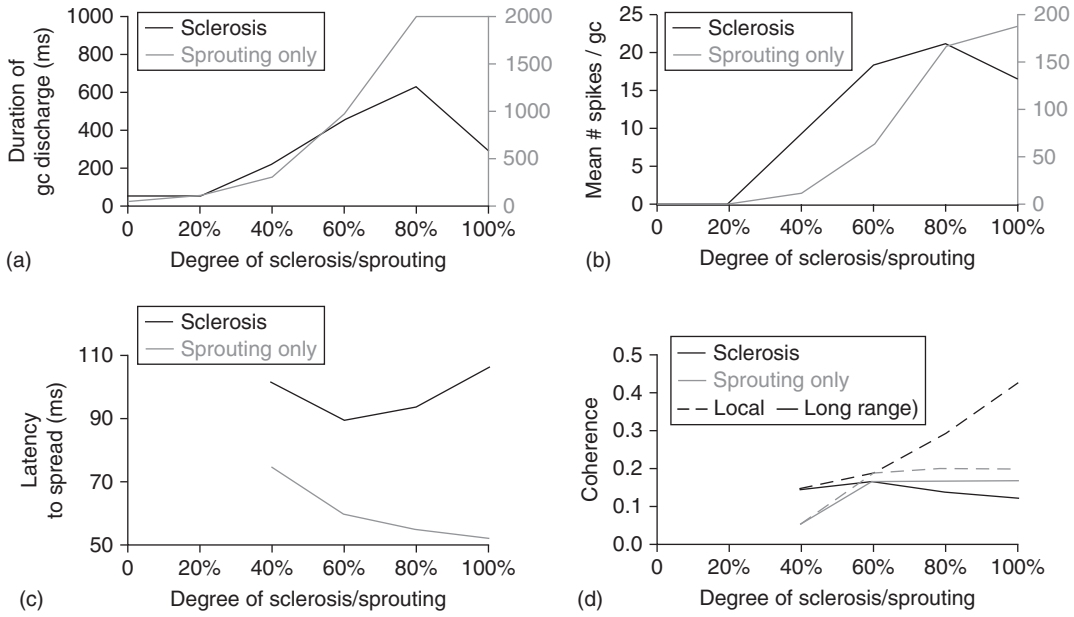


Figure 7 Biphasic changes in functional model network activity during sclerosis, but not during sprouting without hilar neuron loss. Black lines: sclerosis; Gray lines: sprouting without hilar cell loss. (a) Maximal duration of perforant path stimulation-evoked granule cell firing as a function of sclerosis or sprouting without hilar cell loss (note that at 80% and 100% sprouting in the sprouting only case, granule cell firing persisted beyond 2000 ms). (b) Mean number of spikes fired by each granule cell during sclerosis or sprouting alone. (c) Latency to granule cell firing in all 100 bins (only quantified at degrees of sclerosis/sprouting with persistent activity in all bins). (d) Local (dashed line, all-to-all comparison of action potential firing in granule cells #25 000–#25 999) and long range (solid line, pair-wise comparison of the activity in granule cells #25 000–#25 999 to granule cells #45 000–#45 999) coherence of granule action potential firing in the period from 100 ms to 200 ms (only quantified at degrees of sclerosis/sprouting with persistent activity in all bins). Used with permission from *The Journal of Neurophysiology*.

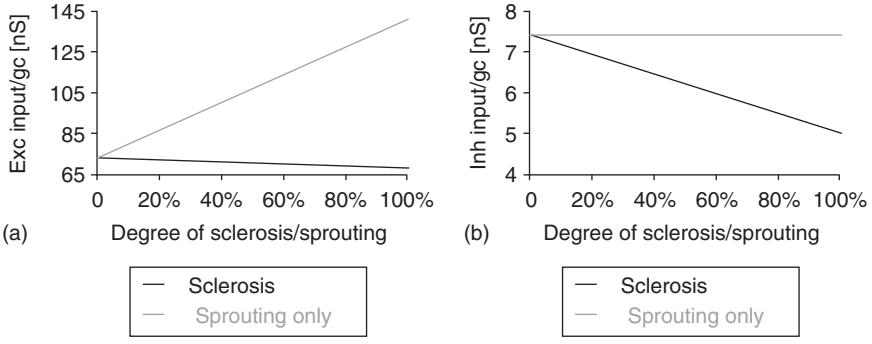


Figure 8 Changes in peak conductance onto granule cells during sclerosis or sprouting without hilar cell loss. (a,b) The average total peak conductance of excitatory (a) and inhibitory (b) synapses onto a model granule cell during sclerosis or sprouting alone. Note that the absence of biphasic changes in **Figure 7(a–d)** during sprouting without hilar cell loss follows the monotonic increase in excitatory drive seen in panel a (gray line). Used with permission from *The Journal of Neurophysiology*.

Figure 8), this pattern could not have been solely responsible for the observed biphasic activity changes.

Activity in Networks with Sprouting but No Cell Loss

Following the approach previously used in determining the mechanism of network structural alterations, the roles of L and C in determining functional network activity were examined using a functional model with sprouting in the absence of cell loss (**Figure 9**). This model provided the opportunity to determine whether biphasic changes in the clustering coefficient could account for biphasic changes in network excitability in the absence of a biphasic change in path length. Again, the structural changes in a 1:20 scale sprouting-only network were determined to ensure biphasic changes in C were accompanied by a stable L (gray lines, **Figure 4**).

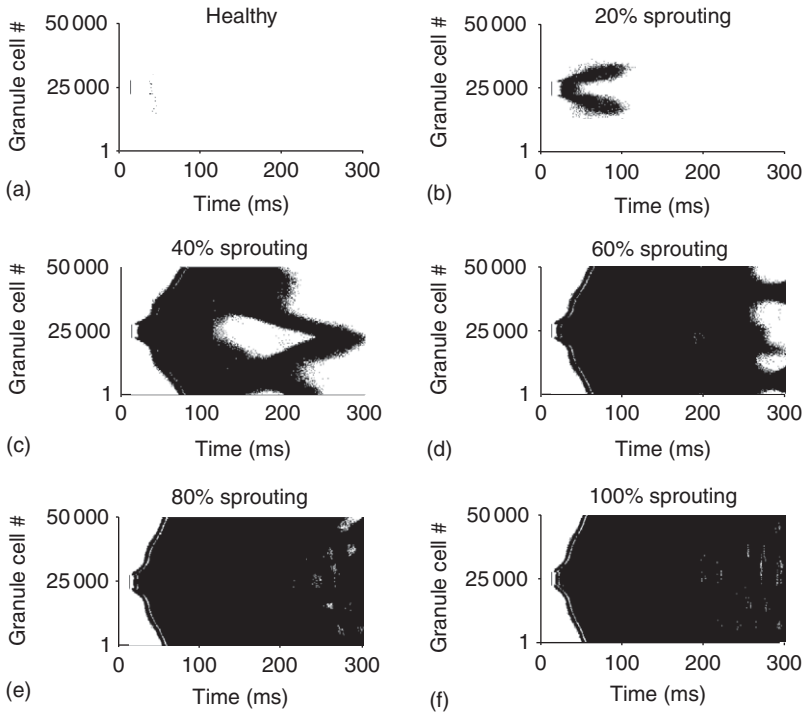


Figure 9 Functional impact on network activity of sprouting in the absence of hilar cell loss. (a–f) Raster plots of granule cell action potential firing in the functional model networks during progressive mossy fiber sprouting. Network activity was initiated as described for **Figure 6**. Note that maximal hyperactivity occurred at maximal sprouting (see **Figure 7(a–d)** for quantification). Used with permission from *The Journal of Neurophysiology*.

Contrasting the activity determined in the sclerotic functional networks described above, all four activity measures showed monotonous changes with increasing sprouting alone in the functional model (gray lines, **Figure 7**), in spite of the remaining biphasic change in clustering coefficient. However, a quantification of the net inhibitory and excitatory drive to the granule cells (**Figure 8**, gray lines) suggested the firing properties in the sprouting-only network simply reflected a monotonic increase in granule cell excitatory drive (68 nS increase per granule cell from 0% to 100% sprouting; gray line in **Figure 8(a)**).

These results demonstrate that both the sprouting and cell loss, initially enhancing small world network properties and subsequently causing the transition to a more regular network structure, are required for the biphasic activity changes seen in the functional model.

Conclusions and Future Directions

The presently discussed study had five major findings: (1) The dentate gyrus network has a small world structure; (2) Moderate sclerosis enhances the small world network characteristics of the dentate gyrus (i.e., L/L_{rand} decreases and C/C_{rand} increases), but severe sclerosis induces a transition to a more regular network structure (L rises rapidly and C declines). (3) Calculations with isolated excitatory and inhibitory networks determined the different roles of hilar cell loss and mossy fiber sprouting in the biphasic structural and functional alterations described. During moderate sclerosis, mossy fiber sprouting compensates for the loss of long range mossy cell connections and enhances small world features, by indirectly connecting granule cells to spared mossy cells. However, at severe sclerosis, hilar neuron loss defines network topology, as mossy fiber sprouting cannot compensate for the maximal loss of long distance connections. (4) Simulations in the functional network model demonstrated that network excitability closely mirrors changes in network topology, with enhanced small world features resulting in network hyperexcitability. With severe sclerosis, however, there is a decrease in hyperexcitability at the transition to a more regular network structure. (5) Simulations of functional sprouting-only networks determined that biphasic changes in the clustering coefficient alone are insufficient to reproduce the biphasic changes in network activity. The increase in relative path length at severe sclerosis caused by maximal loss of hilar neurons results in diminished network excitability, indicating that a small number of surviving, long distance projecting mossy cells can maintain a hyperexcitable dentate gyrus circuit.

The combination of a large number of local and long range connections in a small world network confers the capacity for both fast local computations and efficient

long-range signal relays. In fact, several studies have demonstrated both fast signal propagation and long range synchronization in small world networks. In agreement with these studies, the presently discussed enhancement of small world characteristics of the sub-maximally sclerotic dentate gyrus was accompanied by increasing hyperexcitability and activity propagation. This enhancement of small world structure was made possible by mossy fiber sprouting compensating the massive loss of long distance connections during sclerosis. However, this compensation also led to an increased clustering coefficient and enhanced network hyperexcitability – both very serious and potentially disastrous changes contributing to epileptogenesis.

The surprising decrease of hyperexcitability at maximal sclerosis, caused by the transition of network architecture from an enhanced small world topology to a more regular structure following the loss of the final hilar cells, is in agreement with experimental observations in both epileptic animals and humans. In particular, it is worth noting that studies quantifying hilar cell loss in animal models of epilepsy never report 100% cell loss, and cell counts in surgically removed specimens from pharmacologically intractable human temporal lobe epilepsy patients show a maximal loss of approximately 80% of hilar cells. Interestingly, this number coincides perfectly with maximal hyperexcitability in the functional model networks, observed at 80% sclerosis.

The findings described in this article demonstrate that structural alterations in disease states are crucial for determining neural network function, and computational modeling studies provide a unique way of isolating the impact of multiple pathological changes in epileptogenesis. With the presently established model, the functional effect of, for example, experimentally determined specific changes in intrinsic cellular and synaptic properties can be tested, with only structural changes as the control state. Thus, modeling will continue to be very important for understanding the relative importance of specific pathologies in epilepsy, and potentially help guide efforts towards identifying the optimal targets of improved treatment strategies.

Acknowledgements

This work was funded by the NIH (NS35915 to IS).

See also: **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Models**: Model Characterization in Relationship to Human Disorders; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Neurophysiology**: Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures.

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Ictogenesis in the In Vitro Isolated Guinea Pig Brain

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Introduction and Background

The epileptic condition is characterized by the abrupt occurrence of seizures, usually without prodromic warning. One of the experimental strategies used to understand epilepsy aims at investigating the mechanisms that control the genesis of a seizure – i.e., the events that regulate the transition from the interictal and the ictal state. These mechanisms are still not well characterized and may be specific for different types of epilepsies. Animal models are used to reproduce epileptic discharges and to study the network, cellular, and subcellular alterations that generate seizures. Since the accessibility to brain tissue is limited in the in vivo condition, in vitro experimental models have been developed to analyze systemic and molecular changes associated with epilepsy.

Excessive and hypersynchronous neuronal activity can be precipitated by several means in in vitro preparations. The complexity of in vitro preparations varies from the very simple (cell cultures), to the complex (slices), to the very complex, in which the entire brain or parts of it are functionally preserved. According to their complexity, different in vitro models produce epileptic-like activities that approximate the patterns observed in human focal epilepsies. In the 1980s, an in vitro preparation of the whole brain of the guinea pig was developed in the laboratory of Rodolfo Llinas. The functional and structural preservation of brain structures in this preparation has been analyzed in detail by further studies performed by several laboratories. More recently, acute conditions that mimic brain diseases have been reproduced in the isolated guinea pig brain. Several studies have demonstrated that seizure-like activity can be generated in this preparation by acute application of proconvulsive compounds.

In the last 20 years, ictal discharges in the human temporal lobe have been recorded with invasive intracranial electrodes, implanted in the temporal region of patients suffering from drug-resistant temporal lobe epilepsy. This presurgical diagnostic procedure aims at identifying the epileptogenic area that can be surgically removed to cure epilepsy. One of the prevalent patterns observed at seizure onset in these patients is the appearance of fast activity at 15–30 Hz in the hippocampus and entorhinal cortex; this pattern is usually preceded by a brief enhancement of interictal spiking in these regions. The present article shows that a similar pattern of seizure initiation can be reproduced in the in vitro isolated guinea pig brain.

Methods

Young adult guinea pigs, from 2 weeks to 2 months of age, are commonly used. The implementation of the procedure on older animals prolongs the time required for brain isolation and affects the viability of the preparation. Animals are deeply anesthetized with a sublethal dose of sodium thiopental (80–120 mg kg^{-1}) before starting the dissection. Surgery begins when trigeminal reflex cannot be elicited bilaterally. Thoracotomy is performed to cannulate the aorta at its emergence from the left ventricle, and the animal is perfused with a cold (10 °C) oxygenated (95% O_2, 5% CO_2) solution composed of 126 mmol l^{-1} NaCl, 3 mmol l^{-1} KCl, 1.2 mmol l^{-1} KH_2PO_4, 1.3 mmol l^{-1} $MgSO_4$, 2.4 mmol l^{-1} $CaCl_2$, 26 mmol l^{-1} $NaHCO_3$, 15 mmol l^{-1} glucose, 2.1 mmol l^{-1} HEPES, and 3% dextran (MW 70 000, pH = 7.1). This procedure reduces brain temperature to 15 °C within 3 min. After 3 min of perfusion, brains are carefully dissected out, transferred to a recording chamber, and perfused via a peristaltic pump (Minipulse 3, Gilson, France) with the aforementioned solution (7 ml min^{-1}, pH = 7.3, 15 °C) through a polyethylene cannula inserted in the basilar artery. Brain temperature is raised slowly to 32 °C (over the course of 1 h) before starting the electrophysiological experiment. The brain is positioned with its ventral surface upward, to access the basilar artery for cannulation.

Extracellular recordings were simultaneously obtained with NaCl-filled micropipettes (5–8 μm tip diameter, 5–10 MΩ resistance) positioned in the piriform cortex (PC), in the medial and lateral entorhinal cortex (EC), and in the hippocampus (subfield CA1). Signals were amplified using a multichannel amplifier (Biomedical Engineering, Thornwood, NY) and were digitized via an AT-MIO-64E3 National A/D Board (National Instrument, Milan, Italy). Data were acquired and analyzed with a custom-made software (ELPHO©) developed in Labview by Vadym Gnatkovsky.

The viability of isolated brains was tested by recording the responses evoked in the olfactory–limbic cortex by stimulation of the lateral olfactory tract. Stimuli were delivered through an isolation unit driven by a Grass S88 pulse generator (Grass Telefactor S88, USA). Stimulating and recording electrodes were positioned in the isolated brains under direct visual control with a stereoscopic microscope.

Drugs such as the muscarinic receptor agonist, pilocarpine (100 μmol l^{-1}, 800 μmol l^{-1}, 10–13 mmol l^{-1};

Sigma, St. Louis, MO, USA), histamine, the $GABA_A$ receptor antagonist bicuculline (50 µmol l^{-1}), and kainic acid (4 µmol l^{-1}) were dissolved in the perfusate and were applied through the resident arterial system.

Results

The results reported here demonstrate that complex patterns of epileptiform activity can be generated in the in vitro isolated brain preparation by acute treatment with proconvulsive compounds. Brief (3 min) arterial perfusion of the isolated brain with 50 µmol l^{-1} bicuculline induces a prevalent pattern observed in 80% of the experiments, characterized by the initial appearance of interictal spikes in the piriform cortex (asterisks in Figure 1) that propagate to the lateral part of the EC (the target structure of PC neurons). In 50% of the experiments, these spikes propagate further into the hippocampal region. By evaluating the time delays between the subsequent appearances of interictal spikes along the piriform–entorhinal–hippocampal pathway, we have concluded that a polysynaptic pathway is responsible for the propagation of the interictal spikes that originate in the PC.

A few seconds before the onset of an ictal discharge, a secondary "focus" of interictal spikes, independent from the PC spikes, is activated in the medial and caudal part of the entorhinal cortex (med EC) and in the hippocampus (arrowheads in Figure 1).

In the illustrated experiment, one of these spikes is followed by a discharge pattern switch that correlates with the onset of an all-or-none ictal event, characterized by the appearance of a prolonged sequence of fast activity at 15–25 Hz in the CA1 region of the hippocampus and in the medial EC (Figure 1(a)). In different experiments, this fast activity was initiated from either one of these

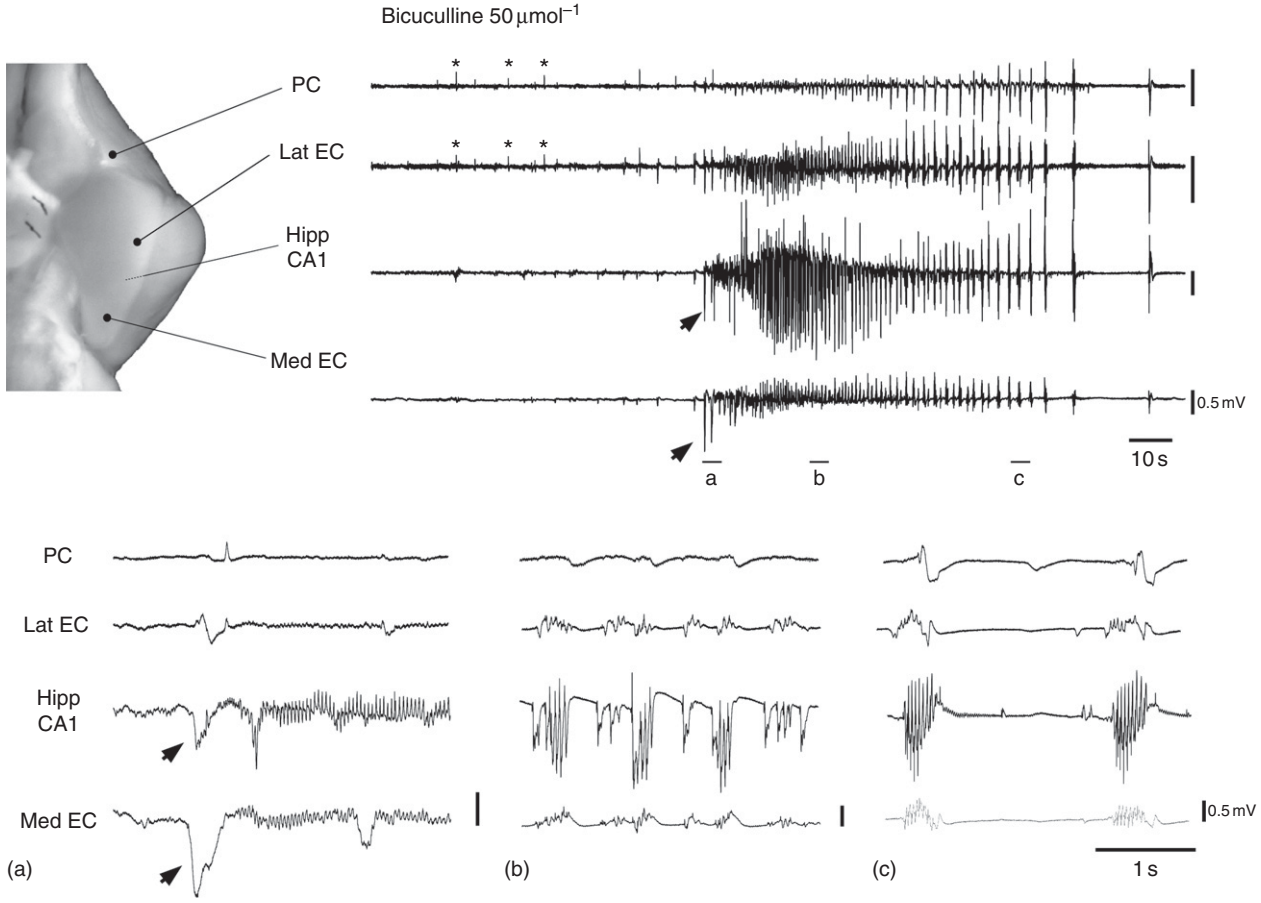


Figure 1 Epileptiform activity generated by a 3-min arterial perfusion of 50 mmol l^{-1} bicuculline in the in vitro isolated brain preparation. The positions of the recording electrodes in the piriform cortex (PC), lateral (lat EC) and medial entorhinal cortex (med EC), and in the CA1 subfield of the hippocampus (hipp CA1) of one hemisphere are illustrated in the panel on the left. Interictal spikes (asterisks) are generated in the PC and propagate to the lat EC. Just ahead of the seizure in the hipp and med EC, large-amplitude interictal spikes (arrowheads) were observed. To enhance visual correlation between activities at different recording sites, slow potentials in all traces were filtered by a 1 Hz high pass filter. Expansions of the recordings at seizure onset, during irregular bursting and during the late-ictal regular bursting (marked by a, b and c, respectively) are shown in the lower part of the figure. Note that fast activity at 15–25 Hz is generated in the med EC and hipp only. Toward the end of the seizure bursting, afterdischarges propagate to more rostral regions.

two regions. Since the recordings were performed from discrete regions within this wide area, no definitive indication on the structure that generates the fast activity can be gained from these experiments. Fast activity at seizure onset remains confined to the hippocampal–medial EC region and does not propagate to the lateral EC and to the PC. Fast activity persists for 3–10 s and is followed by an irregular population bursting pattern (**Figure 1(b)**) that becomes progressively more regular and entrains the lateral EC (**Figure 1(c)**). Late ictal bursting propagates to distant cortical regions, such as the PC, where it may generate secondary interictal spikes (**Figure 1(c)**) or burst responses. Seizures are followed by a postictal depression.

The pattern of activity described earlier was consistently observed during brief and incomplete disinhibition of the whole isolated guinea pig brain with the $GABA_A$ receptor antagonist, bicuculline. A study based on paired-pulse stimulation (not shown) confirmed that inhibition efficacy is reduced only by 20% when this seizure-like activity starts. To verify whether such seizure-onset pattern is specific to bicuculline, other proconvulsive drugs were tested in the in vitro isolated brain. Arterial perfusion of the glutamate receptor agonist kainic acid ($4\,\mu mol\,l^{-1}$) and of the muscarinic receptor agonist pilocarpine ($800\,\mu mol\,l^{-1}$) were examined. As shown in **Figure 2(a)**, kainic acid induced within few minutes of perfusion a clear ictal discharge that also initiated with fast activity (in the 15–25 Hz range) in the medial EC and in the hippocampus. The appearance of this activity correlated with a DC shift of the extracellular trace that typically marks seizure initiation. Pilocarpine alone was not able to induce epileptiform activity, unless the permeability of the blood–brain barrier is enhanced by coperfusion of compounds such as histamine ($100\,\mu mol\,l^{-1}$; **Figure 2(b)**) or bradykinin. In these latter conditions, seizure-like events were consistently observed. Also in these experiments, seizure onset was characterized by the appearance of fast activity (at 15–25 Hz), coupled with a DC shift in the trace, in both the medial EC and the hippocampus. Both kainic acid and pilocarpine + histamine perfusion induced interictal spikes before the onset of an ictal discharge.

These experiments demonstrate that seizure-like activity in the hippocampal–entorhinal area is characterized by the appearance of a sequence of fast activity in the frequency range of the fast activity observed in the same regions in human TLE. The seizure-onset pattern is the same when different proepileptic agents, with different mechanisms of action, are utilized. These findings demonstrate that in an in vitro preparation in which the connectivity between brain areas is functionally preserved, temporal lobe seizure activity is quite stereotyped at onset, suggesting the existence of a prevalent and peculiar pattern generated by the hippocampal–entorhinal network.

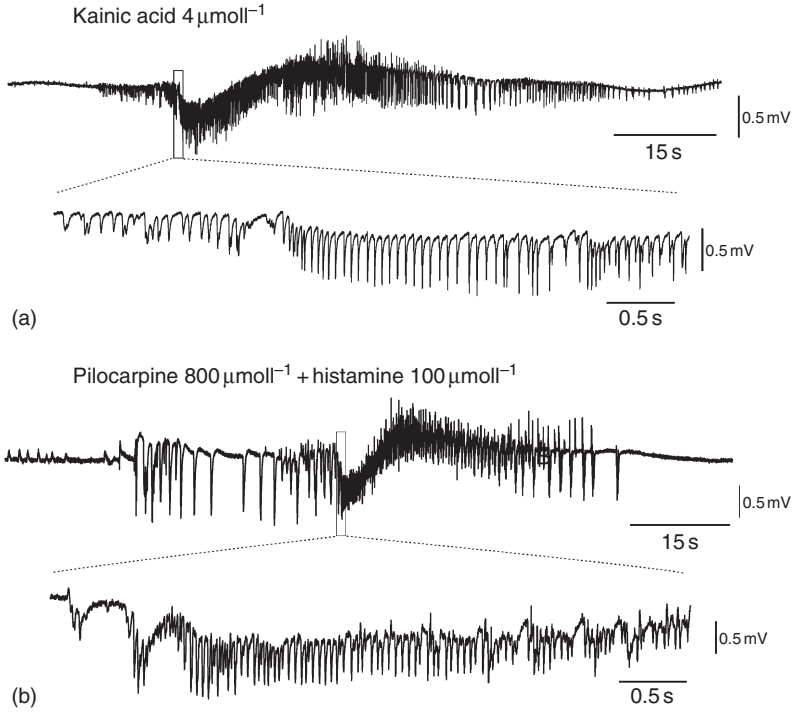


Figure 2 Ictal events recorded in the medial EC during arterial perfusion of kainic acid (a) or pilocarpine and histamine (b) in the in vitro isolated guinea pig brain. Expansions of the recordings at seizure onset are illustrated in the lower part of each panel. High pass filter was 0.02 Hz in these experiments.

Future Goals

The present data, and several reports published in the last 10 years on acute ictogenesis in the in vitro isolated guinea pig brain, demonstrate that this preparation is suitable to study system physiology of the temporal region in conditions of enhanced excitability. This experimental preparation represents a close-to-in vivo condition, but maintains the advantages of an in vitro preparation in terms of mechanical stability, accessibility to different brain regions, pharmacological manipulations, etc. As in other in vitro preparations, intracellular recordings with sharp electrodes can be obtained from principal cortical neurons and putative interneurons in the isolated guinea pig brain. This approach offers the possibility to identify the role of morphologically identified subpopulations of neurons during the interictal and ictal discharges described above. Modification of extracellular ion concentration (such as potassium and protons, i.e., pH) can also be measured in the isolated guinea pig brain during seizure activity. Laminar profiles of extracellular field activity, and current source density analysis of laminar profiles, are currently utilized in our laboratory to analyze evoked potentials and spontaneous event generated in the isolated brain. Finally, optical imaging of the activity-dependent signals generated by voltage-sensitive dyes perfused into the isolated brain has been used to map the tangential propagation of brain activity in the parahippocampal region. The combined use of these techniques can be used to analyze the role of individual variables (extracellular ions, single neuron activity, pH, networks) and their reciprocal interactions in the generation of network and structure-specific interictal–ictal patterns. In addition, further studies in the isolated guinea pig brain preparation will allow us to understand the complex interactions between different cortical (and subcortical) structures, and between hemispheres, in a condition of functional preservation of brain connectivity.

So far, the isolated guinea pig brain has been used to study acute ictogenesis. The in vitro maintenance of an entire adult brain relies on the existence of a good anatomical communication between the vertebrobasilar arterial system and the carotid system. The in vitro perfusion of the guinea pig brain is accomplished via a single access – the basilar artery. In the guinea pig (as well as in other lagomorph, such as the rabbit, but not in the rat and the mouse), the basilar artery bifurcates into two large posterior communicating arteries that allow a proper perfusion of the rostral part of the brain served by the carotid system. In rat and mice, cannulation of the basilar artery is practicable, but the posterior communicating arteries have a small size and do not allow a sufficient perfusate flow into the brain regions supplied by the carotids, via the anterior and medial cerebral arteries. Therefore, the brain of rodents such as rats and mice – in which most models of acquired or genetic-based epilepsy have been developed – cannot be studied using this in vitro in toto preparation. Therefore, to study a whole chronic epileptic brain in vitro, we need to develop chronic models in the guinea pig. A temporal lobe epilepsy model in the guinea pig is presently in development in our laboratory, with the objective of studying the cellular, homeostatic, and network mechanisms that regulate the generation of spontaneous epileptiform discharges in a chronic model of focal temporal lobe epilepsy.

The data reported here support an observation that is well known to clinical neurophysiologists – i.e., that the electroencephalographic correlate of seizure onset in focal epilepsies is the appearance of fast activity. This pattern has been confirmed by intracranial recordings performed in patients with temporal lobe (but also neocortical) epilepsy resistant to the pharmacological treatment, in presurgical evaluation for the excision of the epileptogenic area. Several reports indicated that one of the prevalent patterns of seizure initiation in mesial temporal lobe epilepsy is fast activity, at 15–30 Hz, that may be anticipated by the occurrence of large-amplitude EEG spikes. The reproduction of a similar pattern in homologous brain regions of the in vitro isolated guinea pig brain provides us with an opportunity to identify the mechanisms of seizure generation in the entorhinal cortex–hippocampus. On the basis of on these studies, more specific and detailed hypotheses on the mechanisms that initiate seizure activity in the mesial temporal lobe should be formulated and tested.

Acknowledgement

The study was sponsored by grant RF 06-50 of the Mariani Foundation for Neurosciences and by the Italian Health Ministry.

See also: **Models**: Model Characterization in Relationship to Human Disorders; Pharmacology of Seizure Drugs; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization.

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Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy

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Introduction

Kindling is a specific form of seizure-induced, activity-dependent plasticity which is associated with repeated episodic network synchronization. As an example of activity-induced modification of neuronal and circuit function, kindling shares the common feature of activity-induced long-term change in neural function with other phenomena of activity-dependent plasticity such as long-term potentiation (LTP) and developmental activity-dependent plasticity. However, the distinctive feature that distinguishes kindling from these other activity-dependent phenomena is its association with repeated episodic network synchronization and seizures.

Kindling of limbic structures has been regarded as a model of limbic or temporal lobe epilepsy (TLE). As the cumulative permanent effects of repeated episodic network synchronization eventually result in spontaneous seizures, limbic kindling has features of slowly developing epileptogenesis, and has been used for decades to investigate how repeated seizures modify neural pathways and contribute to the histopathological alterations underlying chronic TLE. The kindling model is also pertinent to understanding how seizure-induced plasticity and repeated seizures can contribute to development of increasingly severe seizures, and provides an opportunity to investigate the underlying neurobiology leading to intractable epilepsy. This latter aspect of kindling has been used to study how the adverse effects of repeated seizures can be modified by therapeutic agents. The well-controlled, gradually evolving chronic epileptogenic process that occurs during kindling has been exploited to study the progression of molecular, cellular, and circuit modifications that are a consequence of repeated seizures. More recently, the kindling model has been used to evaluate novel drugs that may be effective at slowing or stopping the progression of epileptogenesis.

Background

The phenomenon of kindling was initially recognized serendipitously in 1969, by Graham Goddard, during experiments investigating the effects of hippocampal stimulation on a learning task in awake, freely-moving rats. Subsequently, many investigators have used kindling to study a variety of plasticity-related phenomena, at both the electrophysiological and the behavioral/clinical levels. Kindling has been induced in a variety of animal species, with stimulation in many different brain regions. Kindling with stimulation in limbic areas of the brain has been most common, particularly from the amygdala. Clinical responses to limbic seizures were described and classified by Racine according to a scale that is based on severity of the evoked clinical phenomena. Seizure-like activity develops, associated with stereotypic behaviors, as follows: (i) behavioral arrest, eye closure, vibrissae twitching, sniffing; (ii) facial clonus and head bobbing; (iii) unilateral forelimb clonus; (iv) rearing with continued forelimb clonus (bilateral); and (v) rearing with loss of motor control and falling.

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Introduction

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Kindling provides the investigator with an opportunity to test hypotheses during a slowly evolving process of epileptogenesis under rigorous and graded control. This

control of epileptogenesis arises from the ability of the experimenter to determine the number of stimulations or evoked episodes of network synchronization to reach a particular stage of behavioral seizures, and to use the number and timing of stimulations as an experimental variable under rigorous control throughout the chronic kindling process. The episodic network synchronization and afterdischarges (ADs) required for kindling progression, and the eventual emergence of spontaneous seizures, can be elicited via stimulation through chronically implanted electrodes. Stimulation intensities that are initially subconvulsant for eliciting ADs in naïve animals dependably evoke overt secondarily generalized seizures in a fully kindled animal.

Experimental and Methodological Details

Kindled seizures can be induced by any method of stimulation that elicits network synchronization accompanied by ADs. Kindled seizures are typically evoked by electrical stimulation, consisting of trains of pulses 1–2 s in duration. Within the train, the pulses – typically 1.0 ms constant current biphasic pulses – are delivered at 25–150 Hz. Stimulation trains of 60 Hz were initially identified as particularly effective in evoking kindled seizures. Biphasic constant current square wave pulses are generally preferred to minimize possible chronic tissue changes induced by repeated stimulation at the electrode site, but constant voltage and sine wave pulses are also effective. Most kindling protocols use the lowest stimulation intensity that evokes an AD.

The evoked kindled seizures gradually evolve through predictable behavioral stages from partial or focal seizures (classes I–III) to secondary generalized tonic–clonic seizures (class V). For experimental purposes, the behavior observed in classes I, II, and III are compared with the complex partial or limbic seizures found in human epilepsies. The more severe seizures (i.e., classes IV and V) are consistent with secondary generalized seizures (Table 1). Once the animals begin to have class V seizures, they are considered fully kindled. The number of ADs required to evoke the first class V seizure is a highly reliable measure of the rate of kindling progression and varies as a function of stimulation site. As kindling progresses, there is a gradual reduction in the stimulation intensity required to evoke an AD and behavioral seizure, and the repeated evoked seizures induce permanent life-long increases in susceptibility to additional seizures.

Table 1 Classification of kindled seizures

Seizure Class (from Racine, 1972)	*Behavioral Manifestation*	*International Classification of Seizures (from Gastaut, 1970)*
Class 1	Mouth and facial movements	Partial
Class 2	Head nodding	Partial
Class 3	Forelimb clonus	Partial or secondary generalized
Class 4	Rearing	Secondary generalized
Class 5	Rearing and falling	Secondary generalized

In a given animal species, the rate of kindling development to the stage of class V seizures is remarkably consistent, as a function of the site of stimulation. The interstimulus interval that optimizes the rate of kindling progression appears to be under strict control, as repeated brief stimulus trains applied 1–2 times daily (~8–24-h intervals) most reliably induce the gradually evolving behavioral and electrographic seizures and permanently increased susceptibility to additional seizures. 'Massed' stimulation, which refers to delivery of stimulus trains at short intervals, can inhibit kindling development. However, protocols have also been developed that group stimulations more closely than 1–2 per day and still produce permanent increased seizure susceptibility and spontaneous seizures. Although these so-called 'rapid' kindling protocols induce permanent functional modifications, the alterations induced by the different protocols may not be identical in all respects. With sufficient repetition of the evoked seizures (using the conventional once or twice daily stimulation protocol), spontaneous seizures are observed; this kindling stage usually requires 90–100 evoked seizures. At this stage of the kindling process, epilepsy (defined as chronic susceptibility to spontaneous seizures) has been induced.

Although electrical stimulation is most commonly used to evoke kindled seizures, repeated application of chemoconvulsants such as pentylenetetrazol, carbacol, cocaine, bicuculline – any agent that elicits network synchronization accompanied by ADs – also induces kindling. Both focal and generalized application of chemoconvulsants will induce kindling. The regions and pathways of the central nervous system that demonstrate kindling in response to electrical stimulation are diverse, and include neocortex, subcortical regions, limbic system, and brainstem (Table 2). Limbic structures appear to be particularly vulnerable, with fastest rates of kindling (defined by the number or ADs required to elicit a class V seizure) observed with stimulation of the perirhinal cortex > olfactory bulb > amygdala > entorhinal cortex > hippocampus. It is presumed that the rate of kindling, which is remarkably consistent for a given site of stimulation, is a reflection of an evolving sequence of activity-induced neuronal and cellular alterations that gradually propagate into the motor system circuitry underlying evoked secondarily generalized tonic–clonic (class V) seizures. The progression and permanence of kindling in neural pathways 'transfers' across sites

Table 2 Brain regions susceptible to kindling

Brain region	*Mean number of stimuli required to induce first convulsion*	*Relative susceptibility of structure to kindling*
Amygdala	15	More vulnerable to kindling
Globus pallidus	22	
Pyriform cortex	24	
Olfactory area	29	
Anterior limbic field	29	
Entorhinal cortex	37	
Olfactory bulb	44	
Septal area	55	
Preoptic area	63	
Caudate-putamen	74	Less vulnerable to kindling
Hippocampus	77	

Source: Goddard GV, McIntyre DC, and Leech CK (1969) A permanent change in brain function resulting from daily electrical stimulation. *Experimental Neurology* 25: 295–330.

Table 3 Species susceptibility to kindling

Frog	Amphibians
Rats	
Rabbits	
Cats	
Dogs	
Monkeys	
Baboons	Primates

as well as across methods of induction, and kindling can be induced in species ranging from amphibians to nonhuman primates (Table 3). For all of these reasons, kindling is most appropriately regarded as a phenomenon of seizure-induced plasticity.

Recent Results

Cellular and Network Alterations Underlying Epileptogenesis by Kindling

Kindling produces a predictable pattern of evolving molecular and cellular alterations that include (i) changes in composition and biophysical properties of membrane receptors, (ii) alterations in signal transduction pathways, (iii) changes in gene transcription, (iv) neuronal cell loss in the hippocampus and other limbic structures, (v) sprouting and reorganization of neuronal circuitry, (vi) gliosis, and (vii) neurogenesis (Table 4). Previous studies have demonstrated that long-lasting increases in synaptic transmission, resembling the synaptic potentiation induced by LTP, occur early during the acquisition of kindling. Antagonism of NMDA-dependent synaptic transmission during

Table 4 Structural and functional alterations induced by kindling

Number of evoked seizures	*Cellular, molecular, and behavioral alterations induced by repeated kindled seizures*	*Stage of epilepsy progression*
1	↑ NMDA ↑ Inhibition in DG Apoptosis Neurogenesis Gliosis	Epileptogenesis
5	Mossy-fiber sprouting	Epileptogenesis
30	Hippocampal sclerosis Memory loss	Epileptogenesis
90–100	Interneuron loss Reduced inhibition Spontaneous seizures	Epilepsy

application of kindling stimulation blocks the development of progressive cellular and circuit alterations, even when seizures are still evoked. Studies of kindling acquisition in transgenic mice with conditional knockout of the gene for brain-derived neurotrophic factor (BDNF) and its receptor (TrkB) have demonstrated a complete block of kindling progression despite continuing repeated seizures. Other neurotrophins (NT3, NT4) and their associated tyrosine kinase (Trk A/B) receptors may also contribute to the long-term alterations induced by kindled seizures. The NMDA and neurotrophin signaling pathways appear to be directly involved in kindling progression and in the development of chronic and long-term circuit alterations associated with seizure-induced plasticity.

As increasing numbers of seizures are evoked, the gradual progression toward epileptogenesis and spontaneous seizures is associated with continuing morphological reorganization of neurons and neural circuits accompanied by an increasing susceptibility to additional seizures and progressive behavioral changes, including memory dysfunction. The cellular structural/pathological alterations that are induced by repeated episodes of network synchronization (i.e., kindling) have been characterized in considerable detail. These changes occur with a predictable time course, as a function of the number of evoked seizures. These changes include both neuronal cell loss, and aberrant sprouting of mossy-fiber collaterals in the dentate gyrus, which form recurrent excitatory circuits.

Each evoked seizure results in a twofold increase in neuronal apoptosis. Cumulatively, there is a decrease in neuronal density associated with kindling, reflecting neuronal loss, which becomes detectable and progressively increases with increasing numbers of kindled seizures (i.e., after 3, 30, and 150 class V seizures). This neuronal loss is prominent bilaterally in the hilus of the dentate gyrus, and is also noted in CA3 and CA1, in a pattern resembling hippocampal sclerosis – the most common

lesion observed in human TLE. Neuronal loss is also observed in the medial entorhinal cortex, the lateral entorhinal cortex, and the rostral endopyriform cortex, suggesting that the neuronal loss is caused by propagation of repeated seizures and not by damage directly attributable to the stimulation delivered by an electrode. A substantial number of repeated kindled seizures (i.e., 150 class 5 seizures) eventually induce neuronal loss even in relatively 'resistant' brain structures, such as the dentate granule cell layer and CA2 (Table 5). These patterns of cell loss (including a reduction in neuronal density in the temporal pole of the rat) suggest that the progressive pathological changes associated with epilepsy in the kindled rat model parallel many features of hippocampal sclerosis in human TLE – and raise the important question of whether similar mechanisms are involved.

The kindling model enables investigators to perform experiments at different time points obtained during the progression of epileptogenesis. Investigators can carry out correlational studies – e.g., to assess functional alterations associated with seizure-induced progressive structural alterations, such as mossy-fiber sprouting after a specific number of evoked seizures. As noted earlier, kindled seizures induce aberrant granule cell mossy-fiber axon collaterals that sprout into the supragranular layer of the dentate gyrus, as revealed by the Timm staining method (which labels synapses with high concentrations of zinc). Although it is difficult to unequivocally isolate the effects of a given seizure-induced alteration on network function, these studies have revealed that synaptic reorganization and the formation of new excitatory circuits is associated with increases in recurrent excitation that become unmasked by reducing inhibition. There is also a correlation between the degree of mossy-fiber sprouting and (1) the duration of seizure activity in local circuitry, and (2) the propensity of partial seizures to propagate to more distant sites and develop into secondarily generalized seizures. Synaptic reorganization and mossy-sprouting induced by repeated seizures thus are associated with progressive permanent susceptibility to additional seizures, and potentially to development of intractable epilepsy.

The development of spontaneous seizures is associated with loss of specific GABAergic interneuron subclasses, specifically GABA transporter type 1 (GAT-1) and cholecystokinin (CCK) labeled interneurons, which provide critical somatic and axon initial segment control over spike generation. These interneuron subtype-specific alterations are accompanied by loss of GABAergic inhibition assessed by electrophysiological methods, including paired pulse and single-electrode voltage clamp techniques. Paired pulse methods demonstrate that a loss of paired pulse inhibition in the dentate gyrus occurs in association with the emergence of spontaneous seizures in kindled rats (after about 100 evoked class V seizures). Direct measurements of GABAergic inhibition using single-electrode voltage clamp techniques also confirm alterations in the kinetics and reduction in the amplitude of the evoked IPSC in granule cells consistent with reduction in inhibition in animals experiencing spontaneous

Table 5 Neuronal loss induced by repeated kindled seizures

Number of evoked class V seizures	*Brain structure with significant neuronal loss*	*Location*	*Susceptibility of structure to damage*
3	DG hilus	Temporal	More vulnerable to cell death
		Septal	
	CA1a	Temporal	
	CA3a	Temporal	
30	DG hilus	Temporal	
		Septal	
	CA1a	Temporal	
		Septal	
	CA3a	Temporal	
		Septal	
	CA1c	Temporal	
		Septal	
	CA3c	Temporal	
		Septal	
	DG sg	Temporal	
	MEC2	Temporal	
		Septal	
	LEC2	Temporal	
		Septal	
	LEC6	Temporal	
	EN		
100	DG hilus	Temporal	
		Septal	
	CA1a	Temporal	
		Septal	
	CA3a	Temporal	
		Septal	
	CA1c	Temporal	
		Septal	
	CA3c	Temporal	
		Septal	
	CA2	Temporal	
	DG sg	Temporal	
	MEC2	Temporal	
		Septal	
	LEC2	Temporal	
		Septal	
	MEC6	Temporal	
		Septal	
	LEC6	Temporal	
		Septal	
	EN		Less vulnerable to cell death

DG, dentate gyrus; DG sg, granule cell layer of the dentate gyrus; MEC2, MEC6, layers 2, 6 of the medial entorhinal cortex; LEC2, LEC6, layers 2, 6 of lateral entorhinal cortex; EN, endopyriform cortex.

Source: Cavazos JE, Das I, Sutula TP (1994) Neuronal loss induced in limbic pathways by kindling: Evidence for induction of hippocampal sclerosis by repeated brief seizures. *Journal of Neuroscience* 14: 3106–3121.

seizures. In ongoing studies of functional and structural properties of granule cells and hippocampal circuits in kindled rats experiencing spontaneous seizures, it appears that seizure-induced alterations in inhibitory circuitry involving subtype composition of GABA receptors, modifications of proportions of excitatory and inhibitory synapses, and alterations of ion cotransporters influencing Cl^- gradients all contribute to the loss of GABAergic inhibition.

Kindling as a Model for Drug Development

As a model for development of refractory TLE, kindling may potentially have predictive value as a test for new antiepileptic drugs (AEDs) to treat TLE and complex partial seizures. The benefit of the kindling model for the purpose of drug development is that it allows AED testing throughout the well-controlled epileptogenic process, and chronic efficacy studies can be conducted in fully kindled animals that have developed spontaneous seizures. Additionally, if the kindling process is completed rapidly (i.e., 1 day vs. 10 days of repeated stimulation) or if suprathreshold stimulation parameters are used, acute AED testing can be conducted to differentiate between drugs that increase seizure threshold or decrease seizure spread. Efficacy endpoints include: (1) seizure latency; (2) seizure severity based on behavioral seizure classes (see above); (3) seizure duration; and (4) AD duration. Similar endpoints can be assessed during chronic AED testing (in fully kindled animals); daily stimulation is possible at this stage without alteration of seizure parameters. Two main approaches using suprathreshold stimulation in fully kindled animal to test AEDs are possible. One method involves assessing these measures after administration of a single dose of drug (or control substance). This method does not allow differentiation between the affect of drug on partial versus generalized seizures. A second method considers this potential difference by calculating separate ED_{50} values for partial seizures, generalized seizures, and ADs. Phenytoin, carbamazepine, valproic acid, and primidone are effective against the complex partial seizures, as is phenobarbital after prolonged administration. Ethosuximide is ineffective and benzodiazepines are only moderately effective in the amygdala-kindled model. The ED_{50} values calculated for carbamazepine, phenytoin, and phenobarbital show that each of these drugs is more effective at reducing secondarily generalized seizures than at blocking focal seizures. Recent studies have demonstrated that 2-deoxy-D-glucose (2DG), an inhibitor of glycolysis, results in a twofold slowing of the kindling process, and therefore has 'disease-modifying' effects against seizure progression.

AED Responders and Nonresponders

Fully kindled rats are currently the only established model of pharmacoresistant epilepsy, and are therefore of potential use for AED development. The focal and secondarily generalized seizures observed in these animals are less sensitive to traditional AEDs than seizures evoked in the maximal electroshock (MES) test. Repeated testing of traditional AEDs (such as phenobarbital and phenytoin) on the focal seizure threshold (determined by ADs of fully kindled rats) allows separation of the animals into two distinct 'drug-responsive' and 'nonresponsive' categories. The drug-responsive animals exhibit reproducible increases in focal seizure threshold after repeated AED challenges, but nonresponsive animals fail to show any increase in focal seizure threshold. Animals categorized as pharmacoresistant or nonresponders (i.e., phenytoin or phenobarbital nonresponders) have been used for subsequent experiments, in which other established AEDs also show a reduced antiseizure effect in the nonresponsive kindled animals (relative to the drug-responsive group). Kindled rats with drug resistance are now being used to evaluate novel AEDs and to study potential mechanisms of pharmacoresistance.

Slow and Fast Kindling Strains

Racine and colleagues exploited the observation that some animals kindled more readily than others, and used breeding approaches to develop rat strains with reliable phenotypes of fast or slow kindling responses in response to stimulation of the amygdala. The selection of fast and slow strains is consistent with the view that the predisposition of an animal to undergo fast or slow kindling acquisition is under complex genetic influence. 'Slow' kindling animals require a significantly greater number of ADs to experience class V seizures; the converse is the case for 'fast' strain. The discovery that fast and slow kindling strains can be selected by breeding procedures provides an opportunity to examine genes which may be relevant to seizure susceptibility. Whether the 'fast' and 'slow' strains selected in response to stimulation of the amygdala would resemble strains selected by stimulation of other sites is currently uncertain, as is the question of whether a predisposition to one type of epilepsy enables the development of different types of seizures in other circuits.

Current Problems and Future Challenges

Kindling has provided valuable information on seizure susceptibility, the effects of repeated seizures on the brain, and the roles of different structures in initiating and maintaining seizures and regulating seizure propagation. Kindling also offers an opportunity for screening potential new AEDs for partial epilepsy. A practical disadvantage of the model is that it takes such a long time to generate animals with spontaneous seizures. Spontaneous seizures are observed only after numerous (>100) evoked class V seizures, but not in animals that that are typically labeled as 'kindled' – i.e., have exhibited only a few stage V seizures.

Although kindling is undeniably a phenomenon of seizure-induced plasticity, observed across a range of species (including primates) the relevance of the phenomena of seizure-induced plasticity (kindling) to clinical features of human TLE is still debated. One argument against the relevance of the kindling model is that the seizures are induced in *normal* brain. The consequences of seizures in normal animals may not be the same as those observed in chronically epileptic animals with established histopathology – e.g., as observed in kainate-treated rats after status epilepticus.

The practical limitations, and the uncertainty about the general relevance of seizure induced plasticity for human epilepsy must to be considered when kindling is used as a model for studying seizures, epileptogenesis, or chronic epilepsy.

See also: **Interneurons**: Plasticity and Reorganization of GABA Neurons in Epilepsy; **Models**: Characteristics and Mechanisms of Kindling; Model Characterization in Relationship to Human Disorders; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; **Neurotrophic Factors**: Role of BDNF in Animal Models of Epilepsy; **Plasticity**: Circuitry Reorganization, Regeneration, and Sprouting; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy.

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Model Characterization in Relationship to Human Disorders

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Hughlings Jackson, in the mid to late 1800s, identified the anatomic substrates of focal epileptic seizures by observing specific ictal signs and symptoms and correlating them with the location of cortical lesions at autopsy. Localization, however, required confirmation in the animal laboratory – which was obtained when David Ferrier electrically stimulated the same areas of monkey cortex and reproduced the ictal behaviors Hughlings Jackson observed in patients. This work led not only to localization of epileptogenic tissue for purposes of surgical resection (beginning in the 1880s), but to insights into localization of normal function within the human brain. Research on people with epilepsy further contributed to this effort as a result of intraoperative functional mapping with electrical stimulation in the course of surgical resection for intractable epilepsy; in this manner, Penfield and Jasper, at the Montreal Neurologic Institute, in the mid twentieth century, defined the human motor and sensory homunculi, as well as other functional properties of neocortex and mesial temporal limbic structures.

Despite these seminal human investigations, the tremendous advances in our understanding of basic physiologic mechanisms and anatomic substrates of epileptic phenomena that occurred during the latter half of the twentieth century derived almost entirely from animal research. Most of the work at this time involved electrophysiological and morphological investigations that could not readily be performed in patients. Although Crandall and the group at UCLA developed a protocol for chronic in vivo microelectrode recordings from patients undergoing presurgical evaluation for medically refractory temporal lobe epilepsy (results of which were correlated with structural disturbances obtained from en bloc resected tissue), very few centers had the facilities to carry out such research. Furthermore, invasive basic research in the epilepsy surgery setting is difficult because of the absence of control data, the need to modify research protocols to fit individual clinical circumstances, ethical constraints, the limited population that can be examined within a reasonable period of time, and cost. Such human research is essential, however, because the data obtained provide the basis for hypotheses that can then be tested more cost-effectively in the animal laboratory.

In recent years, functional neuroimaging – first with positron emission tomography (PET) and single photon emission computed tomography (SPECT), and then with functional magnetic resonance imaging (fMRI) and magnetic resonance spectroscopy (MRS) – has augmented our enhanced ability to localize the sources of electrical discharges with computerized electroencephalography (EEG) and magnetoencephalography (MEG). These techniques have also greatly increased opportunities to examine basic physiological mechanisms and anatomic substrates of human brain function noninvasively. In addition, the expanding field of genetics has stimulated tremendous research efforts with patients that should ultimately elucidate fundamental mechanisms of brain disturbances causing epilepsy. Animal research, however, will continue to play an essential role in the search for the neurobiological bases of the epilepsies, whether derived from results of human genetic studies or other human research. The development of appropriate animal models for the many forms of human epilepsy, therefore, is central to this effort.

Clinical Epilepsy, What to Model

Epileptic seizures are merely signs and symptoms of brain dysfunction and take many forms, depending upon the nature of the cerebral disturbance, its location in the brain, and preexisting conditions that influence threshold and behavioral manifestations. There are, therefore, many different types of epileptic seizures. The classification of epileptic seizures is described in the Clinical Appendix. Traditionally, ictal events are divided into focal (partial, localization-related) seizures, which begin in a part of one hemisphere, and generalized seizures, which begin on both sides of the brain simultaneously. This division is, however, an artificial dichotomy. It is unlikely that any seizures are truly generalized at onset, and also unlikely that many, if any, seizures are due to a discrete focal abnormality. Rather, most epilepsies, whether focal or generalized, require involvement of diffuse distributed cerebral networks.

Epileptic seizures can be a natural response of the normal brain to a transient insult, in which case they are called acute symptomatic, provoked, or reactive, and do not indicate an epileptic condition. Epilepsy, on the other hand, implies the existence of an enduring cerebral epileptogenic substrate capable of generating spontaneous seizures. With epilepsy, therefore, the brain is epileptogenically abnormal, whether seizures are occurring or not. Just as there are many types of seizures, there are many conditions that cause epilepsy and classification of the great variety of epileptic disorders (see Engel and Berg article on 'Classification' in Clinical Appendix). Traditionally, epilepsies are also subjected to the focal/generalized dichotomy, and in addition are divided into those that are secondary to structural or metabolic brain disturbances (symptomatic or secondary) and those that are epilepsy only, *sui generis* (idiopathic, from the Greek *idios*, or primary). The term 'idiopathic', as used here, does not mean 'cause unknown' as it does in the rest of American medicine, but infers a genetic etiology. Idiopathic epilepsies usually appear at specific periods of brain development and are thus age-related. There are conditions, however, that do not fit the criteria for recognized idiopathic epilepsy syndromes, and therefore, are presumed to be symptomatic although the causes are

unknown, and these are referred to as 'cryptogenic.' The symptomatic/idiopathic dichotomy is also artificial, and the term 'cryptogenic' is considered to be ambiguous. Accurate descriptions of epilepsy syndromes and epileptic phenomena are essential to the development of relevant experimental animal models, and animal research provides critical information for accurate clinical classification.

As there are undoubtedly many different pathophysiologic mechanisms and anatomic substrates responsible for the variety of epileptic seizures and epilepsy syndromes that are encountered clinically, an experimental animal model of one type of seizure or epilepsy may bear no relationship to other types of seizures or epilepsy. Furthermore, there are few, if any, clinical epilepsy syndromes that can be reliably reproduced in their entirety by an experimental animal model. It is helpful, therefore, to break clinical epilepsy down into a number of component parts that can be modeled or studied individually. Such components include the following:

The *interictal state* is characterized not only by a persistent epileptogenic disturbance, which may be a structural lesion or a genetically mediated disturbance, but by homeostatic mechanisms that maintain the interictal state and prevent seizures from occurring continuously.

There are mechanisms within the epileptogenic substrate, as well as normal and pathological cerebral and systemic influences, that are responsible for the *transition from the interictal to the ictal state.*

Once seizures begin, there are *mechanisms responsible for maintenance of the discharge, as well as propagation* locally and to distant structures, including engagement of brain stem systems responsible for generalized tonic–clonic seizures when secondarily generalized seizures occur.

Eventually, seizures are *terminated by multiple mechanisms*, some of which involve active inhibition or desynchronization, while others are passive effects of excessive activity. Clearly, mechanisms that act to terminate seizures are different from those that act to contain the spread of epileptic activity, because the former fail while the latter remain effective in epilepsia partialis continua.

Most (but not all) seizures are followed by *transient postictal disturbances*, which can be more disabling than the seizures themselves, and likely reflect persistent effects of the mechanisms that terminate seizures, although other seizure-induced influences can also contribute to certain postictal behaviors.

Some patients develop *interictal behavioral disturbances*, which can reflect enduring disturbances in cerebral function or, in some cases, even structure – either as a consequence of repeated ictal events or as a side-effect of the homeostatic mechanisms that act to maintain the interictal state. Brain damage results from uncontrolled status epilepticus but might also be caused by repeated, or even single, seizures. Epileptic encephalopathies are conditions where seizures themselves are the cause of progressive epileptic and nonepileptic brain disturbances.

The *development of symptomatic epilepsies (epileptogenesis)*, as a result of epileptogenic insults, occurs over long periods of time and also undoubtedly involves a number of mechanisms that differ from those associated with epilepsy itself. For instance, pharmacological agents that are effective at preventing epileptic seizures may not be effective in preventing epileptogenesis, and risk factors for developing epilepsy are not exactly the same as risk factors for experiencing an epileptic seizure. Furthermore, epileptogenesis does not necessarily stop as soon as spontaneous seizures appear, but in many instances continues, causing the epileptogenic region to increase in size and give rise to secondary epileptogenic areas – resulting in more frequent, more severe, and more intractable seizures. The onset of idiopathic epilepsies may not involve such epileptogenic processes, but rather result from an epileptogenic substrate that requires a specific stage of brain maturation for its manifestation.

Another way to appreciate the multifactorial nature of epilepsy is shown in **Figure 1**, which illustrates three factors. The first – threshold – refers to the ease with which a seizure can be induced in a given brain. Differential susceptibility to acute seizures in humans is influenced by nonspecific predisposing factors such as age, genetics, and acquired insults, which may not themselves be epileptogenic. Threshold is not a static phenomenon – it has a circadian rhythm (usually higher during wakefulness and REM sleep and lower during drowsiness and slow-wave sleep), varies with the menstrual cycle in women, and can be lowered by influences such as fever, sleep deprivation, stimulant drugs, and withdrawal from sedative drugs and alcohol. Most antiepileptic drugs act by nonspecifically modulating excitation, inhibition, and synchronization in the brain to increase threshold, which also can result in unwanted side effects.

A second factor is the specific epileptogenic disturbance, which might be an obvious structural lesion (such as a brain tumor, scar, vascular malformation, cortical dysplasia, infection, or hippocampal sclerosis), or it could be a genetically determined epileptogenic brain dysfunction. The potency of the underlying epileptogenic abnormality may also vary over time. There may be effective treatments for specific epileptogenic disturbances, or they can sometimes be surgically removed.

A third factor is precipitating influences, which determine when seizures occur. In some cases, such as the photosensitive epilepsies, the precipitating stimulus is easily identified, but in most patients these are subtle internal or external events that remain unrecognized. When precipitating factors can be identified, such as flashing lights, they can often be avoided, for instance by wearing colored glasses or patching one eye.

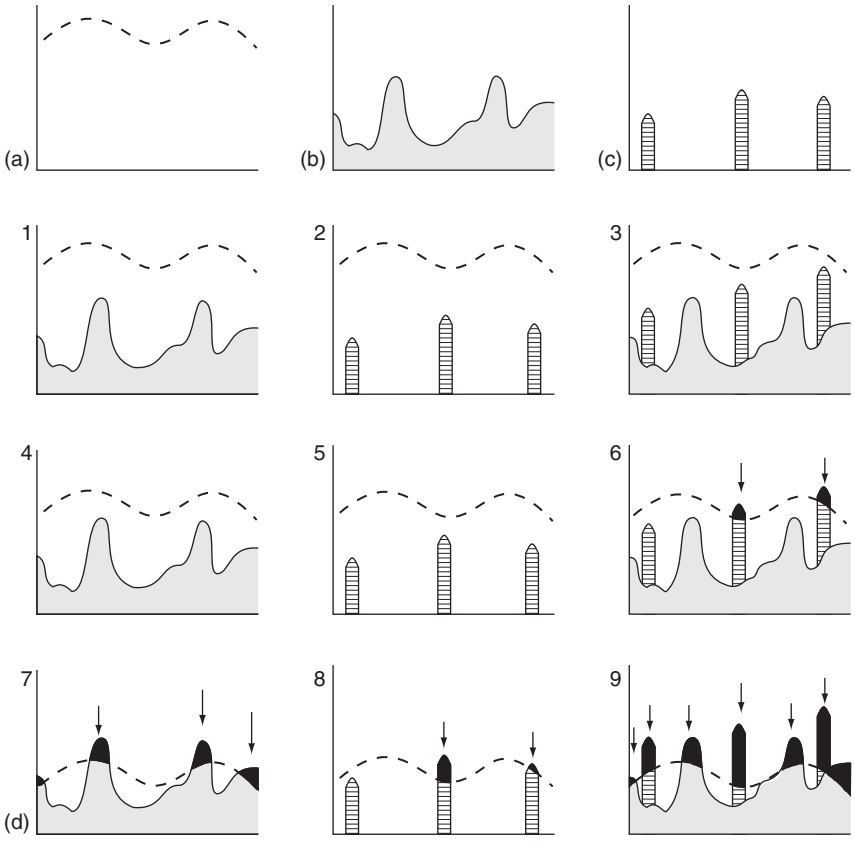


Figure 1 A schematic illustrating the interactions of the fluctuating threshold for seizures determined by *nonspecific predisposing factors* (a); independent fluctuations of a *specific epileptogenic disturbance* (b); and intermittent *precipitating factors* (c). With a high threshold, epileptogenic disturbances and precipitating factors alone (d1, d2) and combined (d3) fail to generate seizures. With an intermediate threshold, epileptogenic disturbances and precipitating factors alone (d4, d5) fail to generate seizures, but seizures (*arrows*) occur when these factors are combined (d6). With a low threshold, epileptogenic disturbances and precipitating factors alone (d7, d8) are each capable of generating seizures, and when combined (d9) generate even more seizures, perhaps constituting status epilepticus. Reproduced from Engel J Jr (1987) *Seizures and Epilepsy*. Philadelphia: F.A. Davis, with permission from F.A. Davis.

Types of Animal Models

Each of the many aspects of epilepsy discussed in the previous section could be studied individually in the animal laboratory by utilizing phenomenological paradigms that mimic a particular component part of the clinical disorder. In their most fundamental form, these may not be models of epilepsy, but rather, epilepsy 'equivalents'. A common component part that was studied effectively for many years is the ictal event itself. Acute experimentally-induced seizure models have been extremely useful for investigating mechanisms of seizure maintenance, propagation, and termination, but they provide no information concerning epileptogenesis, the epileptogenic abnormality that exists in chronic epilepsy, the transition from the interictal to the ictal state, or the consequences of epilepsy. In recent years, research has been more focused on chronic animal models of epilepsy, which come closer to reproducing some of the more common human epilepsy conditions; however, there are currently no experimental animal models for the majority of human epilepsy syndromes.

Whether studying models that represent epileptic equivalents, acute epileptic seizures, or chronic epilepsy, two conditions are important in order to extrapolate results to patients – or to other animal models: age and species. Epileptic mechanisms are different in the neonatal brain than they are in the immature brain; mechanisms during various stages of brain development are different from those of the adult brain and, as we are now learning, from the aged brain. Epileptic phenomena have been studied in a large variety of animal species ranging from single-cell organisms to subhuman primates. Flies, worms, fish, and frogs have been used to create relatively inexpensive experimental preparations. Guinea pigs, cats, and dogs have been popular laboratory animals for epilepsy studies in the past, but most animal research on epilepsy today is carried out on rats, and more recently mice as a result of the availability of mutant and transgenic mice. Even when studying a single species, results obtained from one strain may be different from those of another because of genetic predisposing factors. For instance, rat strains have been developed that kindle rapidly, and

others that are relatively resistant to kindling. Similarly, preexisting genetic epilepsy alters epileptogenic mechanisms; for instance, the photosensitive baboon *Papio papio* kindles much more rapidly than do nonepileptic baboons or monkeys. Most animal research is carried out on males in order to avoid variability introduced by hormonal cycles of females; however, animal models are needed to understand the mechanisms of gender differences that exist in some human epilepsies.

Presumed Epileptic Equivalents

Examples of laboratory phenomena that are believed to reflect mechanisms that contribute to epileptogenesis or ictogenesis are listed in Table 1. Based on responses to specific categories of antiepileptic drugs, posttetanic potentiation is believed to be a model of generalized tonic–clonic seizures, and repetitive stimulation of cat spinal cord to be a model of absence seizures. Long-term potentiation, bursting neurons in vitro, and fast ripples are considered to be components of focal seizures, although they could also contribute to some generalized seizures. Reductionist models have been created in simple organisms that are believed to reflect epileptic equivalents.

Table 2 lists mechanisms believed to underlie epileptogenesis, and that may be targets for the development of surrogate markers. Although epilepsy equivalents are useful for investigating mechanisms of epilepsy, research on target mechanisms could help to develop biomarkers in order to: (1) identify patients at risk for epilepsy following potential epileptogenic insults, who may then be treated with preventive interventions; (2) diagnose epilepsy after a single seizure, in order to institute therapy as early as possible; (3) determine whether an antiepileptic drug, or other therapeutic intervention, will be effective without the need to wait for a second seizure to occur; (4) identify patients whose epilepsy is pharmacoresistant, who should then be considered for early surgical treatment; and (5) localize the epileptogenic region reliably, and cost-effectively, for surgical resection.

Table 1 Examples of epileptic equivalents

Posttetanic potentials (PTPs)
Repetitive stimulus of cat spinal cord
Long-term potentiation (LTP)
Bursting neurons in vitro (e.g., with low Mg^{2+}, NMDA)
Fast ripples
'Seizures' in
Zebra fish
C. elegans
Drosophila

Table 2 Examples of target mechanisms

Cell loss
Axonal sprouting
Synaptic reorganization
Altered gene expression profiles and protein products
Neurogenesis
Altered glial function and gliosis
Inflammatory changes
Angiogenesis
Altered excitability and synchrony

Acute Experimentally-Induced Seizure Models

Examples of acute experimentally-induced seizure models are shown in Table 3. Localized application of electrical stimulation, convulsant drugs (such as penicillin, bicuculline, picrotoxin, and pentylenetetrazol) that block inhibition, and convulsant drugs (such as carbachol and kainic acid) that enhance excitation are commonly used to model focal seizures. Maximal electroshock and systemic convulsant drugs (such as pentylenetetrazol, allylglycene, bicuculline, kainic acid, convulsant barbiturates, ouabain, anticholinesteraces, and thiosemicarbazid) have been used to model generalized tonic–clonic seizures. Certain subcortical electrical stimulations, subcutaneous pentylenetetrazol, intramuscular penicillin in the cat, intracerebroventricular opioids, γ-hydroxybuterate injection, CO_2 withdrawal, and hyperthermia in immature animals are believed to produce seizures that mimic human absences. These different acute seizure models involve a variety of underlying mechanisms, and the relevance of each to acute symptomatic epileptic seizures – or epilepsy – in patients remains to be demonstrated. For instance, the seizures of childhood absence epilepsy appear to have a relatively consistent, well-defined, clinical manifestation, yet the ictal phenomenology appears to be reproduced by a large number of experimental perturbations. Further research is necessary to determine what these different purported models have in common with each other, and ultimately with the human condition.

Experimental models of status epilepticus can be induced by a variety of chemoconvulsants such as bicuculline,

Table 3 Examples of acute experimentally-induced seizure models

Focal electrical stimulation
Topical convulsants
Topical GABA withdrawal
Maximal electroshock
Systemic convulsant drugs
Fluorothyl
Subcortical electrical stimulation
Subcutaneous pentylenetetrazol
Intramuscular penicillin (cat)
Intracerebroventricular opioids
Systemic γ-hydroxybuterate
CO_2 withdrawal seizures
Hyperthermia in immature animals
Status epilepticus (bicuculline, fluorothyl, cobalt-homocystein, kainic acid, pilocarpine, soman, electrogenic)

fluorophyl, cobalt-homocystein, kainic acid, pilocarpine, and soman, as well as by electrical stimulation. These models are important for understanding the breakdown of mechanisms that naturally terminate ictal events, as well as for understanding the evolution of pathophysiological events that appear to change over time, resulting in pharmacoresistant status epilepticus, brain damage, and death.

Chronic Experimentally-Induced Epilepsy Models

Examples of chronic experimentally-induced models are shown in **Table 4**. Freeze lesions, partially isolated cortical slabs, and metals such as alumina, cobalt, tungstic acid, and ferric chloride have been used for decades, particularly to mimic neocortical epileptogenic lesions. Tetanus toxin and immunologic challenges are more recent approaches. Beginning in the 1970s, kindling became the most popular chronic model for the study of epileptogenesis, but technically it is not a model of epilepsy because, as usually performed, seizures do not occur spontaneously, but require electrical stimulation. This approach was most commonly applied to the limbic system and brought the process of epileptogenesis under laboratory control. If kindling is continued long enough, spontaneous seizures do occur; however, this is a difficult experimental model to create and maintain.

Most research on chronic epilepsy today utilizes models of mesial temporal lobe epilepsy with hippocampal sclerosis, which develop after status epilepticus. Systemic pilocarpine, kainic acid, and electrical stimulation-induced self-sustained status epilepticus result in bilateral hippocampal damage and other diffuse lesions that eventually give rise to spontaneous seizures arising from the limbic system. Intrahippocampal kainic acid produces unilateral hippocampal sclerosis with less-diffuse cerebral toxicity and less-frequent seizures, perhaps better modeling the human condition.

With the advent of high-resolution MRI, it has become apparent that malformations of cortical development, particularly focal cortical dysplasia, are common causes of medically intractable focal epilepsy in patients. Several animal models have been developed to study these important pathological conditions in the laboratory.

Spontaneous epileptic seizures, or paroxysmal activity presumed to bear some relationship to epileptic seizures, exist naturally in animals from flies to subhuman primates. **Table 5** lists examples of genetic animal epilepsy models that have been used for investigations into pathophysiologic mechanisms of idiopathic epilepsies. In addition, human gene discovery efforts are yielding candidate genes for epilepsy that can be inserted into mice in order to characterize the aberrant gene products and determine how these proteins eventually lead to the manifestation of epileptic signs and symptoms. Because of species and strain differences, the resultant in vivo models may bear no resemblance to the human condition from which the gene mutation was derived; however, critical information about underlying mechanisms may still be obtained. Aberrant genes, for instance, may not directly influence excitation, inhibition, and synchronization, which predispose the animal to ictal events; rather, they may alter brain development in ways that, in some species but not others, lead to an epileptic condition long after the effects of the mutant gene have disappeared.

The recognition that experimentally-induced ictal events are not epilepsy, and that research into basic mechanisms must involve chronic models with spontaneous seizure generation, was a major paradigm shift. We now know, however, that experimentally inducing an epileptogenic abnormality in a normal animal brain is also unsatisfactory as a model of the human condition, because people who develop epilepsy often, or perhaps always, have underlying predispositions (such as susceptibility genes) that alter their threshold not only to spontaneous seizures but also to epileptogenesis. Clinical research, therefore, must identify these predisposing factors so that they can be incorporated into appropriate experimental animal models of chronic epilepsy.

Table 4 Chronic experimentally-induced epilepsy models

Freeze lesions
Partially isolated cortical slab
Topical metals
Tetanus toxins
Anti-GMI ganglioside antibodies
Kindling
Post status limbic epilepsy
Pilocarpine
Kainic acid (intrahippocampal, systemic)
Self-sustained status epilepticus (SSSE)
Epileptogenic cortical dysplasia
Methylazonymethanol (MAM)
Freeze induced microgyira
Mutant rats with telencephalic internal structural heterotopia (TISH)

Uses of Experimental Animal Models

In addition to the obvious application of experimental animals to understand basic mechanisms of epilepsy – in order

Table 5 Examples of genetic models

Photosensitive *Papio papio* baboon
Epileptic beagle dog
Seizure prone gerbil
Genetically epilepsy prone rat (GEPR)
Genetic absence epilepsy in rats from Strasbourg (GAERS)
The WAG/Rij rat
Spontaneous epileptic rat (SER)
Audiogenic mouse
EL mouse
Serotonin receptor and synapsin-deficient mouse
Tottering, stargazer, ducky, and lethargic mice

to improve diagnoses, treatments, prognoses, preventions, and ultimately cures for the many different types of human epilepsy – models of acute epileptic seizures, and occasionally models of chronic epilepsy, are also used for research into normal brain function, and for screening antiepileptic drugs and other therapeutic and diagnostic approaches. Physiological psychologists, in particular, have investigated the fundamental neuronal substrates of normal cerebral functions such as memory, by perturbing the system with experimentally-induced epileptic seizures (usually electroconvulsive shock) to produce a generalized tonic–clonic seizure. Neuroanatomists have taken advantage of the propagation of focal seizures to trace normal functional pathways in the brain using topical convulsants such as strychnine (strychnine neuronography) and penicillin. These important applications of experimental models of epilepsy, however, are beyond the scope of this article.

Testing Therapeutic and Diagnostic Approaches

New therapies, as well as novel concepts for diagnosis, are tested first in animal models before they can be studied in patients. The most common use of experimental animal models for this purpose is screening potential antiepileptic compounds. There are tens, if not hundreds of thousands, of compounds identified as potential antiseizure medications, and rapid-throughput, cost-effective approaches for screening them are therefore necessary. Maximal electroshock and subcutaneous pentylenetetrazol-induced acute seizures in mice are the screening methods of choice; however, maximal electroshock is a model for generalized tonic–clonic seizures, and subcutaneous pentylenetetrazol is a model for typical absence seizures, both of which are already effectively treated by available antiepileptic drugs. Many seizure types, however, such as atonic seizures and most focal neocortical and limbic seizures, are notoriously pharmacoresistant. This 'pharmacoresistance' may be no coincidence, given that potential antiepileptic compounds are not screened for efficacy against these seizure types in animal models, and many thousands of excellent drugs for these conditions have undoubtedly been discarded because they do not have anticonvulsant or antiabsence properties in the established models for drug evaluation (mentioned earlier).

Although there are many useful animal models of chronic focal epilepsy, these are generally considered to be too expensive and time-consuming for routine screening purposes. Only one antiepileptic drug (leviteracetam) has ever reached the marketplace after failing maximal electroshock and subcutaneous pentylenetetrazol screening, because it was found to be effective against a chronic model of temporal lobe seizures (amygdala-kindled seizures). Although it would be ideal if potential antiepileptic compounds were routinely screened against amygdala-kindled seizures, or seizures of other chronic models of mesial temporal lobe epilepsy, such screening is not likely to happen in the current research climate, for financial reasons; these models tend to be time-consuming and labor-intensive. Consequently, rapid-throughput, cost-effective animal models for pharmacoresistant epilepsies are badly needed.

Animal models have also been important for developing nonpharmacologic therapies, such as the ketogenic diet, vagus nerve stimulation, and deep brain stimulation. Many of these therapies have been devised serendipitously, or by trial and error, and their mechanisms of action are still unknown. Animal research is central to efforts to understand why certain interventions are effective against some seizure types and not others, and to use this information to develop better, more focused interventions with fewer side effects.

There are currently no effective interventions to prevent epilepsy (i.e., antiepileptogenesis), but there has been considerable research in this area. This work requires testing in animal models, such as kindling, where the time course of epileptogenesis is reliably reproducible.

Advances in the diagnosis of epilepsy have also depended, in part, on animal research; for instance, fluorodeoxyglucose positron emission tomography (FDG-PET) was based, to a certain extent, on 2-deoxyglucose (2DG) autoradiography performed on animals with epilepsy. Efforts to identify reliable surrogate markers of epileptogenicity and epileptogenesis, such as α-methyl-typtophan (AMT) and fast ripples, also require extensive animal research.

Understanding Fundamental Neuronal Mechanisms

Most of the seminal research on basic mechanisms carried out in the last century used the neocortical penicillin focus to characterize acute ictal discharges, their patterns of propagation, and (to a lesser extent) mechanisms of seizure termination. Amygdala kindling made it possible to investigate epileptogenesis, secondary epileptogenesis (which previously was studied with the mirror focus phenomenon), and distant pro- and antiepileptic consequences of the epileptogenic process. The current predominance of the use of models of mesial temporal lobe epilepsy with hippocampal sclerosis is not inappropriate given that mesial temporal lobe epilepsy is the most common form of human epilepsy, that hippocampal sclerosis is the most common epileptogenic lesion, and that this condition is among the most medically refractory of all epilepsies – contributing significantly to the total health burden of epilepsy. Now that high-resolution MRI has recognized malformations of cortical development to be a much more important cause of epilepsy than that previously believed (accounting for a large portion of patients previously diagnosed as cryptogenic), there is increasing interest in the development of animal models of these conditions. Treatments for many other human epilepsies, however,

have suffered from the fact that no appropriate animal models yet exist. There are, for instance, no valid models for atonic seizures, or for epileptic encephalopathies such as West, Landau-Kleffner, and Dravet syndromes.

In vitro approaches are primarily used for biochemical, molecular biological, and anatomical research protocols. In vitro slice preparations are also commonly used for electrophysiological studies. Slices can be prepared from chronic animal models, and acute ictal discharges can be induced in slice preparations from normal animals by manipulating the ionic composition of the bath fluid as well as by adding chemoconvulsants and/or applying electrical stimulation. It is important to recognize, however, that although these epileptiform discharges are useful for elucidating neuronal substrates of epileptic abnormalities, they are epileptic equivalents (not epileptic seizures) that lack a behavioral/clinical correlate.

In vivo biochemical studies can be carried out using microdialysis, permitting samples of extracellular fluid to be analyzed for released neurotransmitter and other chemical constituents. Such in vivo experiments are obviously essential for research paradigms that require data related to the behavioral characteristics of the epileptic seizures and/or their consequences (e.g., interictal behavioral disorders). In vivo electrophysiological studies are also important for studying the role of neuronal systems beyond local circuits, when longitudinal studies in the same animal are necessary to evaluate the development of epileptogenesis, and for studying the progression of an epileptic condition over time. Small animal PET and MRI technology now also makes it possible to study structural and functional anatomy, and local biochemical processes such as glucose metabolism, in a longitudinal fashion.

Considerable epilepsy research today involves gene discovery, including studies that employ microarray approaches to identify alterations in gene expression. Identification of epilepsy genes, and of differentially regulated gene expression profiles from epileptic humans and animals (not only from brain but also blood), will lead to hypotheses that must then be tested in experimental animal models. The mechanisms by which epilepsy gene products alter brain development and excitability must be investigated in animal models. The role of specific protein products of regulated genes in the mediation of epileptogenesis and epileptogenicity will need to be studied by inducing epilepsy in genetically manipulated animals.

With increasing ability to carry out invasive basic research on patients with epilepsy in the epilepsy surgery setting, as well as the tremendous advances in structural and functional neuroimaging that also permit mechanistic hypotheses to be pursued with human subjects, there remains an overriding need for parallel reiterative human animal research – i.e., studies using human data to validate the relevance of animal models, and then using these validated models to follow up on aspects of human research that cannot be pursued further (for logistical, ethical, or economic reasons) in patients. The cycle then requires that we return to patients, to confirm the relevance of results obtained in the animal laboratory.

Finally, results of research on animals with seizures and epilepsy are being used to devise and test computer models where variables can be easily altered to carry out investigations of fundamental neuronal mechanisms in the 'dry' lab. This work, however, is not likely to eliminate the need for animal research. Results from computer simulations need to be confirmed by further observations in living organisms; if such confirmation cannot be done in patients, it will need to be done in animal models.

Summary

There are many types of epileptic seizures and epilepsy syndromes. Within each specific epilepsy syndrome or ictal event, there are many component parts involving different pathophysiologic mechanisms and anatomic substrates. Individual component parts can be modeled, and epilepsy equivalents – which may not be epileptic in themselves – can be effective targets for certain types of epilepsy research. In vitro and in vivo models of acute ictal phenomena are valuable, but do not represent epilepsy; models of epilepsy must include an enduring epileptogenic abnormality capable of eliciting spontaneous seizures. Models of chronic epilepsy are essential for research on the epileptogenic process, as well as on mechanisms responsible for the transition from the interictal to the ictal state, and consequences of epilepsy. Epilepsy models can be used for research into normal brain function and for screening antiepileptic drugs and other potential antiepileptic interventions, as well as for testing new diagnostic technologies. They are obviously essential for research into fundamental mechanisms of epilepsy. There remains an overriding need to develop valid animal models for many severe epilepsy syndromes for which no models currently exist. Despite increasing basic research in the clinical setting as well as developments in computer modeling, there will continue to be a need for animal models, and for reiterative parallel human/animal paradigms that use observations from patients to develop hypotheses that can only be pursued in the animal laboratory, and then validated by further studies in patients.

See also: **Classification**: Classification of Seizures and Syndromes; **Glia/Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters; **Models**: Acutely Dissociated Neurons; Characteristics and Mechanisms of Kindling; Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Models of Generalized Seizures in

Freely Moving Animals; Seizure Models in Acute and Organotypic Slices; Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; The Tetanus Toxin Model of Temporal Lobe Epilepsy.

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Models of Generalized Seizures in Freely Moving Animals

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Introduction

Models of generalized seizures are frequently used in the pharmaceutical industry for initial testing of novel compounds for anticonvulsant activity. These models historically include maximal electroshock seizures and minimal pentylenetetrazol seizures. The drugs active against tonic hindlimb extension seen in maximal electroshock seizures were thought to prevent seizure spread; drugs active against minimal pentylenetetrazol seizures (also referred to below as clonic seizures) were thought to increase seizure threshold. These two models still carry some value for routine drug testing, for their simplicity and high throughput.

The current choice of models of generalized seizures is much wider, and more complex models of generalized seizures in freely moving animals have an important use in research. These models may yield interesting information about responsiveness of the nervous system to convulsant drugs – i.e., about propensity of the brain to respond with seizure activity to external stimuli. This seizure susceptibility may be modified by many factors, including genetic conditions, hormonal milieu, previous seizure experience, and environmental conditions (e.g., stress). Models of generalized seizures can be used to determine efficacy of antiepileptic drugs under various conditions. Since different models utilize alterations in different neurotransmitter systems, these studies may help to elucidate mechanisms of antiepileptic drug action. Finally, if action of convulsant drugs is studied during early postnatal development, information can be gathered about nonlinear evolution of seizure propensity of the developing brain.

Many convulsant drugs administered systemically stimulate either the entire brain (bilateral effects) or they activate those brainstem structures projecting bilaterally to upper brain regions. This view is based on current EEG and imaging techniques. The resulting seizures will be perceived as stemming from the entire brain (or at least from the EEG-accessible parts), and considered as primarily generalized. In contrast, some convulsant drugs may create a strong unilateral source (focus) of epileptic activity as identified by current technology, and epileptic activity will spread from this focus bilaterally. These seizures will then be considered secondarily generalized.

Background

Primary generalized seizures – seizures that appear to arise simultaneously over broad regions of the brain – are very common in the human population. Examples of human primarily generalized seizures are absence seizures,

Freely Moving Animals; Seizure Models in Acute and Organotypic Slices; Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; The Tetanus Toxin Model of Temporal Lobe Epilepsy.

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The current choice of models of generalized seizures is much wider, and more complex models of generalized seizures in freely moving animals have an important use in research. These models may yield interesting information about responsiveness of the nervous system to convulsant drugs – i.e., about propensity of the brain to respond with seizure activity to external stimuli. This seizure susceptibility may be modified by many factors, including genetic conditions, hormonal milieu, previous seizure experience, and environmental conditions (e.g., stress). Models of generalized seizures can be used to determine efficacy of antiepileptic drugs under various conditions. Since different models utilize alterations in different neurotransmitter systems, these studies may help to elucidate mechanisms of antiepileptic drug action. Finally, if action of convulsant drugs is studied during early postnatal development, information can be gathered about nonlinear evolution of seizure propensity of the developing brain.

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Background

Primary generalized seizures – seizures that appear to arise simultaneously over broad regions of the brain – are very common in the human population. Examples of human primarily generalized seizures are absence seizures,

generalized clonic seizures, and generalized tonic-clonic seizures. Animal research and improved diagnostic techniques (especially imaging) have recently led to the view that at least some of the seizures previously categorized as 'primary generalized' are actually of focal origin (e.g., in the somatosensory cortex), but spread rapidly (i.e., are actually secondarily generalized). Without question, however, primary generalized seizures constitute a significant proportion of the seizure types seen clinically. Models of generalized seizures in experimental animals contribute a great deal to understanding the mechanisms of seizure spread and control, and also to the search for better antiepileptic therapy; indeed, two generalized seizure models are used for routine testing of newly synthesized compounds (see earlier text). In addition, many epileptic syndromes of childhood involve primarily generalized seizures. Examples of these syndromes are early myoclonic encephalopathies (newborns), infantile spasms (age: 3–12 months), and Lennox-Gastaut syndrome (age: 1–8 years). While current animal models of generalized seizures correspond very well to human generalized clonic, tonic-clonic, and absence seizures, the satisfactory models for many childhood epileptic syndromes involving generalized seizures still do not exist. Recently, better models for infantile spasms have been developed (see Stafstrom and Velíšek), giving the field an opportunity to study underlying mechanisms and more effective therapeutic agents for this catastrophic seizure syndrome of childhood. However, there is a pressing need to develop models for other catastrophic seizure syndromes (associated with progressive mental retardation and, frequently, death) in order to improve current therapy. Appropriate and accurate animal models can provide insights into pathogenesis of these syndromes, and lay the foundation for future mechanistic treatments.

Methodology

Generalized (either primarily or secondarily) seizures can be induced in freely moving animals by systemic injection of chemical substances (convulsant drugs), by direct electrical stimulation of the brain, or they can develop from genetic defects. Models based on systemic injection of convulsant drugs require very simple equipment: a syringe with an appropriate size needle, a convulsant drug in a solution, an observation cage, and a stop-watch. Needle size depends on the density of the injected solution and, of course, on the route of administration. Subcutaneous injection (usually in the skin fold on the back of the neck) requires a fine needle (26 gauge), while administration of a dense solution intraperitoneally will require larger needle, 22–24 gauge. Intravenous administration may be more complicated. The tail vein is commonly used in rats. However, to access this vein, the rat must be well restrained during the entire injection procedure. While very simple, the administration of convulsant drugs by injection produces also significant stress (restraint, pain), which may be hard to standardize for the experimental groups. Alternatively, inhalation of the convulsant ether (flurothyl) can be used to elicit generalized seizures. Flurothyl evaporates easily at room temperature. Since it has convulsant features in both experimental animals and human experimenters, an air-tight cage must be used and the entire system should be placed in the fume hood for additional protection. This arrangement also requires use of a precision microinfusion pump, which delivers flurothyl into the air-tight chamber at a constant rate (although other approaches – e.g., bolus delivery to the air-tight chamber – are also possible).

General electrical stimulation of the entire brain (maximal electroshock) is theoretically possible in freely moving animals, but impractical because the stimulating current is delivered via ear-clip electrodes that easily displace. Therefore, maximal electroshock is generally given with animals restrained. In freely moving animals, electrical stimulation of specific brain sites via implanted electrodes can be used to generate an epileptic focus and secondarily generalized seizures. Additionally, this procedure may be used to model the process of epileptogenesis (i.e., kindling from the focus). Furthermore, some convulsant drugs although injected systemically preferentially affect a restricted part of the brain (because of very high receptor density). These drugs even with systemic administration can be used to create a seizure focus with secondarily generalization.

Pentylenetetrazol

Pentylenetetrazol-induced seizures are elicited most commonly by a bolus dose of subcutaneous or intraperitoneal aqueous solution of pentylenetetrazol, between 40–120 mg kg^{-1} in both rats and mice. Pentylenetetrazol administration results in blockade of GABA(A) receptor (via the TBPS site in the chloride channel). Delivery of graded pentylenetetrazol doses intravenously is also possible; such an approach can be used to determine seizure threshold or threshold of individual components of pentylenetetrazol-induced seizures.

Bicuculline and Picrotoxin

Both drugs are GABA(A) receptor antagonists. Bicuculline competes with GABA on its binding site, while picrotoxin occupies its own binding site in the associated chloride channel. Bicuculline cannot be easily dissolved in water or saline. Those bicuculline salts, which can be dissolved in water, do not cross blood-brain barrier, and therefore cannot be used for systemic administration in adult animals. Therefore, bicuculline is usually dissolved in 0.1 N HCl and pH is titrated back with 0.1 N NaOH. The final pH will be acidic, and thus painful if injected in the area with abundant nerve endings. Hence, bicuculline solution should be

preferentially administered intravenously (around 2 mg kg^{-1}), as a second choice intraperitoneally (6–8 mg kg^{-1}), and only exceptionally subcutaneously. In adult animals, intraperitoneal administration is limited by the 'first-pass' effect, during which the mature liver enzymatic system metabolizes a large portion of the injected drug, resulting in substantially higher dosage required for the same convulsant effects than during intravenous administration. Picrotoxin, on the other hand, can be dissolved in saline. Doses between 3–6 mg kg^{-1} are usually injected intraperitoneally.

Flurothyl

Flurothyl is a liquid ether, usually delivered by a precision microinfusion pump into an air-tight chamber. The rate of delivery depends on the chamber size. We use chambers between 9 and 14 l (volume), with a CO_2 scavenger. For these chambers, flurothyl delivery at 20–40 µl min^{-1} leads to seizure occurrence in mice or rats within 8–12 min. With continuous flurothyl delivery, the latency to onset of seizure marks the seizure threshold. The seizures are the result of flurothyl blockade of GABA(A) receptors, with the addition of opening of sodium channels and activation of the cholinergic system.

Strychnine

Strychnine sulfate can be dissolved in normal saline (at 1 mg ml^{-1}) and doses for adult rats are between 2–3 mg kg^{-1} subcutaneously. Seizures are elicited by blockade of glycine-operated chloride channels, which are abundant in the spinal cord and brain stem.

Kainic Acid (KA)

KA-induced seizures are usually induced by a systemic injection of aqueous solution of KA (with pH adjustment, although the final pH is still acidic), a neurotoxic analog of glutamate with preferential effects in the limbic system. Frequently, a bolus dose of KA is administered intraperitoneally. In rats this bolus dose is between 10–20 mg kg^{-1}, while mice usually require a higher dose, between 20–40 mg kg^{-1}. An alternative approach is to infuse graded doses of KA in the tail vein (in rats, 2.5–5 mg kg^{-1} every 5–20 min) until seizures occur.

N-Methyl-D-Aspartate (NMDA)

NMDA can be dissolved in saline up to 50 mg ml^{-1}. Systemic doses that induce seizures in adult rats range between 150–300 mg kg^{-1}. NMDA activates NMDA subtype of glutamate receptors. NMDA doses for eliciting seizures in developing rats are an order of magnitude lower than for the adult rats, at least in part because of better blood-brain barrier permeability in immature brain. Another reason for this higher sensitivity to NMDA may be the transiently increased NMDA receptor density in the immature brain, and the different subunit composition of NMDA receptors in infancy (high content of the NR2B subunit).

Maximal Electroshock Seizures

For maximal electroshock seizures, a device capable of delivering currents up to 300 mA is required (high voltage!). Current is delivered via ear-clip electrodes and the normal range is between 150–200 mA for seizure induction in rats. The rat is usually restrained during the stimulation protocol.

Kindling

Delivery of short (1–2 s) trains of alternating current (either sinusoid or rectangular pulses), at frequencies 50–60 c s^{-1} (Hz), at regular intervals, which is initially subthreshold for generalized seizure discharge and eventually leads to evoked seizures, is called kindling. The principle of kindling consists in delivery of subthreshold stimulus for motor seizures. With repetitions of this stimulus at appropriate intervals (4–48 h in adult rats) the seizures develop, become more and more severe, and also permanent. While initial stimulations produce focal electrical discharges (focal afterdischarges must be induced by the kindling stimulus), repeated stimulations lead to more severe stages of motor seizures, which represent secondary generalization.

Genetic Models

There are two well developed genetic models of primarily generalized absence seizures. One model was discovered in Strasbourg (Genetic Absence Epilepsy Rat from Strasbourg, GAERS) and the other in The Netherlands (WAG/Rij rat). Both strains are viable and reproduce well. There is also a genetically-based model of generalized seizures precipitated by loud sound (Genetically Epilepsy-Prone Rat; GEPR). In addition, several models of spontaneously occurring seizures in mutant mice are available.

Results

Drugs Affecting $GABA_A$ Receptors

Pentylenetetrazol, bicuculline, picrotoxin and flurothyl significantly block $GABA_A$ receptor function. Therefore, seizures induced by these drugs have many common features: Low doses of these convulsants (or early during the induction phase) elicit behavioral arrest and staring. In the EEG, spindle-shaped trains of spike-and-wave are recorded (Figure 1). Later during induction (or with moderate doses), myoclonic whole body twitches occur, accompanied by individual spikes on the EEG (often

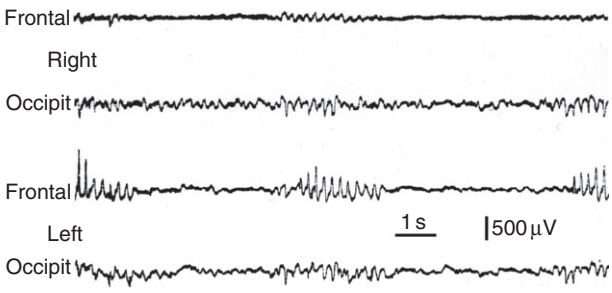


Figure 1 Initial EEG activity produced by low dose of s.c. bicuculline in the adult rat (rhythmic bicuculline-induced activity). Trace legend indicates areas of neocortex, in which recordings have been performed. Spindle-shaped spike and wave activity is marked with arrowheads in the left frontal cortex, yet can be seen in all traces. Calibration 500 μV, time mark 1 s.

Table 1 Developmental specificity of clonic seizures induced by GABA(A) receptor acting convulsant drugs in rats

	Age				
Drug	*P7*	*P12*	*P18*	*P25*	*Adult (P60–90)*
Pentylenetetrazol			- -	—	—
Bicuculline		- -	—	—	—
Picrotoxin	—	—	—	—	—
Flurothyl		- -	—	—	—

Ages indicate postnatal days in rats. Full line marks regular occurrence of clonic seizures, if sufficient dose of the convulsant drug is used. Dotted line indicates infrequent or even rare occurrences.

overlapping with movement artifacts). After myoclonic twitches, clonic seizures of forelimbs and face develop. These seizures last for several seconds to tens of seconds. If the clonic seizure is severe enough to disturb upright posture, the animal immediately and actively regains balance. In the EEG, the seizure presents as a series of spike-and-wave discharges. Finally, with larger doses of convulsant, or after longer time intervals, tonic-clonic seizures occur. These seizures begin with wild running, followed by the loss of righting ability. While lying on the cage floor, the animal displays tonic extension of limbs, concomitant with clonus of all four limbs. On the EEG, seizures usually start with fast polyspikes followed by spike-and-wave complexes. All features occur throughout development except for clonic seizures, which are developmentally specific and also dependent on the convulsant drug (Table 1).

Strychnine

This drug produces myoclonic twitches and tonic-clonic seizures with loss of righting. During the seizures, extensions of the trunk, limbs, and tail are prominent. The EEG correlate of these strychnine-induced seizures are predominantly spikes and sharp waves.

Kainic Acid

KA induces developmentally specific seizure syndromes. In adult rats, after automatisms consisting of rapid and alternating head rotations ('wet dog shakes') or rarely of scratching, clonic seizures of face and forelimbs occur with preserved righting. These convulsions are sometimes termed 'limbic seizures,' a term that emphasizes the focal initiation of epileptic activity. At the time of clonic seizure development, epileptic activity is already secondarily generalized and the seizure pattern is similar to the pattern described above (i.e., the clonic seizures seen in flurothyl, pentylenetetrazol, bicuculline or picrotoxin seizure models). In adult animals, tonic-clonic seizures after KA are rare; however, clonic seizures may last for many minutes to hours, developing into status epilepticus (continuous seizure activity lasting 30 min and more). Severity of the status epilepticus episodes correlates with the degree of resulting neuronal damage in the hippocampus. Epileptic EEG activity precedes motor seizures, and can be recorded earlier in limbic structures than in the neocortex.

NMDA

In adult animals, NMDA induces periods of wild running followed by generalized clonic seizures with complete loss of righting ability. This seizure pattern is similar to the tonic-clonic seizure pattern described above after pentylenetetrazol, flurothyl, bicuculline or picrotoxin. However, tonic seizure phase follows, rather than precedes, the clonic phase. Usually the occurrence of the tonic phase indicates a lethal ending for the seizure. Cortical EEG associated with NMDA injection is very nonspecific, consisting of periods of EEG suppression and polyspike-and-wave discharges.

Maximal Electroshock Seizures (MES)

Maximal electroshock delivered via ear-clip electrodes immediately produces tonic-clonic convulsions with the loss of righting. If the current is strong enough, the following sequence occurs: tonic flexion of forelimbs, tonic flexion of hindlimbs, tonic extension of forelimbs and tonic extension of hindlimbs. Prolonged tonic hindlimb extension frequently marks the end-point of the experiment.

Kindling

Stimulation of a sensitive brain structure, such as hippocampus, amygdala, entorhinal cortex, etc., delivered at low intensity, produces an EEG afterdischarge (Figure 2). With repeated stimulations, the duration of afterdischarges increases and behavioral seizures develop. These seizures have a character of clonic seizures of face and forelimbs, with preserved righting ability, as described above for

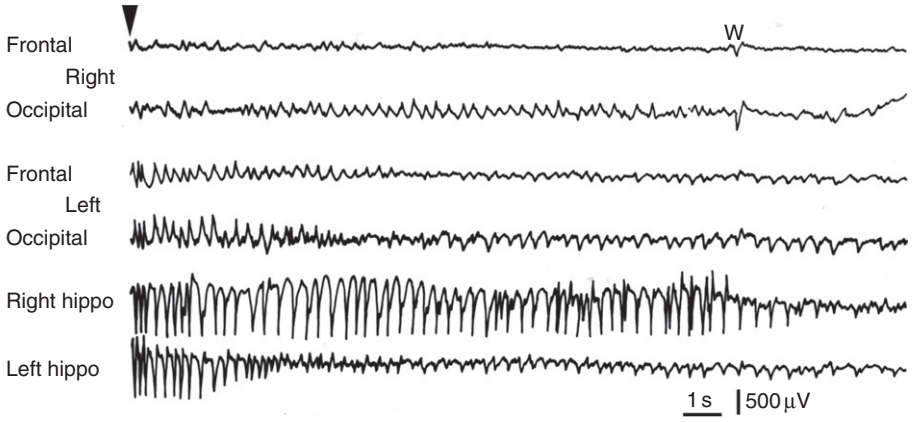


Figure 2 Afterdischarge following hippocampal stimulation in the adult rat. Trace legend indicates areas of neocortex, in which recordings have been performed. Left and right hippo indicate recordings from the left and right hippocampus. Arrowhead points to the end of stimulation (electrode in the left hippocampus). The afterdischarge consisting of spike-and-wave complexes follows (well seen in the trace recorded in the right hippocampus) with cessation about 2 s after a 'wet dog shake' (W) occurred. Calibration 500 μV, time mark 1 s.

convulsant drugs acting at $GABA_A$ receptors. Behavioral seizures also evolve, and can be classified according to the widely accepted Racine scale (Table 2). Once the stage 4 seizures occur, the seizures are considered secondarily generalized.

Genetic Models

Both GAERS and WAG/Rij rat strains develop EEG activity consisting of periods of spike and wave rhythms, with crescendo-decrescendo amplitude (spindle shaped) resembling the 3 Hz spike-and-wave discharges in human absence epilepsy. Behaviorally, these EEG episodes are accompanied by motionless stare. Another rat model, the GEPR, comes in two variants: GEPR-3s show moderate clonic seizures, and GEPR-9s exhibit severe tonic-clonic seizures with the behavioral seizure patterns very similar to those described above for $GABA_A$ receptor acting drugs.

Future Goals

Our discussions in this article should convince the reader that in the current era of molecular and cellular neurobiology, there is still significant room for models of generalized seizures in freely moving animals. Sometimes it is difficult to determine what these models model, what the underlying mechanisms might be, and what the consequences of these seizures are for the brain. However, there are precise fits for some models – e.g., the genetic absence models in rats provide important insights into the mechanisms of absence seizures in humans, most recently suggesting focal cortical origins of spike-and-wave absence epilepsy.

Table 2 Racine scale for kindling-induced generalized seizures in adult rats

Stage	*Behavioral expression*
1	Mouth and facial movements
2	Head nodding
3	Clonus contralateral to the stimulus site
4	Symmetrical forelimb clonus with rearing
5	Rearing and falling (yet regaining upright position)

There is a need to investigate old models of generalized seizures, and to create new ones. There are several reasons why new models are necessary: (1) New models may better reflect the human condition, and thus help to improve studies of the disease mechanisms. (2) For some human seizure syndromes, there are no corresponding animal models. For example, our understanding and treatment of Lennox-Gastaut syndrome would be immeasurably helped by research on a relevant animal model. Similarly, early infantile epileptic encephalopathies have no available models though there is an urgent need to improve their therapy and find underlying mechanisms. Thus, further exploitation of existing models of generalized seizures and development of new models will play a major role in future epilepsy research.

See also: **Aging**: Effects of Aging on Seizures and Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; Pharmacology of Seizure Drugs; Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy.

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Pharmacology of Seizure Drugs

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Introduction and Background

Epilepsy is clinically manifested in recurring, self-sustaining bursts of abnormal electrical activity (epileptic seizures) in the brain. In contrast to reactive seizures, which occur in response to 'environmental stimuli' (e.g., fever, hypoglycemia, presence of seizure-inducing agents), epileptic seizures occur at unpredictable intervals. With only few exceptions (e.g., reflex epilepsies), seizures appear in the absence of any clear identifiable triggers. In contrast, isolated seizures can be induced in many different ways, even in intact and healthy brain without any previous experience of epileptic activity or behavior. Under experimental conditions, seizures can be induced chemically, using seizure-inducing or convulsive drugs (chemoconvulsants). These drugs do not fall into any specific pharmacological category, and they act through many different mechanisms. Chemoconvulsants can be used to induce seizures in various animal species, including mammals, invertebrates, fishes, or birds. For many experimentally used drugs, seizure induction has also been described in humans, for example, after overdose or accidental intoxication. Chemoconvulsants involve drugs of different origin, including plant toxins (strychnine), weapon-grade organophosphorus substances (soman, sarin), or bacterial toxins (tetanus toxin). Many drugs that are used clinically for various indications can induce convulsions/seizures when overdosed or administered in a way that leads to nonphysiological concentrations in the brain. Some of these drugs are used experimentally, such as aminophylline (theophylline and elhylenediamine; drug used to treat asthma), isoniazid (antimicrobial agent used in chemotherapy of tuberculosis), lidocaine and cocaine (local anesthetics), and penicillins (antibiotics).

In experimental epileptology or pharmacology, seizures induced by chemoconvulsants represent frequently used models, induced to study generation or generalization of epileptic activity and to search for new anticonvulsant drugs. Such models also represent powerful tools to study effects of sex, age, genetic background, or additional pathologies on seizure susceptibility or development. In addition, chemoconvulsants with known mechanisms of action can be used to study postnatal development and functional maturation of neurotransmitter systems and ion channels, and to investigate the role of specific systems (e.g., neurotransmitter receptors or metabolic pathways) in brain excitability or seizure development.

Chemoconvulsant models are inexpensive and widely used. Their major advantage is that the procedure is simple and convenient, and models are usually well described. For many of these models, there are standard methods of evaluations, with relatively standard scoring systems developed to quantify seizure severity and pattern. Thus, the results obtained from these models are usually highly reproducible and comparable across different laboratories. There are, however, some disadvantages that should be

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Chemoconvulsant models are inexpensive and widely used. Their major advantage is that the procedure is simple and convenient, and models are usually well described. For many of these models, there are standard methods of evaluations, with relatively standard scoring systems developed to quantify seizure severity and pattern. Thus, the results obtained from these models are usually highly reproducible and comparable across different laboratories. There are, however, some disadvantages that should be

emphasized. First, chemoconvulsants are xenobiotics, and as any other systemically administered drug, they must penetrate through barriers, be appropriately distributed, metabolized, and eliminated. Pharamacokinetics of chemoconvulsants can be affected by other drugs that are present in the organism at the same time (including tested anticonvulsant drugs), and also by sex, age, or genetic predisposition. Thus, a different efficacy of chemoconvulsive drugs in individual strains of rats or mice can reflect genetically related differences in seizure susceptibility, as well as differences in pharmacokinetic parameters. Also, these models usually mimic isolated (reactive) seizures, not epilepsy as a chronic disease. There are however some exceptions. For example, some chemoconvulsants (e.g., GABAergic antagonists, local anesthetics) can be used for chemically induced kindling, a model of progressive enhancement of seizure susceptibility or epileptogenesis. In addition, there are drugs with various mechanisms of actions (e.g., the cholinomimetic drug pilocarpine, agonists of excitatory amino acid (EAA) receptors such as kainate), which induce convulsive status epilepticus (SE), a highly destructive and epileptogenic brain insult. In great majority of surviving (adult) animals experiencing drug-induced SE, spontaneous recurrent seizures develop within days or weeks. Thus some chemoconvulsants induce reactive seizures (i.e., they have ictogenic potential) and also epilepsy as consequence of seizure-induced brain damage (i.e., they also have epileptogenic potential).

Methods

Chemoconvulsants can be administered systemically or focally onto/into various brain structures. Systemic administration (subcutaneous (s.c.), intraperitoneal (i.p.) or intravenous (i.v.)) is most widely used, because administration is simple and does not request any special equipment or procedure. Focal administration/injection is technically more difficult, but intraparenchymal injection of convulsive drugs can create specific models of seizure activity, including acute or chronic seizures with known site of seizure onset. Infusion of chemoconvulsants into the brain ventricular system is also used. With this procedure, however, there is no control of the focus of seizure onset, because all structures surrounding ventricular system can be affected. Focal or intracerebroventricular administration generally involve the use of much lower doses of chemoconvulsants than those used with systemic injections, by-passing problems with hematoencephalic barriers (some convulsive drugs do not cross the blood–brain barrier (BBB)) or with systemic metabolism of the drug. In general, water insoluble chemoconvulsants, or drug solutions with pH different from physiological values, are not optimal for focal delivery, because organic solvents or local changes of pH may affect tissue excitability independently of the convulsant drug.

Results – Mechanisms of Actions and Seizure Types Induced by Chemoconvulsants

To provoke reactive seizures, drugs suppressing inhibition (GABAergic or glycinergic) or potentiating EAA transmission are used most frequently. There are, however, many other drugs with different mechanisms which can induce reactive seizures, including agents that interact with the opioid system (morphine), with acetylcholine neurotransmission (pilocarpine), or have direct interaction with ion channels (4-aminopyridine). Depending on their mechanisms of action, chemoconvulsants induce various types of seizure activity, with different EEG and behavioral correlates. Seizure pattern is also affected by dose, route of administration, or additional pharmacological manipulation. In addition, seizure features can change with age of experimental animals, with some chemoconvulsants (e.g., NMDA, homocysteine, homocysteic acid) inducing age-specific seizures types. Because of limited space, I will focus only on effects of some drugs studied in our laboratory. Because we have studied primarily developmental epileptology for many years, specific age-related features of chemically induced seizures will be stressed in the following paragraphs.

Suppression of GABAergic Inhibition

Drugs interacting with the GABAergic system are probably the most frequently used type of chemoconvulsant. This group of drugs involves $GABA_A$ receptor antagonists and also drugs that block GABA synthesis (**Figure 1**). The antagonists bind to different binding sites of $GABA_A$ receptors, but despite these differences, they tend to induce similar seizures – with quantitative rather than qualitative differences in their effects. GABA-related chemoconvulsants induce several types of epileptic activity, dependent on dose, means of administration, and latency after administration. After systemic administration, myoclonic twitches appear with short latency, accompanied by isolated spikes in the EEG. Most GABAergic chemoconvulsants induce 'absence-like' seizures, reflected in freezing behavior and spike and wave spindles (frequency around 5 Hz; **Figure 2**) in the EEG. These seizures can often only be detected in EEG recordings, since the behavioral manifestation is rather subtle. Clonic seizures occur after higher dose of these chemoconvulsnts or with longer latency after the administration. Behaviorally, these clonic seizures are expressed as clonus of forelimb and head muscles, with hindlimbs usually widely abducted, and tail erect; rearing and/or falling frequently occurs during convulsions. Righting reflexes are preserved. In the EEG, clonic seizures are typically seen as spike-and-wave activity (**Figure 2**). Generalized tonic–clonic

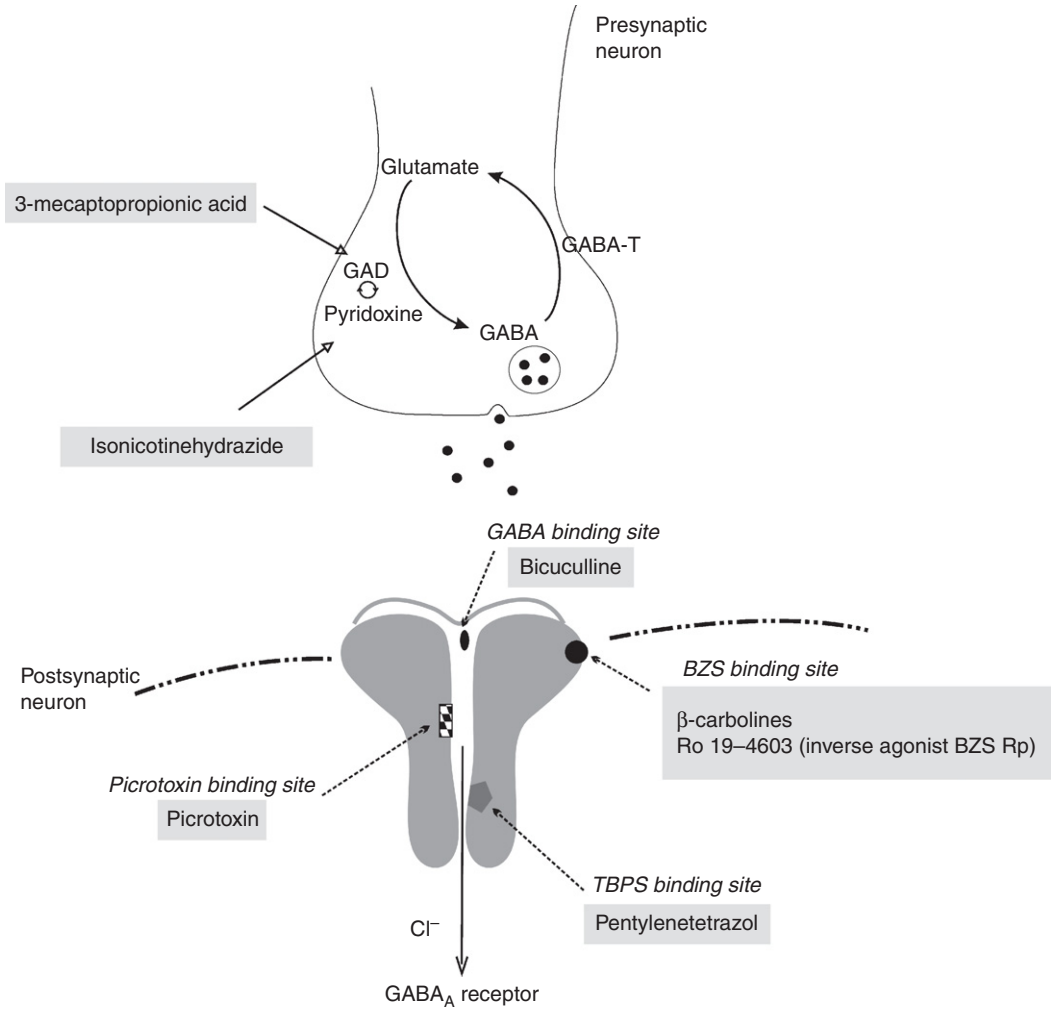


Figure 1 Schematic drawing illustrates different sites of action (pointed by arrows) of GABAergic seizure drugs mentioned in the text. GABA is synthesized from glutamate by glutamic acid decarboxylase (GAD). Inhibition of this enzyme (direct (3-MPA) or through blockade of the GAD coenzyme pyridoxine (isonicotinehydrazide)) results in decrease of GABA synthesis, failure of inhibition and development of seizures. Inhibitory effects of GABA can be antagonized also by blockade of different binding sites at postsynaptic $GABA_A$ receptors. For details, see text.

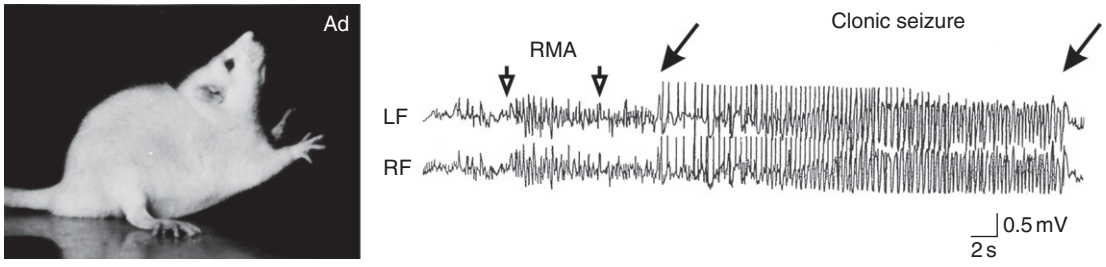


Figure 2 Photograph on the left demonstrates clonic seizure induced by systemic administration of PTZ in adult rats. The elecrocorticogram on the right site illustrates the typical pattern of clonic seizure (the onset and the end are marked by black arrows). In ECoG, clonic seizure is preceded by spike + wave discharge (RMA – rhythmic metrazol activity; empty arrows) which represents absence-like seizures. In behavior, discharge is accompanied by behavioral arrest.

seizures (GTCS; 'grand-mal' type) are the most severe type of epileptic seizures induced by chemoconvulsants. In rodents, they are expressed as a short initial phase of wild running, followed by loss of righting reflexes, then by a tonic phase (usually lasting for 5–15 s), and finally a clonic phase (lasting for tens of minutes). Incomplete, generalized clonic seizures, without a tonic phase, can also be induced. Such seizures are most frequently seen in pharmacological studies,

since some antiepileptic drugs specifically block only tonic phase of generalized tonic–clonic seizures. In EEG recordings, generalized tonic–clonic seizures are represented by polyspikes or polyspikes and waves activity.

After focal injections, GABAergic drugs can induce focally generated seizure. Both EEG and behavioral pattern of focal seizures depend on the exact site of injection, and as well as dose and age of animals.

Pentylenetetrazol (pentamethylenetetrazol, metrazol, PTZ) was formally used as a nonspecific stimulant of the nervous system. It is easily water soluble and it crosses BBB. PTZ noncompetitively antagonizes the $GABA_A$ receptor, likely through a benzodiazepine binding site and an allosteric interaction in the chloride channel. PTZ injected subcutaneously, together with maximal electroshock, are the most frequently used models employed to search for new antiepileptic drugs. As mentioned earlier, PTZ induces various seizure patterns. The clonic seizures induced by PTZ were historically used as a model against which antiabsence drugs were tested. These seizures can, however, be suppressed by some antiepileptic drugs, which aggravate absence seizures in human patients (e.g., barbiturates or gabapentin). Thus, it has been suggested that PTZ-induced clonic seizures model myoclonic (rather than absence-like) seizures. Low doses of PTZ induce absence-like nonconvulsive seizures, which may represent a better model of human absences than seen with higher dose PTZ, but this low-dose model is not frequently used. To test antiabsence efficacy of new drugs, genetic models with spontaneously occurring absence seizures are now preferred over chemically induced models. Generalized tonic–clonic seizures induced by PTZ represent a model of primary or secondary generalized seizures in humans. Repeated intraperitoneal administration of low doses of PTZ may be used to induce SE. In subthreshold doses, repeated PTZ administration leads to the progressive development of kindled seizures.

The behavioral and electrical patterns of PTZ-induced seizures are highly age-related. In rats, both absence-like nonconvulsive seizures and clonic seizures cannot be reliably induced during the first 2 weeks of life. In contrast, generalized tonic–clonic seizures are observed starting in the first postnatal days. The behavioral pattern of these seizures, however, reflects the level of maturation of the motor system; for example, **Figure 3** demonstrates differences in the tonic phase in P7 vs. adult rats.

The $GABA_A$ receptor antagonist bicuculline, and the Cl-channel blocker picrotoxin, induce similar seizure phenomena as seen with PTZ. In contrast to PTZ, however, bicuculline induces clonic seizures as early as P12. Both bicuculline and picrotoxin cross the BBB; however, in contrast to picrotoxin, bicuculline is not easily water soluble. For focal (intracerebral) administration, the use of a water soluble derivative of bicuculline, bicuculline methiodide, is recommended. Notably, the ability of bicuculline methiodide to cross the BBB decreases with age. Systemically administered bicuculline methiodide induces clonic or generalized tonic–clonic seizures in rats up to

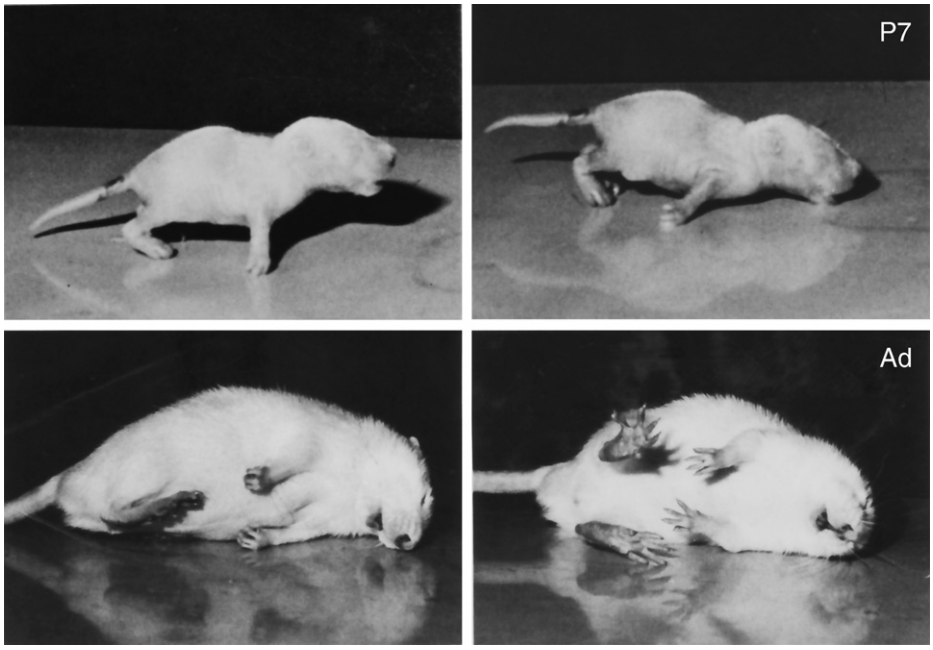


Figure 3 Generalized tonic–clonic seizure in P7 and adult rats. Tonic phase is demonstrated on the left site, beginning of the clonic phase on the right site. The difference in tonic phase on both forelimbs and hindlimbs (left site) between both age groups reflect level of maturation of the motor system.

P18, but not in older animals, probably due to gradual maturation of the BBB.

Drugs acting as inverse agonists on the benzodiazepine binding site of $GABA_A$ receptors (β-carbolines and Ro 19–4603) have been shown to induce seizures. β-Carbolines (methyl-6,7-dimethoxyl-4-ethyl-β-carboline (DMCM) and methyl-β-3-carboxylate (β-CCM)) induce both clonic and generalized tonic–clonic seizures, but they are less frequently used than the previously mentioned chemoconvulsants. The spectrum of seizures induced by Ro 19-4603 (imidazothieno-diazepinone (*tert*-butyl5,6-dihydro-5-methyl-6-oxo-4H-imidazol [1,5-*a*] thieno [2,3*f*] [1,4] diazepine-3-carboxylate) is the same as seen with PTZ, except that clonic seizures can be successfully induced as early as P7 in rats.

In addition to drugs affecting directly $GABA_A$ receptors, glutamic acid decarboxylase (GAD) inhibitors, which inhibit GABA synthesis, are potent chemoconvulsants. The inhibition of GABA synthesis decreases GABA concentration, thus both $GABA_A$ and $GABA_B$ receptors are less likely to be activated. The relatively specific inhibitor of GAD, 3-mercaptopropionic acid (3-MPA), elicits clonic as well as generalized tonic–clonic seizures as early as the first week of life. In this model, clonic seizures last for several minutes (in the PTZ model, the duration of clonic seizures is tens of seconds). Sensitivity to 3-MPA changes with age; the CD_{50} for different age groups of rats is shown in Figure 4. Also, the initial phase of GTCS – wild running – is longer and more intense with 3-MPA as compared with PTZ. GABA synthesis can also be inhibited by other drugs (allylglycine or isonicotinehydrazide); none of these drugs, however, are routinely used in experimental epileptology or in pharmacological screening.

Penicillin in low doses acts as $GABA_A$ antagonist. The penetration of penicillin through the BBB is, however, very limited. When administered topically, penicillin induces focal seizures, with seizure pattern depending on exact location of application. Behaviorally, ictal activity may be associated with clonic seizures. In the past, models of epileptic seizures induced by focal administration of penicillin were frequently used to study various cellular epileptic phenomena; however, it is currently used much less frequently.

Taken together, seizures induced by drugs suppressing GABAergic inhibition represent well-described models of several types of epileptic activity. Both seizure pattern and severity are highly age and dose dependent. Some of these models are routinely used for drug screening.

Drugs Suppressing Glycinergic Inhibition

The suppression of glycinergic inhibition, by the alkaloid strychnine, induces generalized clonic or tonic–clonic convulsions as early as P3 in rats. Rats that are 3-weeks old (P18–P25) are the most sensitive age group in this model. Typically, the tonic seizure phase is accompanied by apnotic pause and high mortality. Strychine-induced seizures are highly pharmacoresistant, and are therefore considered as a possible model of therapy-resistant seizures by some authors. Currently, the use of strychnine-induced seizures in drug screening is limited.

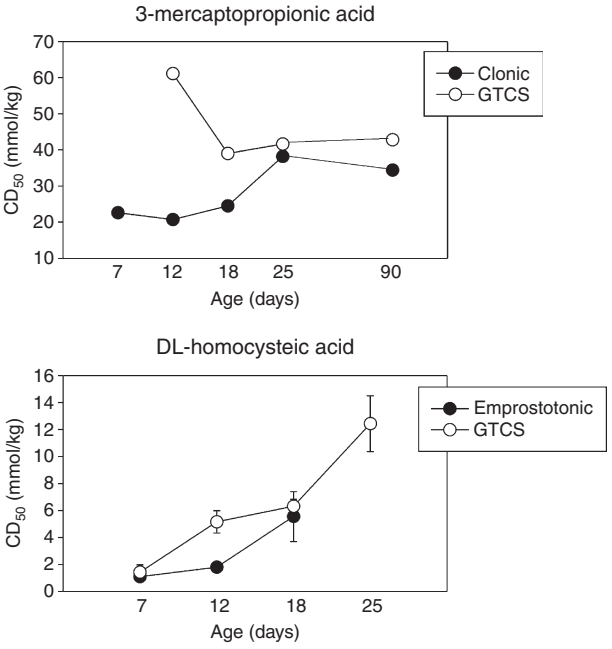


Figure 4 Age-related changes of CD_{50} (50% convulsive dose i.e., estimated dose which induces seizures in 50% of animals in experimental group) for two types of seizures induced by two seizure drugs with different mechanisms of actions. Note differences in age-related susceptibility to clonic and generalized tonic–clonic seizures after administration of 3-mercaptopropionic acid (on the top). Such differences cannot be simply explained by maturation of BBB or pharmacokinetics of used drugs. They rather reflect development of target structures or seizure generators. CD50 for both seizure types induced by D,L-HCA acid increases with age of animals. Flexion seizures however cannot be induced in animals older than P18.

Enhancement of EAA Excitation

Agonists of EAA receptors can induce both clonic and generalized tonic–clonic seizures. In the EEG, spike, spike and wave, or polyspike activity is usually recorded after administration of these chemoconvulsants. Shortly after administration, or after low-dose administration, various behavioral automatisms (scratching, wet dog shakes, washing, chewing, etc.) occur. Typically, behavioral features are drug specific and their pattern is age and dose dependent. Importantly, some EAA agonists (i.e., agonists of NMDA receptors) can induce age-specific seizure types.

Kainic acid (kainate or KA; agonist of KA EAA receptors) represents the most frequently used agonist of EAA

receptors. Usually, kainate is used to produce convulsive SE, which subsequently results in chronic seizure discharge. To induce SE, KA can be administered systemically (due to limited penetration through the BBB, the necessary dose increases with age) or intracerebrally. The development of KA-induced SE and its consequences are highly specific to the strain of rat or mouse used for the experiment. Seizure features are also affected by age of animals. In 2-week-old rats, generalized clonic–tonic seizures represent the most frequent seizure type; their incidence, however, decreases with age of animals. Also, intense scratching is a typical automatism seen within the first 2 weeks of age; starting with the third week of life, scratching is gradually replaced by wet dog shakes.

After systemic administration of *N*-methyl-D-aspartate (NMDA; agonist of NMDA type of EAA receptors) to rats of all age groups, there is development of clonic–tonic seizures, preceded by marked hyperlocomotion and tail flicking. Generalized clonic–tonic seizures are accompanied by very high mortality. In rats younger than 3 weeks, systemic administration of NMDA induces a very characteristic seizure pattern, so-called flexion (emprostotonic) seizures (**Figure 5**). Because of their highly age-specific occurrence, flexion seizures may represent a valuable model of infantile spasms. The relevance of this model must be further validated. After NMDA administration, flattening of the electrocorticogram (ECoG) is typically seen; however, in spite of an almost isoelectric EEG, various seizure behavioral phenomena are observed. Permeability of the BBB, as well as sensitivity of NMDA receptors, decreases with age; thus, much higher doses of drug are necessary to induce generalized seizures in adult rats.

Intracerebrally administered NMDA induces severe excitotoxic brain damage and development of generalized clonic or tonic–clonic seizures in freely moving animals. Mortality of experimental animals is much lower compared with systemic administration. This lower mortality makes it possible to follow animals for long periods. Thus, intracerebral injection of NMDA has been suggested as a model of traumatic or ischemic brain injury by some authors.

Effects of homocysteic acid as well as homocysteine are at least partially mediated by NMDA receptors, even though other mechanisms must also be considered. D,L-Homocysteic acid (HCA) and its stereoisomers induce seizures similar to NMDA (**Figure 5**). There are certain differences between effects of D- and L-HCA. Both stereoisomers induce flexion as well as generalized tonic–clonic seizures, but there are marked differences in the initial signs of actions. The behavior after L-HCA resembles behavioral changes induced by NMDA (tail flicking, hyperlocomotion), whereas D-HCA administration induces behavioral features that can be classified as epileptic automatisms (sniffing, chewing). Also, EEG suppression is induced by L-but not D-HCA. This difference may reflect different mechanisms of action of the two stereoisomers. Sensitivity to D,L-HCA acid changes with age; the CD_{50} for different age groups of rats is shown in **Figure 4**. When administered intracerebrally, HCA results in the development of severe and long-lasting SE, and massive excitotoxic brain damage.

Homocysteine (HC) acts through multiple mechanisms, including the interaction with ionotropic and metabotropic glutamate receptors. In addition to flexion and generalized tonic–clonic seizures, HC also induces clonic seizures similar to those induced by PTZ or other GABAergic drugs; such seizures do not develop after administration of NMDA or HCA. Automatisms (sniffing, washing, and chewing) precede seizure development. Both clonic and generalized tonic–clonic seizures occur in all age groups of rats. Flexion seizures are frequently seen in up to the second week of life, but they are uncommon in older animals.

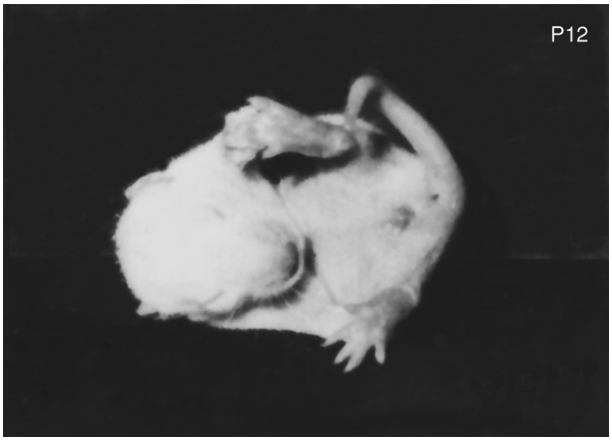


Figure 5 NMDA-induced flexion (emprostotonic) seizure in P12 rat.

Future Goals

Chemoconvulsants have been used for many years to study the general properties of neuronal tissue as well as specific features of seizures, or to screen for new antiepileptic drugs. There is, however, still great potential for future development. Despite recent progress, there are no relevant and widely accepted models of specific seizure types – e.g., age-specific or pharmacoresistant seizures – that can be used for drug screening. Recently described flexion seizures represent a promising possibility, but verification and pharmacological analysis are still needed. Further, in parallel with development of such seizure models, there is also a need for relevant models of chronic diseases, such as age-related epileptic syndromes and pharmacoresistant or progressive epilepsies. Such models should mimic morphological and functional features of the disorder of interest, including development of spontaneous seizures, seizure pattern, and pharmacological

sensitivity. Chemoconvulsants are likely to play an important role in development of such models, as seen in the recently described model of epilepsy induced by the intrahippocampal infusion of tetrodotoxin in developing rats.

See also: **Glia/Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters; **Models**: Ictogenesis in the in vitro Isolated Guinea Pig Brain; Model Characterization in Relationship to Human Disorders; Models of Generalized Seizures in Freely Moving Animals; Seizure Models in Acute and Organotypic Slices; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model.

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Seizure Models in Acute and Organotypic Slices

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Introduction

In vitro preparations permit investigators to undertake studies of the principles of generation, spread, and the termination of seizures – using methods which are difficult to employ under in vivo conditions. In vitro approaches provide precise control of temperature and ionic environment, stable recording conditions for electrophysiology (e.g., for recordings from subcellular compartments such as dendrites, axons and presynaptic terminals), and pharmacological and visual access that are not available in in vivo studies of mammalian brain. Recent development of fluorescent dyes now enables studies on various aspects of metabolism and changes in ion concentrations, in different intracellular compartments (including mitochondria). Occurrence and spread of seizure-like events (SLEs) can be monitored with extracellular recording techniques (e.g., multiple electrodes), voltage sensitive dyes, or even intrinsic optical signals. In vitro models of seizure activity must, of course, be validated – and such validation can be accomplished by assessing these models with respect to their sensitivity to antiepileptic drugs (which should be similar to that of the human condition being modeled). Since about 30% of epileptic patients do not become seizure free with classical anticonvulsant drugs, there is a need to develop in vitro models that mimic drug-resistant epilepsy.

Background

Features of Seizure-like Events

Although the behavioral aspects of seizures are lacking in in vitro preparations, many treatments which induce seizures in vivo induce similar electrical events in vitro. These activities can be termed seizure-like events (SLEs) if they last more than 10 s; are associated with a sustained depolarization of neurons and glia; and are accompanied by a negative extracellular DC field potential shift superimposed by clonic- or tonic-like events. Tonic-like

sensitivity. Chemoconvulsants are likely to play an important role in development of such models, as seen in the recently described model of epilepsy induced by the intrahippocampal infusion of tetrodotoxin in developing rats.

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Introduction

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discharges are characterized by relatively fast small transients with a frequency above 8 Hz. Clonic-like discharges consist of relatively large, 50–200 ms duration field potential transients. Following a seizure-like event, there is usually a postictal depression period. Interictal discharges then reappear, eventually leading to the next seizure-like event. Interictal discharges can be 'pro-ictogenic,' but there are also examples where the frequent occurrence of interictal discharges appears to prevent seizure generation. Criteria to discriminate between pro- and antiepileptic interictal discharges in the clinical environment have not yet been worked out, and can only be determined experimentally.

Intracellular recordings from neurons reveal transient depolarizations superimposed by bursts of action potentials during the initial phase of a seizure-like event, a sustained depolarization (about 30 mV) during the tonic phase, and depolarization shifts which again evoke bursts of action potentials superimposed on a slowly declining membrane potential during the clonic phase. During the tonic phase, intrasomatic recordings show frequently aborted spikes or no action potentials at all, due to depolarizing inactivation of sodium currents. However, as the depolarization propagates electrotonically into the axon, axonal action potentials will occur where the depolarization is weak enough to prevent inactivation of sodium currents; hence, high frequency axonal firing can be observed during the tonic phase of seizure-like events. Following a SLE, a long-lasting (~30 s) after-hyperpolarization is often observed, with reduced synaptic noise and initial suppression of interictal discharges.

Seizure-like events are typically accompanied by rises in extracellular potassium concentration ($[K^+]_0$) to levels near 12 mM (with an undershoot to below baseline following the seizure-like event), decreases in extracellular calcium concentration ($[Ca^{2+}]_0$) by up to 0.6 mM, decreases in sodium and chloride concentrations ($[Na^+]_0$ and $[Cl^-]_0$), and changes in the size of the extracellular space. Changes in pH are also characteristic, with an initial alkalotic shift followed by acidosis.

Special Features of In Vitro Models

It must be noted that treatments which induce seizures in animals and humans often induce only relatively short recurrent discharges in slice preparations. This 'discrepancy' is most notably the case with agents which interfere with GABAergic or glycinergic synaptic transmission, such as strychnine, bicuculline, penicillin, and picrotoxin. By contrast, agents which block potassium currents, such as 4-aminopyridine, barium, or cesium, are more likely to induce seizure-like events. Conditions in which changes in Mg and Ca bathing medium concentrations are used to induce seizure-like events bear some relationship to the eclamptic seizures in humans. In such cases, opening of the blood–brain barrier as a consequence of sudden surges in blood pressure leads to a hypomagnesaemia and a hypocalcaemia in the interstitial space of the CNS – which in turn may contribute to ictogenesis. Delayed inactivation of sodium currents, as observed with veratridine, can induce ictal events both in vivo and in vitro. Based on these observations, it appears that interference with intrinsic neuronal excitability is more likely to result in seizure-like activity in vitro than is interference with inhibitory synaptic transmission.

It should also be noted that most seizure models are used in otherwise healthy tissue. Less information is available about seizure characteristics in tissue from chronically epileptic animals or humans. For example, it remains unclear why we were unable to reliably induce seizure-like events in slices from human epileptic tissue with low Mg bathing medium, bicuculline, 4AP, and other agents – but elevating potassium successfully evokes seizure-like events in these tissues. Since 4AP has also been reported to fail to induce seizure-like events in the entorhinal cortex of kainate treated rats with spontaneous frequent limbic seizures, it is possible that adaptive homeostatic plastic changes develop in chronically epileptic tissue which prevent frequent occurrence of seizures. Understanding such mechanisms might open new strategies for the cure of epilepsies.

Methods

Two types of in vitro preparations are easy to use for studies of seizure generation: acute slices and organotypic slice cultures. Slice cultures can be made from rat or mouse tissue slices if prepared under sterile conditions early after birth. The most useful method seems to be the procedure described by Stoppini *et al.* More intact preparations of early postnatal neocortex or hippocampus, which correspond to perinatal human tissue, offer advantages for studying epilepsy features in immature systems. An isolated brain preparation offers additional research options. Increasingly, preparations are used which preserve interarea connectivity – for example, between the entorhinal cortex and the hippocampus, between the amygdala and the entorhinal cortex–hippocampal formation, or between the neocortex and the thalamus. Acute slice preparations exploiting brain stem tissue, involving regions often involved in generation of myoclonic seizures and in secondary generalization of seizures, have been much less studied.

Use of acute slice preparation requires attention to a number of problems. Such preparations have undergone a period of ischemia. Further, many fiber projections entering and leaving the brain slice are severed. While recording from slices, synaptic reorganization is likely to occur. Moreover, the thickness of the preparation requires

supply of nutrients which is dependent on exchange by diffusion from an artificial cerebrospinal fluid (ACSF) (and thus indirectly dependent on perfusion rate). In the intact brain, the average distance between capillaries is only 50 μm, providing efficient exchange of oxygen and nutrients to the neural tissue. In acute slices, diffusion processes are much less efficient. Exchange of the extracellular solution in acute brain slices takes about 60 min when investigations are performed under interface conditions, and about 20 min when investigations are performed under submerged conditions. The induction of seizure-like events in submerged slices requires perfusion rates of more than 3 ml min^{-1} in order to provide for sufficient supply of oxygen. Due to the limitations of diffusion, and in order to provide a sufficient oxygen and glucose supply, solutions saturated with 95% of oxygen and high glucose content are typically used for maintaining acute slices.

The ionic contents of the artificial cerebrospinal fluid should be close to physiological levels. Presently, few investigators add amino acids (determined to be 0.6–2 mM glutamine and about 20 μM GABA and taurine in normal CSF) to the ACSF; the addition of e.g., glutamine to ACSF for perfusion of slices from chronic epileptic animals may uncover spontaneous epileptiform discharges. Our laboratory uses ACSF containing (in mM): NaCl 124, KCl 3, $MgSO_2$ 1.8 or 1.2 (the latter being close to physiological Mg concentration in CSF), $CaCl_2$ 1.6, NaH_2PO_4 1.25, $NaHCO_3$ 21 (26 at room temperature) and glucose 10. The solution is equilibrated with carbogen gas (95% O_2/5% CO_2) to maintain a pH of 7.4 and the osmolarity of the solution should be about 290 mOsm. Ca^{2+}, Mg^{2+}, and K^+ concentrations must be carefully adjusted, as small variations in concentrations of these ions have marked effects on neuronal activity. The extracellular Ca^{2+} concentration ($[Ca^{2+}]_0$) in vivo is about 1.2 mM which is mimicked in slices by 1.6 mM. The reason for this difference is the chelating effect of HCO_3 on Ca^{2+} (HCO_3 precipitates about 25% of the free $[Ca^{2+}]_0$). An issue in preparation of slices is the choice of anesthetic (and depth of anesthesia) used in initial sacrifice/decapitation. Pups are frequently anesthetized by putting them on dry ice, a procedure that exploits the anesthetic effect of high carbon dioxide levels combined with hypothermia. Many labs use gas anesthetics such as ether or halothane. This choice gives reliable anesthesia with a reasonable therapeutic gap between deep and irreversible anesthesia. It should be noted that very deep anesthesia leads to circulation arrest and hypoxia, and causes cessation of protein synthesis (slices prepared in this way often do not exhibit long-term plasticity). Gas anesthesia works well in both sexes. Barbiturate anesthesia has the drawback that females often require more anesthetic to obtain an adequately deep state – but frequently are put down so deeply that they die before decapitation. Ketamine anesthesia interferes with NMDA receptors, an effect not always desirable in studies on seizures; in addition, long term effects on synaptic plasticity have been reported.

Our procedures for preparing hippocampal slice cultures follow published protocols. In brief, hippocampal slices (400 μm) are cut using a tissue chopper from 7–9 day-old Wistar rats, under sterile conditions. Slices are immediately transferred to ice-cold, oxygenated (95%) minimal essential medium (MEM) at pH 7.3. Slices with intact anatomical structure are maintained on a biomembrane surface (0.4 μm, Millicell-CM, Millipore) at an interface between culture medium (1 ml, containing: 50% MEM, 25% Hank's balanced salt solution, 25% horse serum (Gibco), and 2 mM L-glutamine (pH 7.4)) and humidified atmosphere (5% CO_2, 36.5 °C) in an incubator. Half of the culture medium is replaced every second day. Slices cultures are used after 7 days in vitro.

Results

Generally, before studying seizure induction, the viability of the tissue should be tested; experiments should be carried out only on preparations which present with 'normal' physiological responses – i.e., which show no signs of epileptiform alterations such as multiple population spikes. A good test of viability (in addition to that described earlier) is whether LTP or LTD can be maintained beyond 20 min. Slices exposed to prolonged ischemia lose the capability for protein synthesis and therefore cannot maintain long-lasting changes in synaptic plasticity. The induction of seizure-like events in such preparations yields atypical discharge patterns, disturbances in ionic homeostasis, and rapid decline of the preparation, once SLEs are induced. Often it is impossible to record spontaneous activity.

Low Ca Induced Epileptiform Activity

Recordings in vivo have shown that extracellular Ca concentration can drop to levels as low as 0.2 mM during seizure activity. Studies initiated in vitro showed that lowering extracellular Ca^{2+} (i.e., in the bathing medium) could induce regular seizure-like events which lasted for about 30 s and recurred in a clock-like fashion. Similar events can also be induced when the hippocampus is treated with Ca chelating agents in vivo. This pattern of activity depends on low (0 mM) Ca treatment and simultaneous elevation of $[K^+]_0$ to 5.5 mM. The induction of seizure-like events coincides with blockade of evoked synaptic transmission. It is a result of reduced surface charge screening (which increases neuronal excitability) and reduced Ca-dependent K currents. In addition lowering of Ca activates a cationic current which may contribute to the generation of the epileptiform discharges. Electrographically, the events appear to mimic tonic-like discharges, with slow negative shifts upon which a series of population spikes are

superimposed. The appearance of population spike activity is somewhat delayed with respect to the onset of the negative field potential shift. Clonic-like after-discharges are not apparent. Intracellular measurements reveal a membrane depolarization of about 30 mV (corresponding to the negative shift in the field), with superimposed action potentials. When normal Ca levels are restored in the bathing medium, the tissue behaves normally, with little signs of neuronal deterioration.

Interestingly, the low Ca activity spreads with a low speed similar to that observed for seizure-like events in the entorhinal cortex induced by low Mg and 4-AP – a preparation in which synaptic transmission is maintained. These results suggest that nonsynaptic mechanisms – likely potassium diffusion – are involved in seizure spread. As evoked transmitter and peptide release is blocked under conditions of low Ca, this model is particularly well-suited for studies on mechanisms of nonsynaptic seizure spread, and also for studies of whether/how anticonvulsants directly modify neuronal excitability. The low Ca model works well in rat hippocampal area CA1 where neurons are densely packed. Tissue from juvenile animals appears to be more sensitive to the low Ca condition (i.e., SLEs are more readily generated). The low Ca model does not work in human tissue (even when potassium concentration is elevated) or in other brain areas with low cell packing density – unless the size of the extracellular space (ES) is reduced by treatment with hypoosmolar solutions (which, however, also affects transmembrane Cl concentration gradients). For example, in the dentate gyrus, low Ca-induced seizure-like events may require simultaneous application of augmented potassium concentration (~10 mM) and/or modification of extracellular osmolarity. Under submerged conditions, the induction of low Ca seizure-like events appears to be facilitated by lowering recording temperature (for still little-understood reasons), but in interface chambers lowering of temperature blocks this activity. Pharmacologically, low Ca seizure-like activity responds to carbamazepine (CBZ), phenytoin (PHT), and valproate (VPA) in clinically relevant concentrations. The activity can also be blocked by the application of DC fields. Application of veratridine, which removes fast inactivation of sodium channels, causes SLEs similar to those seen with low Ca, and these events persist when synaptic transmission is blocked.

Low Mg Induces Seizure-like Events

Epileptiform activity can be induced in virtually all epilepsy-sensitive regions of the brain by omitting Mg from the ACSF. The induction of this activity depends on increased neuronal excitability due to reduced surface charge screening, facilitated transmitter release, and the activation of NMDA receptors due to the removal of the Mg block of NMDA receptor-operated ion channels. Initially these events are sensitive to the application of NMDA receptor antagonists – which is consistent with the idea that the activation of NMDA receptors is critical for the induction of such events. However, NMDA receptor antagonists may lose their efficacy with increasing time after application of low Mg. In such cases, a form of long-lasting synaptic potentiation is observed. In slices of isolated rat hippocampus, lowering Mg induces recurrent, short duration discharges which share properties of interictal discharges. Shorter events are reminiscent of sharp wave ripple complexes. They are initiated in area CA3 and propagate to area CA1. The dentate gyrus does not normally participate in this activity, although these recurrent epileptiform discharges do involve the hilus. These interictal-like discharges last for 60–150 ms, are characterized by positive-going field potential transients in stratum pyramidale of area CA1 and CA3, and are superimposed by bursts of population spikes. Low Mg seizure-like events can be induced in rat hippocampus by simultaneously applying baclofen or elevating potassium. In mice, omitting Mg from the ACSF can cause seizure-like events which are similar to those observed with low Mg in rat limbic structures such as temporal neocortex, entorhinal cortex, and subiculum. Each of these regions can produce seizure-like events when isolated from the remaining structures of the hippocampal formation. The seizure-like events in subiculum, EC, and temporal neocortex are characterized by negative potential shifts superimposed by high frequency small amplitude oscillations followed by clonic-like afterdischarges. Intracellular recordings reveal depolarization shifts of up to 30 mV, upon which are superimposed prolonged bursts of action potentials. During clonic-like after discharges, depolarization shifts are observed which trigger a series of action potential discharges. In combined hippocampal – entorhinal cortex slices, the seizure-like events most frequently start in medial entorhinal cortex but can also begin in temporal neocortex, lateral entorhinal cortex, or the subiculum. The seizures spread from the site of origin with a low speed to neighboring areas. The speed of spread is slightly lower than that for low Ca-induced seizure-like events. The spread of activity can be monitored by multiple DC recording electrodes or by use of intrinsic optical signals. Low Mg seizure-like events possess the same pharmacological properties as seizures induced in vivo – that is, they are sensitive to CBZ, PHT, and VPA, and also to benzodiazepines and barbiturates in clinically relevant concentrations. However, when seizure-like events are induced by low Mg in organotypic hippocampal slice cultures, they do not respond to standard antiepileptic drugs and hence may represent a valuable model of pharmacoresistant epilepsy.

Interestingly, seizure-like events induced by low Mg may show different properties in frontal cortex slices. In this brain region, the epileptiform pattern does not include afterdischarges. Further, the activity spreads more

rapidly than in temporal/limbic structures and the episodes are much shorter than in the entorhinal cortex, amygdala, and hippocampus. This feature of low Mg activity suggests that the relatively short, frequent, seizure episodes often observed in clinical frontal lobe epilepsies are dependent on the intrinsic properties of frontal cortical tissue.

Low Mg-induced seizure-like events show different characteristics of the spread when induced in chronic epileptic tissue. In both pilocarpine-treated animals and kindled animals, the seizure spread is facilitated. Interestingly, there are two routes of low Mg seizure spread in pilocarpine treated animals – one through the subiculum and one through the dentate gyrus. In contrast, the seizure spread in kindled animals is preferentially through the dentate gyrus, suggesting that the DG has lost some of its filtering properties. A low Mg seizure spread is also facilitated in preparations from rat pups, where the hippocampus is variably involved.

Importantly, seizure-like events disappear some time after inducing low Mg, and are replaced by shorter events which most frequently represent tonic–like discharges. These events can be mistaken for interictal discharges. However, since the ionic change and the intracellularly recorded signals accompanying these activities differ from the properties of interictal discharges in duration and amplitude, we believe that these events correspond to activity patterns observed during prolonged status epilepticus. As the application of GABA and GABA-uptake inhibitors reverse these events to seizure-like patterns, a loss of GABAergic functions seems to be involved in this activity. Since muscimol blocks this activity at low concentrations, the change is likely not due to alterations in GABA receptor affinity but more likely due to the consumption of GABA in the GABA shunt of the tricarboxic acid cycle. These late recurrent discharges are of particular interest, as they are not blocked by standard anticonvulsants even when high concentrations of these agents are applied. The induction of low Mg activity involves omission of Mg sulfate from the perfusate. The first epileptiform discharges appear after 40 min under interface recording conditions, and after 20 min under submerged conditions. Transition to the late recurrent, pharmacoresistant discharges occurs between 30 and 90 min after the onset of epileptiform activity. The low Mg model works also in human neocortex but not in hippocampal slices from most patients with temporal lobe epilepsy.

4-Aminopyridine Induced Seizure-like Events

4-Aminopyridne (4-AP) is a blocker of transient potassium currents. At low concentrations, it affects potassium currents containing specific Kv1 or Kv3 subunits, while at higher concentrations (millimolar range) it also affects other potassium currents of the Kv 4 family. In rat hippocampus, 4-AP usually induces recurrent discharges. In parahippocampal structures such as the entorhinal cortex, subiculum and temporal neocortex, and also in the amygdala, 4-AP elicits seizure-like events. Similar discharge is produced organotypic hippocampal slice cultures. The seizure-like events start with an initial bursting period, and give rise to a tonic-like episode and clonic afterdischarges superimposed on slow negative potential shifts; these electrographic events are associated with ionic changes characteristic of seizure-like events. In interface recordings, the activity commences about 40 min after the application of 50 or 100 μM 4-AP. Transition to late recurrent discharges – after more than 1.5 h of ongoing seizure-like activity – may or may not occur. These seizure-like events respond to all standard anticonvulsants. Late pharmacoresistant activity can be evoked by combining bicuculline with 4-AP. In slice cultures, 4-AP induced seizure-like events are usually resistant to standard antiepileptic drugs. Human tissue and entorhinal cortex from kainate treated rats with limbic epilepsy appear to be less sensitive to 4-AP. In pilocarpine treated epileptic rats, 4-AP fails to induce seizure-like events in about 50% of slices; the efficacy of 4-AP in these slices correlates with seizure frequency prior to preparation of slices.

Seizure-Like Events Induced by Elevating Potassium Concentration

Early in vivo studies in different species suggested that elevating potassium concentration can induce seizure-like events in hippocampus. This effect is readily mimicked in vitro by raising extracellular potassium in ACSF from 3 mM (standard in most laboratories) to 7.5 mM. This activity also responds to standard anticonvulsant drugs. When $[K^+]_0$ is elevated to 11 mM, and Ca lowered at the same time, seizure-like events can be induced in the dentate gyrus of Sprague–Dawley rats. Interestingly, in Wistar rats, the induction of seizure-like events with high K and moderately lowered Ca and Mg is difficult to reproduce. The high K model is very reliable in human tissue. Different forms of seizure-like activity can be induced by high K in all studied regions, including temporal neocortex. If the slices in which seizure-like events are induced come from patients who are resistant to carbamazepine or valproate, the seizure-like events induced by high K are also generally resistant to VPA and CBZ; however, occasionally one slice (or one region) may respond to CBZ or VPA, suggesting that the area of brain which determines pharmacoresistance may be smaller than the epileptogenic area.

Stimulation Induced Seizures

Short trains of repeated high frequency electrical stimuli have also been used to induce ictaform activity, in the form

of afterdischarges. In combined hippocampal–entorhinal cortex slices from young Sprague–Dawley rats (P21–P30), repeated trains (100 Hz, 0.1 ms pulses, 1 or 2 s train duration) in the presence of 0.5 mM Mg eventually resulted in self-sustained status epilepticus. In slices from older Sprague–Dawley rats, ictaform afterdischarges have been induced by stimulus trains when slices were bathed in low Mg and low Ca concentrations and elevated K, or with 1.3 mM Mg and 5 μM 4-AP bathing media. Trains of repetitive stimulation in organotypic slice cultures are also, in some cases, able to induce afterdischarges and spreading depressions.

Focal Induction of Seizure-like Events

Although the majority of in vitro models for seizures are thought to mimic the aspects of focal or partial epilepsies, few attempts have been made to induce seizure-like events by focal manipulations. Seizure-like events in the models described in this article usually have focal onset – most frequently in the entorhinal cortex, but sometimes in area CA3 and more rarely in area CA1. An interesting alternative approach involves the focal application of bicuculline (50 μM) and high K (10 mM) to the EC. In such investigations, experimenters can study the spread patterns of epileptiform activity, for example, from the EC to the hippocampus in normal and kindled rats, and in pilocarpine-treated rats. Another method of focal induction involves discrete application of carbachol (10 mM) from a pipette, with short pressure pulses (10–200 ms).

Directions for Future Research

At present many in vitro studies are limited to preparations from healthy animals. In these preparations, one major question is not yet fully resolved. Preictal discharges are often noted, which are generated by the release of GABA, preceding seizure-like events. This observation has led to the hypothesis that GABAergic synaptic transmission is required for the induction of seizures. However, an avalanche of inhibitory activity precedes seizure-like events when they are spreading from the site of ignition into neighboring areas; this preictal inhibition was recently shown to slow seizure spread. Thus, the preictal GABAergic signals may represent an anticonvulsant mechanism. To resolve this debate, studies are required which focus on the role of inhibition at the sites of seizure onset.

A second general problem is that most in vitro studies are done in normal tissue (treated as described in this article) and not on tissue derived from patients during surgery or from epileptic animals. In fact, in such epileptic tissue, mechanisms might be discovered which counteract ictogenesis. In any case, adaptive changes in cellular and synaptic properties, as well as in neural circuits, are rarely taken into account and certainly are not reproduced in manipulations of normal tissue slices.

Finally, in vitro preparations are restrained to minimal circuits. Depending on the orientation of the preparation, these circuits are differently disturbed. Systematic studies on effects of circuit maintenance are still lacking. Importantly, the onset of seizures is often controlled by a rather distributed network which cannot be maintained under in vitro conditions. Therefore, many findings from in vitro preparations await confirmation from in vivo studies.

See also: **Imaging**: Optical Monitoring of Circuitry Activity In vitro; **Models**: Ictogenesis in the in vitro Isolated Guinea Pig Brain; Model Characterization in Relationship to Human Disorders; Pharmacology of Seizure Drugs; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Non-Synaptic Mechanisms**: Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; **Pharmacoresistance**: Mechanisms of Pharmacoresistance; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury.

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Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats

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Introduction

Investigations undertaken in genetically epilepsy-prone rats (GEPRs) have contributed appreciably to an understanding of the epilepsies and of their comorbidity with the affective disorders. Although genetic determinants of rare forms of monogenic epilepsies have been identified in some cases, most genetic epilepsies, including those in the GEPR, are complex polygenic disorders with or without known experiential contributions. Whether sustained before, during, or after birth, brain injury is believed to be etiologically important in some epilepsy cases. Many people or other mammals with epilepsy are not known to have sustained injury or to have ontogenetically dependent structural defects. These and other factors suggest that epilepsy is a diverse group of neurological disorders with few, if any, unifying causal features. Nevertheless, evidence supports the concept that genetically determined seizure predisposition may link multiple epilepsies and some depressive disorders into a partially common diagnostic category. Overall, comparatively little investigative scrutiny has been focused on the etiologic role of genetically determined seizure predisposition in the polygenic epilepsies. Until the latest iterations of the definition of epilepsy, prominent roles for seizure predisposition have been often ignored. We propose future steps that may produce a positive shift in experimental epileptology and in clinical medicine.

Background

Predisposition

The subject of seizure predisposition has received considerably less emphasis in the medical sciences literature than the subject of seizure initiation. The disparity between these subject areas is evident in data that we collected on December 5, 2007 in a search of the Ovid Medline database. During the 30-year interval of 1978–2007, our Ovid search identified 599 publications on the subject of seizure predisposition versus 2261 on the topic of seizure initiation. This disparity is striking, particularly given the fact that during the last three decades, investigations – of the GEPR, other polygenic models, and neurologically normal mammals – have provided increasing evidence that seizure predisposition is the fundamental neurological dysfunction that distinguishes many, if not most, brains with epilepsy from those of healthy subjects. In GEPRs, seizure predisposition is present in the seizure naïve condition (i.e., in animals that have never had a seizure).

Seizure predisposition is a condition of the brain wherein the paroxysmal episodes can be triggered by endogenous neuronal events and/or exogenous stimuli that fail to ignite seizures in the 'normal' animal. Such neuronal liability is exhibited by the moderately epileptic GEPR-3, the more severely epileptic GEPR-9, humans with epilepsy, and other epileptic mammals. Moreover, predisposition typically includes exaggerated seizure responsiveness to stimuli that also cause seizures in nonepileptic subjects. For example, GEPR-3s and GEPR-9s kindle more rapidly than nonepileptic controls, with the highest rate being a strikingly evident trait of the GEPR-9. Such acceleration is also evident in the 'fast kindling rat.' With seizure predisposition, the brain may exhibit abnormally low thresholds to systemically administered convulsant chemicals. However, the pharmacokinetics of chemoconvulsants may be abnormal in some genetically epileptic mammals, and little research has been carried out to explore this possibility.

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Seizure predisposition is a condition of the brain wherein the paroxysmal episodes can be triggered by endogenous neuronal events and/or exogenous stimuli that fail to ignite seizures in the 'normal' animal. Such neuronal liability is exhibited by the moderately epileptic GEPR-3, the more severely epileptic GEPR-9, humans with epilepsy, and other epileptic mammals. Moreover, predisposition typically includes exaggerated seizure responsiveness to stimuli that also cause seizures in nonepileptic subjects. For example, GEPR-3s and GEPR-9s kindle more rapidly than nonepileptic controls, with the highest rate being a strikingly evident trait of the GEPR-9. Such acceleration is also evident in the 'fast kindling rat.' With seizure predisposition, the brain may exhibit abnormally low thresholds to systemically administered convulsant chemicals. However, the pharmacokinetics of chemoconvulsants may be abnormal in some genetically epileptic mammals, and little research has been carried out to explore this possibility.

The concept of seizure predisposition does not demand the coexistence, or the experientially induced development, of seizure circuitry that is specific to epilepsy. Two convulsive seizure circuitries have been demonstrated in mammals, whether seizure naïve or experienced, or whether genetically normal or epileptic. One network is denoted as the forebrain seizure circuitry. This rostral substrate generates episodes that are known clinically as cortical/limbic seizures. The other network is caudal to the forebrain circuitry, and is designated as the brainstem seizure circuitry. This second brain substrate drives episodes known clinically as generalized tonic/clonic seizures (GTCSs). Even though both the rostral and caudal seizure substrates are subject to modification by locally directed electrical currents or other types of kindling (including overt seizures per se), neither set of circuitry is created de novo by kindling processes.

The forebrain and brainstem seizure circuitries share evolutionary outcomes of mammalian genetics in neurologically normal brains. Both seizure substrates also participate in normal neurological functions. Additional and anomalous seizure circuitries may be genetically determined. For example, a unique seizure circuitry may underlie infantile spasms in some cases of West syndrome. Also, the GEPR appears to exhibit a genetically determined anomalous and localized seizure circuitry within the inferior colliculus. The brainstem seizure circuitry can be triggered by a localized seizure within the inferior colliculus in response to the acoustical activation of cochlear hair cells. The inferior colliculus rapidly becomes a component of the circuitry of the generalized brainstem seizure in GEPR-9s and 10s, and probably other GEPRs, with field potential electrographic characteristics that match those of the other caudal structures. Brainstem seizures in GEPRs can also be elicited by electrical stimuli despite surgical removal of the inferior colliculus. Moreover, the highly exaggerated brainstem seizure responsiveness characteristic of the GEPR is retained in the absence of the inferior colliculus, although a seizure can no longer be activated by sound stimulation.

In the foregoing discussion, we have emphasized the concept that seizure predisposition is a condition wherein some animals exhibit a greater level of seizure predilection than is typical of their neurologically normal counterparts. We now turn to the idea of relative seizure responsiveness of different subdivisions within the mammalian brain. More specifically, we refocus on the relative propensity of the forebrain versus brainstem seizure circuitry, whether in nonepileptic or epileptic mammals. Although in GEPRs, and probably humans with epilepsy, the neuroanatomical extent of genetically determined seizure predisposition encompasses both the forebrain and brainstem seizure networks, the degree of predilection differs between the two parts of the brain. Also, in neurologically normal mammals, the seizure propensity of these two seizure networks differs appreciably.

In nonepileptic mammals, the greatest degree of seizure predilection appears to exist in the rostral seizure circuitry. Accordingly, the threshold for seizure ignition in the forebrain is below that of the brainstem (the forebrain–brainstem threshold ratio being less than 1). The accentuated seizure propensity of the rostral brain has also been demonstrated in the moderately epileptic GEPR-3 strain. However, despite the similarity of the propensity ratio between GEPR-3s and their nonepileptic counterparts, both sets of circuitry in GEPR-3s exhibit exaggerated seizure responsiveness when compared with the anatomically corresponding circuitry of nonepileptic subjects. In the more severely epileptic GEPR-9 strain, both the rostral and caudal thresholds are below the neuroanatomically corresponding threshold of the GEPR-3. In contrast to the GEPR-3, the threshold ratio in the GEPR-9 is greater than unity. Consequently, GEPR-9s are likely to exhibit brainstem seizures (generalized tonic/clonic seizures) in lieu of, or as a consequence of, forebrain seizures (cortical/limbic seizures).

Relation of Forebrain and Brainstem Seizures to Human Seizures

Based on a substantial body of electrophysiological and behavioral evidence – from studies in GEPRs and primates (including humans) with epilepsy – we have previously advanced the hypothesis that forebrain seizures in multiple mammalian species are equivalent to seizures subsumed under the terms 'cortical/limbic' and 'partial seizures' in human medicine. Seizures that are commonly evaluated in limbic kindling paradigms are produced by a part of or all of the forebrain seizure circuitry. Forebrain seizures, including every category of kindled seizure in the Racine classification system, occur in nonkindled and nonepileptic mammals (including humans) in response to an appropriate electrical stimulus, whether delivered by intracerebral or transcorneal electrodes.

In kindled or seizure-naïve mammals, the magnitude of a single electrical stimulus determines the behavioral seizure class exhibited. Prior kindling stimuli are not needed to produce any of these seizure classes. A relatively weak stimulus causes class 1 or 2 forebrain seizures (Racine classification system); a stimulus of intermediate magnitude causes forebrain seizures of moderate severity (class 3); and a relatively strong stimulus triggers class 4 or 5 seizures. The neuroanatomical extent of seizure activity throughout the forebrain seizure circuitry is an important determinant of the severity of the seizure as indicated by seizure class. Although the Racine class 4 and 5 seizures occur in response to activation of most or all of the forebrain seizure circuitry, these episodes are not equivalent to a generalized tonic/clonic seizure in humans or other mammals as some investigators have argued. Rather, these 'Racine' episodes are appropriately viewed as manifestations of maximally

severe, pervasive, or generalized seizures driven by forebrain circuitry in mammals. Use of the term 'maximal seizure' (without qualification) contributes to confusion because it does not distinguish these forebrain episodes from maximal brainstem-driven seizures.

In experimental epileptology, electrically induced brainstem seizures of maximal severity are commonly designated as maximal electroshock seizures (MES). A maximally severe brainstem seizure is a GTCS, whether in a rodent, nonhuman primate or human. Such seizures are essentially alike behaviorally and electrographically in humans, GEPR-10s, and other mammals. In GEPR-9s and GEPR-10s, such GTCSs have been observed to occur spontaneously and in response to multiple sensory stimuli. However, prodromal running episodes occur in the sound-induced submaximal forms of GTCSs in rodents, but not in humans.

Importantly, the term 'brainstem-driven seizure' does not imply absence of a simultaneously occurring seizure in the forebrain circuitry. Indeed, most brainstem seizures also drive an electrographically prominent seizure within the forebrain, although examples of brainstem seizures that are not paralleled within the forebrain have been fully documented in GEPR-9s and GEPR-10s. When driven by a brainstem seizure, the forebrain circuitry does not exhibit electrographic morphology similar to a cortical/limbic seizure. Rather, the forebrain appears to mimic in essentially every detail the electrographic morphology and temporal qualities of the overpowering brainstem seizure.

Forebrain or cortical/limbic seizures are well known as triggers for GTCSs in many humans with epilepsy and in GEPR-9s and GEPR-10s. This phenomenon appears to be extremely rare or absent in nonepileptic rats and (perhaps) in humans without epilepsy or seizure predisposition. In GEPRs, the pervasiveness of the forebrain seizure is unrelated to the probability that a GTCS will be triggered by a seizure within the rostral circuitry. Accordingly, a unilateral forebrain seizure and a bilateral forebrain seizure generalized throughout the forebrain circuitry are equally likely to ignite GTCSs in GEPR-9s and 10s. Clinically, people who experience what is now believed to be a brainstem seizure triggered by a forebrain seizure have commonly been designated as exhibiting a partial seizure secondarily generalized to a tonic/clonic seizure.

Scope of Seizure Predisposition

The scope of seizure predisposition may be extensive but not unlimited. In some people with epilepsy, seizures are triggered by visual stimuli, thinking, music, speaking, writing, and/or eating. Many individuals with epilepsy experience spontaneous seizures. People with idiopathic or generalized or focal epilepsies reportedly exhibit abnormally low pentylenetetrazol seizure thresholds. Predisposition in GEPRs also includes spontaneous seizures, exaggerated responses to the seizure inducing properties of pentylenetetrazol, flurothyl, aminophylline, barbiturate withdrawal, morphine via intracerebroventricular administration, hyperbaric conditions, transcorneal or transauricular electrical current, intracerebral limbic kindling, and forebrain seizure-induced brainstem seizures. Stimuli that produce seizures in GEPRs but not in their nonepileptic counterparts include acoustic stimuli, hyperthermia (only adults have been evaluated), and less dependably handling and postural alterations.

Seizure predisposition is sometimes assumed to include a predilection to all seizure inducing modalities. However, broad the scope of seizure predisposition may be, data demonstrate limitations. For example, although supratherapeutic doses of many antidepressant drugs induce seizures in humans, GEPRs and other mammals, the GEPR (and probably humans with epilepsy) do not exhibit predisposition to such seizures. Nevertheless, the medical literature contains many warnings that physicians should be especially cautious about using these medications in patients with epilepsy.

While medical science literature may be devoid of direct claims that predisposition to one convulsant is tantamount to predisposition to all other seizure-inducing modalities, this view seems to be a common one. However, for the antidepressive treatments, this notion is erroneous. Almost all antidepressive agents appear to exert anticonvulsant effects in doses that are approved by the FDA for treatment of affective disorders. This beneficial property of the antidepressive treatments was first demonstrated decades ago in multiple seizure and epilepsy models, including GEPRs and epileptic baboons. Confirmation studies in humans with epilepsy include a double blind crossover investigation, other methodologically less rigorous studies, and a recent analysis of seizure incidence data from FDA Phase II and III clinical trials of antidepressants approved between 1985 and 2004. The absence of seizure liability for therapeutic-range doses of most of the antidepressive medications probably stems from multiple factors. For example, these treatments augment noradrenergic and serotonergic neurotransmission. Activation of these two transmitter systems produces marked anticonvulsant effects in the GEPR and other species including primates.

Methodological Considerations and Results

Generalized Convulsive Seizures

We have previously proposed three types of generalized convulsive seizures. One occurs throughout the forebrain seizure circuitry. The second is generalized through the brainstem seizure circuitry. The third is a brainstem-driven seizure that is mimicked electrophysiologically

in the forebrain. Our concept of generalized convulsive seizure departs sharply from the term 'generalized epileptic seizure' as defined in 1973 by Gastaut and his collaborators (*Dictionary of Epilepsy*). This 1973 definition emphasized the difference between unilateral versus bilateral seizures, did not distinguish between the rostral and caudal seizures, and did not distinguish between a seizure that is generalized throughout the forebrain and the one that is bilateral, but within only a part of the forebrain circuitry in both hemispheres. Also, the classification did not provide a framework to distinguish between a focal and a generalized brainstem seizure. Finally, the earlier concept did not set forth a classification for brainstem seizures that drive a seizure in the forebrain.

In our approach to classification, both forebrain and brainstem seizures have the potential to involve the entire musculature of the body. However, generalized forebrain seizures are characterized by behaviors that are clearly distinct from those of generalized brainstem seizures. Behaviors of brainstem seizures that are concomitantly mimicked in the forebrain may be indistinguishable (or perhaps only slightly different) from brainstem seizures restricted to the caudal circuitry. Our system of classification also runs completely counter to modern concepts that define seizures as disordered, high amplitude rhythmic discharges of populations of neurons that arise only from the cerebral cortex. The corticocentric concept essentially rules out the possibility of seizures being driven from the brainstem circuitry. While electroencephalographic field potential determinations in multiple species show that seizures occur in the cerebral cortex, measurements are rarely made in the brainstem structures (especially in humans) and so one cannot conclude that seizures do not arise within (and are not driven by) caudal structures.

Detailed electroencephalographic and behavioral measurements in multiple mammalian species, including GEPRs and primates, provide strong evidence that GTCSs are brainstem phenomena. Nevertheless, any shift away from cerebral cortex dogma is thwarted by the several impediments. First, electroencephalographic measurements in humans are primarily made via scalp electrodes. Information regarding brainstem field potentials is therefore unattainable. Only if the scalp electrodes reveal the absence of a cortical seizure during GTCS behaviors might an investigator infer seizure activity in another deeper part of the brain. But, even this inverse approach to the question is unlikely, since in the vast majority of GTCSs, the brainstem seizure circuitry drives seizures within the forebrain that mimic those in the brainstem.

Second, electroencephalographic measurements in humans are almost always made under conditions that are known (from studies of nonhuman species) to disallow any detection of seizures that occur in the brainstem, rather than the cerebral cortex. For example, electroencephalographic measurements during seizures in humans are almost always made after, rather than during, the first seizure in previously seizure naïve individuals. Ethical considerations, rather than scientific imperatives, may dictate the exclusion of first seizure measurements. From studies of seizure naïve GEPRs, it is clear that the initial GTCS may occur in the absence of electroencephalographic seizure in the cerebral cortex. Whether the first GTCS in humans with epilepsy occurs in the absence of a cortical seizure will probably remain unknown.

Third, other evidence demonstrates that the window of opportunity for observing only brainstem electrographic seizures is vanishing in the intact GEPR. With continued selective brother/sister inbreeding for specific traits of epilepsy, GTCS in the brainstem and the forebrain began to occur almost simultaneously, with the initial high amplitude spiking first appearing in the brainstem reticular formation and subsequently in the cerebral cortex. Later in strain development, rostral and caudal spiking began to occur seemingly at the same time throughout the brain, even upon first seizure. These observations suggest that with a sufficient level of genetic seizure predisposition, the onset of initial high amplitude spikes in the caudal brain may become indistinguishable from those in the rostral subdivisions of the brain. The mechanisms of coordinated seizure onset throughout the brain warrant intensive investigation.

Seizure Expression

Both the electrophysiological and behavioral characteristics of forebrain-driven seizures differ from those of brainstem-driven seizures. Forebrain seizures are characterized by one or more of several behaviors: immobility with maintenance of muscle tone and posture; facial, neck, and forelimb clonus; head and neck turning, and gradual extension of the hip joints. If in a sitting posture at the onset of the seizure, a biped may partially rise. A quadruped may rise from a four legged posture so that it sits on its haunches with its vertebral column oriented in a position largely vertical to the floor. Bilateral forelimb clonus is typical during these rearing episodes. In both bipeds and quadrupeds, 'rising' may be interrupted by slumping to the floor. A forebrain seizure characterized by the full range of these behaviors is commonly known as a class 5 episode according to the Racine system that was developed through studies in rodents for grading the magnitude of cortical/limbic seizures.

Maximally severe brainstem seizures in GEPRs and humans are characterized by behaviors that differ sharply from those of maximally severe forebrain seizures. These caudally determined behaviors include initial massive limb thrusts, followed immediately by loss of righting posture, and then tonic rigidity throughout the body with the superimposition of clonus, a behavior that increases in amplitude and decreases in frequency as tonic rigidity

subsides. Postictal flaccidity is common following the termination of tonic and clonic features of maximally severe brainstem seizures.

Both rostral and caudal seizure types include some behavioral components that might be mistaken as volitional because they are typical behaviors known to occur in the absence of a seizure. 'Scratching' is part of the cortical/limbic seizure repertory, but also commonly occurs in normal mammals in the absence of a seizure. Similarly, running behavior is produced in quadrupeds by a sound-induced brainstem seizure, but is nevertheless common in the absence of a seizure. In contrast, the sequential and repetitive elements of a seizure generalized throughout the rostral circuitry (class 5 in the Racine categorization) are probably seizure- and circuitry-specific, as are the tonic/clonic behaviors driven by a brainstem seizure that is generalized throughout the caudal circuitry (Class 10 in the Jobe and colleagues scale).

A brainstem seizure commonly drives an electrographic seizure in the forebrain. Nevertheless, such a rostral seizure is correctly designated as a brainstem seizure because the seizure determinants are within the brainstem. Accordingly, during a brainstem seizure, forebrain neuronal activity is entrained by the seizure within the brainstem so that the rostral electrographic morphology and rhythm mimic those of the brainstem. At the close of a brainstem seizure expressed throughout the brain, a condition of seizure refractoriness ensues. This transitory postictal anticonvulsant condition of the brain probably prevents the expression of forebrain-determined seizures in direct response to a brainstem-driven seizure of maximal severity. During a generalized tonic/clonic seizure, a forebrain-determined seizure is excluded by the aggressive pervasiveness and overwhelming dominance of the brainstem seizure.

Submaximal brainstem seizures have the capacity to trigger forebrain-driven seizures. However, the maximal class 9 and class 10 brainstem seizures do not appear to have this feature. Rather, they drive brainstem electrographic seizures in the forebrain. Adult GEPR-9s or -10s that experience a sufficient postictal anticonvulsant effect against forebrain seizures will not exhibit a forebrain seizure at the close of the brainstem seizure. At a specific stage of development, GEPR-3s also exhibit brainstem seizure-induced forebrain seizures.

Noradrenergic and Serotonergic Pathophysiology as a Determinant of Predisposition

Genetically generated noradrenergic and serotonergic deficits are major determinants of seizure predisposition in GEPRs. The moderate magnitude of predisposition in GEPR-3s arises at least partly from moderate deficits in both types of monoaminergic transmission. The more marked level of seizure predisposition in GEPR-9s is caused by a more pronounced noradrenergic deficiency.

Data from cell culture experiments support the concept that the noradrenergic deficiencies of GEPR-9s are characterized by deficits in the density of noradrenergic neurites in terminal fields innervated by the locus ceruleus, in the concentration of norepinephrine per noradrenergic neuron, and in challenge-evoked norepinephrine release within noradrenergic terminal fields. The deficits in noradrenergic morphology of the GEPR-9 appear to arise from causative factors located within both the region of the locus coeruleus and its terminal fields. Similar investigations show that the noradrenergic deficiencies of the brains of GEPR-3s are less pronounced than those of GEPR-9s. Serotonergic deficits are characteristic of both GEPR-3s and GEPR-9s.

As previously described, noradrenergic and serotonergic neurons are located outside the forebrain and brainstem seizure circuitry. Neither type of monoaminergic neuron initiates or maintains a seizure within either set of circuitry. However, both noradrenergic and serotonergic neurons innervate these units of seizure initiation and continuation, and control the concentrations of norepinephrine and serotonin in the extracellular fluid surrounding (and within) the circuitry. A deficiency of these two transmitters in the extracellular fluid increases the likelihood that fluctuations in neuronal activity within the circuitry will initiate a seizure. Conversely, elevation of norepinephrine and/or serotonin concentrations produces anticonvulsant effects, thereby providing a 'shield' to protect or defend the circuitry against the onset of dysfunctional neuronal initiatives. Within the circuitry, sufficient decrements in GABAergic and increments in glutamate transmission initiate and continue convulsive seizures. In the intact mammal, such dysfunctional episodes of neuronal activity can be prevented by sufficient increments in noradrenergic and serotonergic transmission. In contrast, complete discontinuation of monoaminergic transmission does not initiate a seizure. The excitatory and inhibitory components located within the circuitry per se continue to function, but the anticonvulsant properties of monoaminergic transmission have been lost. Freedom from these external constraints allows excessive responses to excitatory influences initiated internally within the circuitry and/or delivered to the circuitry by other parts of the brain.

Future Directions and Recommendations

Predisposition Model for Preclinical Development of Treatments and Preventions

We recommend that a seizure and epileptogenesis predisposition paradigm be created for the preclinical development of epilepsy treatments and preventions. Such a paradigm was first proposed by Jobe and Laird more

than 20 years ago, and has the potential to foster the discovery of novel treatments that are exceptionally effective in the common polygenic epilepsies. We anticipate that the models of polygenic pathophysiology of seizure predisposition will, in some instances, enable the detection of antiepileptic effectiveness in exquisitely small nontoxic doses or magnitudes. We believe that in some cases, the use of nonepileptic animals in the traditional seizure screening protocols of the past, has resulted in the detection of potentially useful treatments, but only in toxic dose in these animals. If we are correct, the past 60 years of antiepileptic drug screening has included multiple cases wherein potentially major advances have been systematically and mistakenly eliminated from the discovery process. The cumulative 'failures of the past' may be one of several rich sources for future achievement. An important goal should be to shift from anticonvulsant screening to an antipredisposition (antiepileptic) programmatic emphasis.

Clinical Model for Detection of Surrogate Indices of Predisposition

We recommend the development of an imaging-based neurochemical and -receptor analysis program (e.g., PET) designed to detect surrogate indices of predisposition to seizures and epileptogenesis in humans. Surrogate indices are essential to obviate the need for convulsive seizure-inducing drugs or electrical stimuli to establish seizure predisposition in humans without clinically relevant symptoms. Because ethical practice of medical science prohibits the induction of seizures in healthy people, predisposition cannot be directly evaluated. Initially, our proposed program for the identification of surrogate markers would be structured to detect presynaptic and postsynaptic indices of noradrenergic and serotonergic deficiencies at an early stage, before genetically determined predisposition becomes exacerbated by the superimposition of experientially dependent epileptogenesis.

Because of the comorbidity between epilepsy and depressive disorder, this program could initially target seizure and depression naïve siblings (and parents) of patients with these disorders. This approach would capitalize on the concept that noradrenergic and serotonergic deficiencies are common causes of genetic predisposition in both disorders. These and other discoveries provide a plausible basis for using noradrenergic and/or serotonergic deficits in humans as surrogate indices of seizure predisposition. Several investigations already provide support for noradrenergic and/or serotonergic deficits in humans with epilepsy. These studies have led to the discovery of α-receptor noradrenergic deficits in epilepsy patients undergoing brain surgery; however, these patients have a history of seizures, so that the pristine condition of seizure naivety remains unknown in humans.

We propose that seizure naïve siblings, parents, and progeny of documented epilepsy patients be evaluated with PET to determine the status of both the noradrenergic and serotonergic systems of the brain. Investigations of seizure naive patients with major depressive disorder might be an especially fruitful group for such investigations. Multiple factors support the use of these categories of people. Pertinent elements of the rationale underlying our proposed studies have been described in other parts of this manuscript. Individuals identified with monoaminergic deficiencies would become eligible for prophylaxis against epileptogenesis. This potential preventative would be designed to restore their transmitter function towards that of appropriately matched people without epilepsy or depressive disorder.

A Model for Spontaneous Seizures in the GEPR

We recommend further investigations with the GEPR as a predisposition model. These investigations should include the development of three major types of spontaneous seizure GEPRs: GTCSs, cortical/limbic seizures, and cortical/limbic seizures with secondary ignition of GTCSs. Infrequent spontaneous seizures have been observed in GEPRs as in epileptic baboons and in people with epilepsy. Although weeks and months of continuous monitoring of field potential EEG in GEPRs has not been undertaken, interictal spikes or spike/wave complexes may occur as they do in epileptic baboons and humans. Moreover, since spontaneous seizures in GEPRs are not known to be caused by identifiable exogenous stimuli or by focal lesions, they are likely to be initiated by interictal events or by other endogenous vicissitudes in neuronal activity that have not been defined (and would not be capable of producing seizures in normal animals). The initial goal of these proposed studies would be to identify these electrographic events.

As a corollary to the above proposal, we recommend that the National Institutes of Health develop a funding mechanism for maintenance and further development of the GEPR models of epilepsy, depressive disorder, and of comorbidity between epilepsy and affective illnesses. A major impediment to scientific investigations with the GEPR pertains to the absence of a vender/advocate. Neither GEPR-3s nor GEPR-9s are commercially available and they cannot be obtained from the NIH. Presently, both the GEPR-3 and GEPR-9 strains are facing imminent extinction.

See also: **Genetics**: Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Sixty Years in the Making: A Multifactorial Mouse Model of Seizure Susceptibility; **Models**: Model Characterization in Relationship to Human

Disorders; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; Role of the Noradrenergic System in Seizure Regulation; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures; Interictal Anxiety in Temporal Lobe Epilepsy; Interictal Behavioral Comorbidities in a Model of Epilepsy; Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model

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Introduction: Rationale for the Model

The importance of cholinergic mechanisms for epilepsy was already suspected by neurologists at the turn of the nineteenth century. The convulsant potential of acetylcholine and physostigmine was experimentally demonstrated, using electrocorticographic monitoring, as early as the late 1930s. In the following years, the use of acetylcholine, mainly in in vitro models, contributed to the understanding of electrophysiological phenomena involved in epileptic spike discharges, neuronal depolarization, and burst generation. In the 1980s, investigators demonstrated that periodic administration of carbachol effectively induced kindling, and that administration of high doses of pilocarpine – a cholinergic agonist – may lead to the acute induction of status epilepticus (SE) and to the subsequent onset of spontaneous seizures.

The pilocarpine model of epilepsy, as it is now called, thus emerged shortly after the kainate model. The kindling model, pilocarpine model, and kainate model constitute the three most widely used animal models of epilepsy. Over the years, the particular features of the pilocarpine model, such as a high frequency of spontaneous recurrent seizures and robust supragranular mossy-fiber sprouting, have contributed to its wider acceptance and use.

The pilocarpine model of epilepsy relates both to a model of acutely induced seizures culminating with a prolonged episode of SE, as well as to a model of chronic spontaneous seizures that follows – at some delay – the initial acute seizure period. The acute induction of seizure activity is characterized by long-lasting limbic SE associated with sustained electrographic discharges in limbic structures. Several parameters have been used to assess subsequent chronic epileptogenicity, which appears

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after the acute induction stage. These parameters include thresholds for fluorothyl-induced seizures, kindling facilitation, and response to paired electrical pulses. However, the best criterion for the epileptic condition is the occurrence of actual spontaneous seizures. As in temporal lobe epilepsy (TLE) patients with prolonged febrile convulsions, spontaneous recurrent seizures occur after a seizure-free interval that consists of apparently normal behavior and electrographic activity. Accordingly, the phenomenological descriptions and pathological changes after SE-induced damage are critical for understanding the process of epileptogenesis.

Background: Natural History

The administration of pilocarpine to rodents induces ictal and interictal epileptic activity in hippocampal and cortical electrographic recordings. These electrical phenomena are correlated with a sequence of behavioral alterations that includes akinesia, ataxic lurching, facial automatisms, progressing to motor seizures, and SE. After a single application of these drugs, SE can last from 6 to 12 h. After spontaneous remission of SE, animals are comatose and both hippocampal and cortical recordings show a depressed EEG with occasional high-voltage spiking activity. These events have been called the 'acute period' of this model, and metabolic studies performed during this period have revealed increased glucose utilization – mainly in the hippocampus and other limbic structures, thalamus, and substantia nigra. The pattern of neuronal loss observed in these animals closely corresponds to the areas that are metabolically activated during SE. The cytopathology following SE does not mean that the cholinergic system has excitotoxic potential. It is rather believed that the cholinergic system may play a role in triggering and maintaining seizure activity. It is, indeed, the excessive synaptic release of glutamate that leads rapidly to neuronal depolarization and breakdown of membranes. The synaptic release of acethylcholine is thought to provide a sustained drive that keeps neurons firing (as seen in SE), but without causing any neuropathological damage per se. According to this hypothesis, the function of the cholinergic system is in the maintenance of sustained seizure activity that drives excitatory glutamatergic mechanisms responsible for neuronal damage. Accordingly, experiments have shown that anticholinergic drugs could prevent sustained seizures but are not useful for seizure arrest.

Animals surviving the acute period of SE proceed to a latent 'seizure-free' period with apparently normal behavior – except for some aggressiveness if manipulated and toward other rats if they are maintained in groups. This latent period lasts 1–8 weeks, depending on the animal strain and the method used to induce SE, and ends with the occurrence of the first spontaneous seizure. The recurrence of spontaneous seizures during the 'chronic period' may vary according to the details of the model, ranging from 1 seizure per month to several seizures per day.

The evolution of spontaneous seizures follows the behavioral and electrographic stages of kindling. This aspect of epileptogenesis has been better characterized for the pilocarpine model. In the majority of Wistar rats subjected to the pilocarpine, the first seizure is normally characterized by paroxysmal discharges localized in the hippocampus or amygdala, without changes at cortical recording sites. Behavioral correlates of this limbic seizure consist of motor arrest followed by eye-blinking, chewing, and head nodding – resembling kindling stages 1 or 2. The following seizures show gradual synchronization of neocortical and hippocampal regions, and longer duration of ictal events. Forelimb clonus, and rearing with falling (as in kindling stages 4 or 5) are the hallmarks of these later generalized seizures. The progression of spontaneous seizures tends to be quite variable, in terms of duration of paroxysmal discharges and sequence of behavioral stages, from animal to animal. Several seizure stages of similar or lesser intensity can be observed before a generalized motor convulsion is reached. Once the generalized convulsion (i.e., like a stage 5 kindled seizure) occurs, then the vast majority of the following seizures are also generalized. These observations made in the pilocarpine model of epilepsy suggest that the development of epilepsy, as a consequence of SE, follows the stages of kindling.

Methods: Generation of the Model

The acute model was originally described in rats and subsequently in mice. Pretreatment of the animals with scopolamine was shown to prevent the development of convulsive activity caused by pilocarpine. The original descriptions of the model included the subcutaneous administration of methyl-scopolamine, at the dose of 1 mg kg^{-1}, to reduce the peripheral effects associated to the autonomic activation caused by pilocarpine. Studies have clearly demonstrated that activation of M1 muscarinic receptors is critical for the induction of SE by systemic administration of pilocarpine. Pilocarpine has been shown to effectively produce acute seizures in Wistar and Sprague-Dawley rats. The initial characterization of the model in Swiss mice was later followed by its description in C57BL/6, CD1; CF1, kinin B1 and B2 receptor knockout mice; and 129SV background strains. Administration of pilocarpine to a different rodent species, *Proechimys* sp., has also been shown to be effective at triggering acute seizures. Finally, systemic administration of pilocarpine to the common marmoset (*Callithrix jachus*) promotes acute seizures and SE.

Pilocarpine has been shown to trigger acute seizures after intracerebroventricular, and intrahippocampal administration. In addition, while the intrahippocampal administration of pilocarpine in rats triggers spontaneous recurrent seizures in just some of the injected animals, supragranular mossy-fiber sprouting ipsilateral to the injection is found in all animals.

Regarding age sensitivity, the pilocarpine model can be induced in rats starting from postnatal day 11 to animals of ~12 months of age. Administration of pilocarpine to postnatal day 3–6 rats leads only to minor electroencephalographic changes characterized mostly by flattening of the background activity and occasional low-amplitude, low-frequency spikes with no clear seizure behavior. In 7–10-day-old rats, more frequent spikes, with grater amplitude, are seen in both cortex and hippocampus, together with infrequent spike and wave complexes – but still without clear motor seizures. Intense behavioral signs, evolving (in some animals) to limbic seizures and SE, occurs when pilocarpine is administered to animals older than 11 days. Animals ranging from 15 to 21 days of age appear to have the greatest sensitivity to pilocarpine-induced SE; at this age, SE develops with a shorter latency, and more often results in death as compared with adult animals. It is important to note that young animals usually have a greater susceptibility to the acute induction of seizures by pilocarpine, often requiring doses that are 30% lower than that used for adult animals. From the morphological point of view, the pattern of neuronal loss and ensuing plastic changes, however, is markedly less in these young animals than that seen in adult animals. In contrast, induction of multiple episodes of SE in 7–9-day-old rats pups results in important plastic changes, leading to long-lasting epileptogenesis, as manifested by electrographic epileptiform discharges, behavioral deficits, and in vitro hyperexcitability of hippocampal networks.

For the adult animal, susceptibility to the convulsant effects of pilocarpine increases with age; the threshold dose for generalized seizures is essentially constant from 30 to 70 days, but decreases thereafter, particularly beyond 100 days. Older (and thus heavier) rats not only require a smaller dose of pilocarpine to develop SE but also have a greater mortality rate. Indeed, pilocarpine-triggered seizures in adult animals often have an intense tonic phase followed by respiratory arrest and death, even when produced by small doses of pilocarpine. In this sense, for experiments aimed at studying the functional and morphological consequences of pilocarpine-induced SE the ideal age range for rats is between 2 and 3 months of age at the time of status induction.

Although most studies have been conducted with male animals, it has been shown that female rats – both young (starting at postnatal day 7) and adults (usually 2–3 months of age) – can have acute seizures induced by pilocarpine. The induction of SE with pilocarpine in female rats leads to an increased diestrous, suggesting an anovulatory estrous cycle and an inadequate luteal phase. In addition, pregnant and lactating rats show a clear effect of the hormonal changes that characterize such conditions, with animals having less-frequent spontaneous seizures.

As described earlier, the susceptibility of different animals to pilocarpine-induced seizures is influenced by a number of different factors. Indeed, even for the same animal species, strain, gender, and age (in this case 2–3–month-old, male, Wistar rats), the dose required to induce SE across a significant number of animals has been shown to range from 400 to 320 mg kg^{-1}. Therefore, for anyone using the model, it is useful to assess the sensitivity of the particular animal batch to be used for experimentation.

The remaining issues associated with the induction of SE depend on the intended use of the model. The use of 400 mg kg^{-1} of pilocarpine will most likely result in a 100% chance of induction of severe SE associated with a high mortality rate in the few hours after status onset. In addition, depending on the animal species and strain, this high dose may result in a number of animals having severe tonic seizures followed by respiratory arrest as a very early seizure manifestation. With the reduction of the injected dose, seizures are likely to be less severe and associated with a greater survival rate. This reduction, however, also results in a smaller number of the animals developing SE – and thus with a less-efficient use of the animals (often requiring a greater initial number of animals to achieve a given set of animals with SE per group). This 'negative' effect of lower dose pilocarpine can be used to advantage to generate a control group that, despite being injected with pilocarpine, did not develop SE. Such controls may more effectively allow investigators to assess the effects of SE induction, as opposed to effects associated with administration of pilocarpine per se.

For studies aimed at inducing SE in a great percentage of the animals, and with a high and long-term survival rate, the best strategy is to titrate the administered dose of pilocarpine. With this protocol, the administration of a pilocarpine dose yielding SE in ~40% of the animals is followed; for those animals not developing clear seizures, by the subsequent administration pilocarpine at 30% of the initial dose within 1 h after the initial administration. This procedure can be continued for one or two additional doses. Animals not responding to these subsequent doses probably comprise a category of resistant animals.

In the pilocarpine model, induction of SE is critical for the later development of spontaneous seizures. Pilocarpine-induced SE, however, is usually associated with a large mortality rate in the initial 3-day period. Mortality rate is effectively reduced by controlling the severity of the status episode. It has been shown that achieving complete control of SE in the first hour after its onset results

in a large number of animals not developing spontaneous seizures. Good survival rate associated with the development of epilepsy (i.e., spontaneous seizures) in all of the animals can be accomplished by titrating the severity of the SE; after 90 min of free-running SE, the various drugs can be used to diminish SE without actually abolishing it. Thus, diazepam at a dose of 5 mg kg^{-1} or thionembutal at a dose of 25 mg kg^{-1} are good alternatives for this purpose. Both the dose used for the induction of SE and the duration of this episode should be carefully monitored to yield the greatest frequency of animals developing intense SE episodes and surviving to exhibit spontaneous epileptic seizures.

In the following hours after SE induction, animals often become comatose and unable to care for themselves. Animals to survive 12 or more hours after SE induction should remain in the laboratory overnight (not returned to the vivarium). Administration of saline, glucose, and electrolytes, by subcutaneous or intraperitoneal route, can help to minimize loss of animals in this period. By the following day, some animals are already alert and able to care for themselves, while others are still in a poor condition. For the latter group, hand feeding is an alternative and can have good results (fruit juices mixed with sweetened milk and mashed rat food pellets). In addition, leaving fruit slices in the floor cage also helps in a faster recovery.

For rats subjected to free-running SE, with no use of anti-SE medication, the chance of eventually developing spontaneous seizures is 100%. Controlling status 90 min after its onset, as described above, results in a similar yield of animals with spontaneous seizures. Earlier control of status, initiated before 90 min, or its more complete control, achieved with higher doses of anti-SE medication, are likely to reduce the percentage of animals developing spontaneous seizures. Once developed, spontaneous seizures do not remit for the animal's lifetime.

Recent Results: Characteristics and Defining Features

Behavioral and Clinical Features

The sequential pattern of electrographic changes during the acute phase, immediately following the injection of pilocarpine, is characterized by a significant theta rhythm that replaces the background activity in the hippocampus and low-voltage fast activity in the cortex. This activity progresses to high-voltage fast activity with spikes in the hippocampus. The spiking activity spreads to the cortex and evolves into electrographic seizures. Ictal periods recur every 3–5 min and finally lead to sustained discharges 50–60 min after the injection of pilocarpine. This pattern of electrographic activity lasts for several hours and may evolve to a pattern of periodic discharges on a relatively flat background.

Seizure frequency in the chronic period may vary considerably among epileptic rats, and several seizure patterns have been observed. Some pilocarpine-injected rats may present with a low seizure frequency throughout several weeks or months; others may have daily seizures; and some may present clusters of seizures in short periods of time. Such variability in seizure frequency patterns may represent a drawback for behavioral or antiepileptic drug (AED) studies. To assemble a homogeneous group, it is necessary to identify – through baseline monitoring – a group of rats with regular consistent seizure frequency.

It is important to stress that most behavioral or AED studies rely upon video monitoring to establish seizure frequency. Therefore, class 3–5 limbic seizures are preferentially detected, and less-severe ('asymptomatic') seizure stages (class 1 and 2) are frequently overlooked. This point is particularly relevant because many intractable complex partial seizures in humans rarely generalize, even when AEDs are tapered during video-EEG monitoring – and thus are the 'equivalent' of class 1 and 2 limbic seizures in rats. High-resolution video capturing coupled with EEG is necessary to detect subtle behavioral seizures correlated with, for example, focal ictal activity in the hippocampus or amygdala.

Response to AEDs and the Usefulness in Screening Tests

Several drugs have proved to efficiently suppress or attenuate pilocarpine-induced SE when injected either before or right after pilocarpine administration. For this reason, benzodiazepines and barbiturates have been widely used to prevent the relatively high mortality associated with prolonged seizures. Studies in which SE is attentuated (by both drugs) after different durations of SE have shown that early SE suppression can prevent spontaneous recurrent seizures. In addition, neuron loss is prevented if treatment is established with 30 min of SE. When administered before pilocarpine, clonazepan (90.35 mg kg^{-1}), phenobarbital (23.4 mg kg^{-1}), and valproic acid (286 mg kg^{-1}) prevent limbic seizures and protect against seizure-related neuron damage. Pretreatment with trimethadione (179 mg kg^{-1}) results in a moderate protection against pilocarpine-induced seizures, while phenytoin (10–200 mg kg^{-1}) and carbamazepine (10–50 mg kg^{-1}) have no effect and do not prevent pilocarpine-induced brain damage. The noncompetitive NMDA receptor antagonist MK-801, when given 20 min prior to pilocarpine (4 mg kg^{-1}), has no effect in blocking the onset of pilocarpine-induced SE; however, MK-801 pretreatment prevents later spontaneous recurrent seizures and protects against CA1 pyramidal-cell loss. Ketamine, another noncompetitive NMDA receptor antagonist, at a dose of 100 mg kg^{-1}, i.p., exerts a time-dependent protection of hippocampal cells and prevents late spontaneous seizures and specific motor deficits in the Morris water maze.

Drug testing in the latent period has a fundamental importance because several molecular changes occur in this phase that may contribute to the process of epileptogenesis. Thus, a compound with efficacy in inhibiting the development of spontaneous seizures may be considered a true antiepileptogenic drug. There is a growing body of evidence that suggests that there are differences between the mechanisms underlying the development of epileptogenesis and the mechanisms involved in maintaining the expression of seizures after they have established. Hence, it is reasonable to assume that compounds that prevent epileptogenesis may be quite different from classical AEDs. Drugs that regulate the expression of trophic factors involved in neuroprotection or plastic changes induced by damage may have an antiepileptogenic effect.

AEDs that have efficacy in suppressing pilocarpine-induced SE are not the same drugs that are effective in controlling spontaneous limbic seizures. Diazepam, phenobarbital, valproic acid, and trimethadione protect against pilocarpine-induced SE, while phenytoin and carbamazepine are ineffective. In the chronic phase, carbamazepine and phenytoin are effective against spontaneous seizures. Valproic acid is also effective at high doses (600 mg kg^{-1}), and ethosuximide does not prevent spontaneous seizures.

Neuropathology

Induction of SE by pilocarpine leads to severe and widespread cell loss in several brain areas. Dying cells can be assessed via a number of different techniques that characterize a given biochemical or structural aspect of the degenerating cells. Differences in the biochemical and morphological profile of dying cells may indicate whether the cell is suffering from an apoptotic or a necrotic degenerating process. As with a number of different pathological conditions, cell damage in the pilocarpine model has been described as to its necrotic or apoptotic nature. However, classifying cell damage according to these two categories may be confusing, and will therefore be avoided here. A more fruitful perspective is to consider that the excitotoxic insult triggered by pilocarpine-induced SE leads both to immediate cell damage – that takes place minutes to a few hours after its onset – and also results in a protracted process of neurodegeneration that may take weeks and months to develop.

The initial damage, occurring a few hours after the onset of SE, is most intense in the superficial layers of some neocortical areas, hilus of the hippocampus, endopiriform nucleus, piriform cortex, and claustrum. Eight hours after SE onset, damage has further intensified in those areas, and in addition, becomes significant in entorhinal cortex, amygdaloid nuclei, ventromedial nucleus of the hypothalamus, subiculum, and the bed nucleus of the stria terminalis. Damage in these distinct brain areas is time specific.

Damage is not restricted to the initial hours and days after SE, and tends to progressively involve other areas in the following months. To this end, damage to the thalamus is often found in animals sacrificed many months after the onset of pilocarpine-induced SE but is notably less intense (or even absent) in some thalamic nuclei at shorter survival times.

In addition to cell loss, there is also clear injury resulting in both morphological and functional pathology. Evidence of altered cell morphology in the pilocarpine model has been provided mostly for the hippocampus. Altered distribution of dendritic spines in dentate granule cells and distorted dendritic trees in putative GABAergic hippocampal interneurons are some of these changes. Additional morphological changes are more likely to be reactive rather than a direct consequence of the initial insult. In this sense, the emergence of axonal sprouting – the most notable being the supragranular mossy-fiber sprouting, granule-cell dispersion, increased rate of neurogenesis, and development of granule-cell basal dendrites – are among the morphological changes that are likely to represent a reactive response.

As with any other lesional model, pilocarpine-induced SE does not uniformly damage different cell groups. Here again, cell type-specific vulnerability has been best studied in the hippocampus. There is greater damage to principal cells, that is pyramidal and granule cells, in the hippocampal complex, but interneurons located in the other strata can also be damaged. Most notably, hilar mossy cells can be markedly damaged by pilocarpine-induced SE, but with large variation in the extent of damage between different animals. Damage to GABAergic neurons is also extensive throughout the hippocampus. However, not all GABAergic neurons in the hippocampal complex are equally vulnerable, with specific populations in different strata showing different rates of loss. In a recent study, the density of GAD65 mRNA-positive neuron profiles in layer III of the entorhinal cortex was similar in control and post-SE rats evaluated between 3 and 7 days after pilocarpine. Similar evaluations have not yet been provided for other brain areas, with the exception of a qualitative assessment of the neocortex.

Glial pathology in the pilocarpine model has not received the same level of attention as neuronal pathology. Nevertheless, there have been descriptions regarding the proliferation of astrocytes, as shown by the increased expression of glial fibrillary acidic protein (GFAP) and other glial markers. In addition to glial proliferation, it has been shown that in the CA1 area of the hippocampal complex of animals subjected to pilocarpine-induced SE, glial cells 'adapt' to permit rather large increases in extracellular potassium accumulation. Microglia and other

markers of inflammatory tissue reaction have also shown to be present in the early phases after pilocarpine-induced SE.

Challenges for the Future

Although there are many types of epilepsy, both genetic and acquired forms, TLE with hippocampal sclerosis is probably the most common human epilepsy and the one most intensely studied. Despite a wealth of descriptive data obtained from patient histories, imaging techniques, electroencephalographic recording, and histological studies, the epileptogenic process remains poorly understood. Progress toward understanding the etiology of an acquired neurological disorder is largely dependent on the degree to which experimental animal models reflect the human condition. The pilocarpine model of epilepsy has the potential to provide useful information about the pathophysiologic mechanism of human temporal epilepsy. In addition, demonstration of interictal disturbances of learning and other behaviors, as seen in this model, could be used to develop therapies for the interictal impairments that occur in human TLE. Finally, resistance to medical therapy is recognized as one of the major problems in epilepsy treatment today. The pilocarpine model of epilepsy could facilitate the use of modern state-of-the-art techniques in neurobiology to address critical questions in the pharmacoresistance of epilepsy.

See also: **Models**: Characteristics and Mechanisms of Kindling; Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Model Characterization in Relationship to Human Disorders; The Tetanus Toxin Model of Temporal Lobe Epilepsy; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Oscillatory Activity**: Neuronal Networks Generating Pathological High Frequency Oscillations; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures.

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The Tetanus Toxin Model of Temporal Lobe Epilepsy

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Introduction

Temporal lobe epilepsy is the most common type of epilepsy in adults, and often proves resistant to drug treatment. Good experimental models provide a means to develop theories about the neural malfunctions responsible for the development of epileptic foci, and to test novel treatments. They have the important advantages over direct investigations in patients of being reproducible, and accessible to experimental procedures that would be unethical in humans. A major challenge is to find an appropriate model for each clinical condition of interest and specific research question.

The research outlined here on the intrahippocampal tetanus toxin model is aimed at:

1. Comparing the model with human temporal lobe epilepsy.
2. Identifying structural and functional abnormalities in the epileptic focus – that is, how the structure and function of tissue in the model differ from those of normal tissue.
3. Determining how epileptic seizures and interictal discharges are generated – and in particular, elucidating what mechanisms lead to the hypersynchrony and hyperactivity of hippocampal and related circuits.
4. Testing antiepileptic treatments.

Background

Experimental models of epilepsy have been around for over a century. Progress in the identification of the neuronal dysfunction that leads to epileptic activity gained real momentum in the last few decades of the twentieth century. Much of this work relied on models in which epilepsy-like activity was produced by applying convulsant drugs to normal brain tissue. This approach models human symptomatic seizures, but not epilepsy. Chronic models are needed in order to characterize cellular abnormalities associated with an epileptic focus. Such models also provide an experimental approach to understanding how epilepsy impacts on the whole organism.

Intracerebral injection of tetanus toxin was one of the earliest reported methods of triggering epileptic seizures, dating back to the late nineteenth century. In its modern incarnation as a model of temporal lobe epilepsy, a very small dose of toxin is injected directly into the hippocampus. The animal (usually a rat) will start to experience spontaneous, repeated, intermittent epileptic seizures after ~1 week. This model differs from the kindling model of temporal lobe epilepsy in readily producing spontaneous epileptic discharges. The appearance of spontaneous seizures is shared with the models that depend on an initial period of prolonged epileptic activity – status epilepticus – induced by injection of a drug (kainic acid – intracerebral or systemic, or pilocarpine (usually systemic)) or by prolonged electrical stimulation. Tetanus toxin differs from these other models in that it: lacks an initial period of severe/prolonged seizure activity, does not exhibit early gross lesions (e.g., as produced directly by a period of status epilepticus), and has a low mortality rate. The absence of early gross lesions following intrahippocampal tetanus toxin suggests that it is a particularly good model for nonlesional temporal lobe epilepsy in humans. The observation that about 10% of tetanus toxin-injected rats do develop hippocampal sclerosis suggests that the model is also relevant to lesional temporal lobe epilepsy – and that the hippocampal sclerosis seen clinically in many cases is a consequence of epileptic activity rather than its initial cause. If one accepts this possibility, then the tetanus toxin model may provide a good approximation of the early stages of mesiotemporal lobe epilepsy, and offer a useful system in which to study the mechanisms underlying the development of hippocampal sclerosis.

Practical Methodological Issues

Tetanus toxin is one of the most potent toxins known. It is the agent that causes spastic paralysis following infection of peripheral injuries with *Clostridium tetani* (a condition also known as 'lockjaw'). Tetanus toxin is a protein of about 150 kDa, comprising a light and a heavy chain. The light chain is a protease selective for a synaptic protein called vesicle associated membrane protein (VAMP, or synaptobrevin), and as a result of its action at this site, blocks synaptic transmission. However, the whole toxin is needed to induce experimental epileptic foci, which depends on the internalization, and probably the intraneuronal transport, of the toxin as well as the protease action.

To induce a temporal lobe epileptic focus, a few nanograms of toxin are injected, under stereotaxic control, directly into the hippocampus of an anesthetized animal. The injection target is near the end of the hippocampal fissure to ensure exposure of the CA3 region; dorsal and

ventral injections produce similar results. Injecting the toxin into the neocortex also produces an epileptic focus, but with different characteristics, resembling medically refractory epilepsia partialis continua. Recording electrodes may be implanted to monitor electrical activity from the hippocampus and other regions. After recovery from anesthesia, the animal is housed under normal conditions. Handling the toxin takes some care, both because of its toxicity and because it can be degraded in transit or by careless handling in the laboratory.

Investigations performed on these epileptic animals exploit a wide range of neuroscience methods. Much of the work outlined here uses hippocampal slices (usually 0.4 mm thick) prepared from the epileptic rats and maintained in vitro for several hours to allow electrophysiological investigation with microelectrodes. Chronic electrical recordings from implanted electrodes allow detection and characterization of electrographic seizures, while direct visual observation or video recording allows investigators to describe the behavioral correlates of seizures, and assess seizure severity and seizure types. Animals experiencing the fully developed epileptic syndrome may be used to test the behavioral consequences of the epilepsy, or serve in trials of antiepileptic drugs. At the end of the experiment, tissue should be prepared for terminal physiological, histological, and molecular investigations.

Results

Epileptic Syndrome

About a week after intrahippocampal injection, the rats start to have spontaneous and repeated seizures, developing a full-blown syndrome of temporal lobe epilepsy. The earliest electrographic abnormalities are occasional large spikes or brief burst discharges. When seizures start, they typically consist of a cluster of partial seizures, over a period of 1–2 days that culminate in a secondarily generalized seizure with motor signs. Seizure frequency typically peaks during the second week, at several to 20+ seizures per day. The electrographic picture in this model is characterized by interictal discharges and seizure activity of various patterns. In particular, seizure onsets can be either hypersynchronous or low-voltage fast, as in humans. Seizures usually start in the CA3 area – of either the injected or uninjected hippocampus – and spread into the entire hippocampus and to contralateral hippocampus and other parts of the brain (including cingulate and entorhinal cortices). Electrical activity during the seizure normally follows a sequence of frequencies, generally slowing as the seizure progresses (**Figure 1**); about one-third of seizures terminate with a period of 2–3 Hz activity. The behavioral correlates of the electrographic seizures resemble those described for other models of temporal lobe seizures, and mimic the seizures observed in human patients with mesiotemporal lobe epilepsy. Behaviorally, these seizures can be classified as complex partial, with behavioral arrest, staring, oroalimentary automatism (chewing), sniffing, vibrissal and facial twitching, and wet-dog shakes. Seizures can be followed by postictal confusion. Some seizures can progress to become secondary generalized, and are then characterized by fore-limb clonus, and rearing and falling convulsions. The seizures rarely last more than 2 min, and the rats maintain themselves in good condition. During the periods between seizures, modest behavioral disorders (e.g., hyperreactivity) can be found, but are less severe than in some other models, thus simplifying handling. Cognitive changes are discussed in the following sections.

Once the epilepsy is established in adult animals, then seizures recur intermittently for 6 weeks or so, after which the rats usually gain remission from seizures. This remission appears to be an active process for most hippocampal foci. In contrast, neocortical foci induced by tetanus toxin continue to discharge for the life of the animal – as do hippocampal foci if they are induced at postnatal day 10 (and also in a minority of hippocampal foci induced in adults).

In a pioneering series of studies on animals with hippocampal tetanus toxin foci, Mellanby found that the rats had enduring impairments on a range of tests of learning and memory, particularly on tasks known to depend on hippocampal function. These impairments cannot be attributed to epileptic seizures interrupting brain function, because they could be demonstrated after the rats gained seizure remission. They cannot be attributed also to medication (as corresponding changes in patients often are) because they occurred in the absence of any drugs. Finally, they cannot be due to gross morphological changes in hippocampus (such as neuronal loss or hippocampal sclerosis), since these pathologies are not present in the majority of animals. Therefore, these changes are primarily functional – and argue that the tetanus toxin model provides a unique opportunity to gain insights into the mechanisms of cognitive and memory dysfunctions observed in patients with mesiotemporal lobe epilepsy.

Cellular Mechanisms

Hippocampal slices and synaptosomes prepared from injected animals reveal a loss of inhibitory postsynaptic potentials (IPSPs) and calcium-dependent GABA release for ~1 week post toxin injection. Subsequently, the IPSPs and GABA release recover, at least in response to direct activation of inhibitory neurons or their terminals. However, inhibitory function continues to be impaired, in that stimulation of inhibitory neurons through excitatory synaptic pathways evokes very small 'disynaptic' IPSPs (**Figure 2**). This effect has parallels with the proposed function of 'dormant' basket cells, but differs in the absence of related neuronal loss; in the case of the tetanus

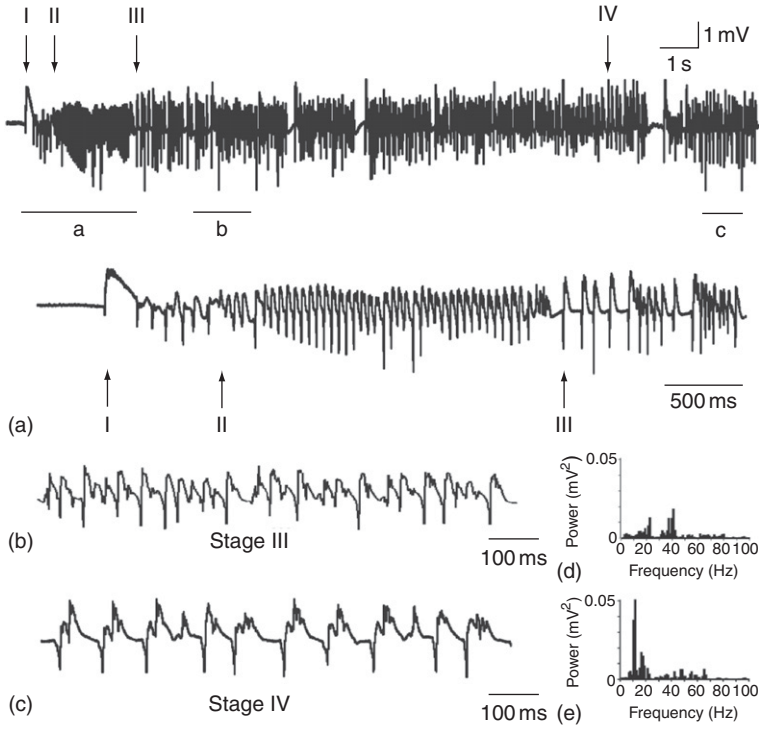


Figure 1 Recordings from a freely moving rat during a spontaneous epileptic seizure. Note the relatively high frequencies at the beginning and the progressive slowing as the seizure progresses. The top panel shows the full seizure until just before the onset of the motor component of the secondarily generalized seizure. The segments labeled (a), (b), and (c) are reproduced below. Panels (d) and (e) are power spectra of the signals in panels (b) and (c) respectively. Note the progressive slowing as the seizure develops. Reproduced from Finnerty GT and Jefferys JGR (2000) 9–16 Hz oscillation precedes secondary generalization of seizures in the rat tetanus toxin model of epilepsy. *Journal of Neurophysiology* 83: 2217–2226, with permission from American Physiological Society.

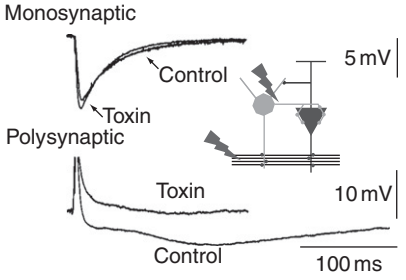


Figure 2 Functional dormancy during the active seizure phase of tetanus toxin model. 'Monosynaptic IPSPs' evoked by direct excitation of inhitory neurons, when excitatory synapses were blocked by drugs, were the same in tissue slices from epileptic and control rats, showing that GABA release mechanisms had recovered by (in this case) 2 weeks. 'Polysynaptic IPSPs' were substantially smaller in the epileptic rats when EPSPs were not blocked, that is before the glutamate receptor antagonists were added to the slice; the smaller stimuli used in these experiments activate inhibitory neurons via excitatory synapses. Cartoon illustrates mono- and poly-synaptic activation. Adapted from Whittington MA and Jefferys JGR (1994) Epileptic activity outlasts disinhibition after intrahippocampal tetanus toxin in the rat. *Journal of Physiology* 481: 593–604, with permission from Blackwell Publishing; see also Vreugdenhil M, Hack SP, Draguhn A, Jefferys JGR (2002) Tetanus toxin induces long-term changes in excitation and inhibition in the rat hippocampal CA1 area. *Neuroscience* 114: 983–994.

toxin model, 'dormancy' is functional rather than structural. Remarkably, this apparent interneuron malfunction persists for over 5 months, despite the rats gaining remission from their spontaneous seizures within that timeframe. Other changes found at the late stage of tetanus toxin-induced epileptogenicity, such as weakened synaptic excitation and reduced cellular excitability, may be part of the remission mechanism. These changes may also be responsible for the enduring impairments of learning and memory found in these animals (see explanation given earlier). Notably, a form of synaptic plasticity associated with learning and memory, long-term potentiation, is preserved in the epileptic rats, suggesting that impaired learning and memory in this case is due to a reduction in hippocampal responsiveness rather than in mechanisms of synaptic plasticity.

Histological changes in the tetanus toxin model are modest, at least when it is injected in the adult rat. About 10% of animals experience focal loss of hippocampal neurons with associated gliosis (not the same 10% that fail to gain remission from seizures). These structural changes often involve CA1 and extend up to 1–2 mm along the hippocampus. Selected classes of interneuron also can be lost, typically after many spontaneous seizures

over ~2 months. One consistent structural change is axonal sprouting, both in the dentate gyrus (mossy fibre sprouting shown by Timm staining) and in CA1 (shown electrophysiologically – antidromic responses can be evoked from stimulation in stratum radiatum which normally does not contain CA1 pyramidal cell axons). Axonal sprouting is one of the most consistent structural changes in temporal lobe epilepsy.

Tetanus toxin injected at a neonatal stage (postnatal day 10) produces a more intense seizure syndrome and disrupts cellular organization, but like the adult version of the model, results in minimal neuronal loss, recurrent seizures, and learning deficits.

The effects of antiepileptic drugs have been tested on both the neocortical and hippocampal foci induced by tetanus toxin. Neocortical foci appear medically refractory, at least to diazepam and phenytoin. Hippocampal foci do respond, with a significant reduction in seizure frequency, to both carbamazepine and levetiracetam. It remains to be determined whether these apparent differences in drug response are due to the site of the focus, to the drugs tested, or to other factors such as strain differences in the rats. It is worth noting that the intrahippocampal tetanus toxin model presents some difficulties if one plans to use it as a screen for antiepileptic drugs. While the spontaneity of the seizures makes the model more 'realistic,' it also makes quantifying the efficacy of antiepileptic treatments more difficult than in models with frequent seizures or seizures that can be triggered at will, (as is the case for kindling).

Future Challenges

Like other chronic models of focal epilepsy, the big challenge posed by the intrahippocampal tetanus toxin model for the neurophysiologist is the nature of the dysfunction of the neuronal circuits that leads to the generation of spontaneous epileptic seizures. The mechanisms that impair the excitation of inhibitory neurons need resolution and their interactions with other aspects of the focus (including axonal sprouting) need rigorous analysis. A distinctive feature of this model is the seizure remission gained by most rats. Previous work has provided circumstantial evidence of changes in excitatory transmission and in neuronal excitability, but these changes need to be investigated in more detail; in particular, it is important to determine which are missing in the minority of rats that do not gain seizure remission.

The main purpose, and challenge, for research on experimental models of epilepsy is to find improved treatments for epilepsy in humans. Understanding the cellular and local network mechanisms responsible both for establishing the epileptic focus and for seizure remission will provide clues for new treatments. The question of refractoriness to existing anticonvulsants needs to be addressed – in particular, whether rats fall into responder and nonresponder groups that can be used to determine mechanisms of drug resistance and methods of overcoming it. Finally, this model can be used for developing novel treatments, such as focal drug administration, local inactivation, and therapeutic electrical stimulation, and for developing improved methods for seizure prediction or detection.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; **Models**: Model Characterization in Relationship to Human Disorders; Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; **Neurotransmitter Function**: Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Oscillatory Activity**: High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures; **Plasticity**: Axon Sprouting in Epilepsy; Circuitry Reorganization, Regeneration, and Sprouting; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus.

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The use of Cell Culture Models to Study Mechanisms Related to Epilepsy and Antiepileptic Drugs

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Introduction and Background

In order to understand the role of cell culture models in epilepsy research, it is useful to briefly examine the understanding of central nervous system (CNS), cellular physiology, and pharmacology that existed prior to the time that cell culture models were first 'invented' for the study of mammalian CNS. Much work had been performed in the 1960s and early 1970s, investigating the cellular phenomena occurring in the mammalian brain during interictal discharges and seizures. This research was developed in parallel to studies of neuronal and synaptic function in general. At that time, there was a basic understanding of neuronal physiology, still based on the Hodgkin and Huxley model of the squid giant axon, although even such elementary discoveries as the presence of Ca-dependent action potentials in mammalian neurons had not yet been made. In addition, there was a rudimentary understanding of synaptic physiology and pharmacology. The identities of the inhibitory neurotransmitters were just being discovered, and the identities of the excitatory transmitters were still somewhat of a mystery, as was even the basic physiology of excitatory synaptic potentials. All of the epilepsy research, until that time, had been performed in intact animals in which seizures were induced by either chemical or electrical stimulation. Mechanisms underlying the seizures were inferred from these in vivo experiments, and were based on the then understanding of neurophysiology. At the same time, relatively little was known about the mechanisms of action of any available antiepileptic drugs (AEDs).

In the early 1970s, it became clear that in order to begin to understand neurobiology at a cellular, and ultimately molecular level, in vitro preparations of mammalian CNS were needed. Almost simultaneously, a small number of investigators began to grow and maintain embryonic mammalian CNS in dissociated cell cultures, while others learned how to maintain viable sections of more mature CNS tissue in slice chambers (see Further Reading section). These discoveries led to a virtual explosion of new information about the physiology and pharmacology of mammalian CNS and, in parallel, to a great expansion of our knowledge of epilepsy mechanisms and the mechanisms of action of antiepileptic drugs. Thus, it is impossible to separate pure 'epilepsy research' using cell culture models from basic neurobiological research that led to major insights into epilepsy mechanisms.

This article briefly reviews techniques for establishing dissociated cell cultures of mammalian CNS that are most relevant to epilepsy studies. It then discusses the kinds of questions about epilepsy mechanisms and AEDs that can be best answered by studies employing this model system, using examples from the literature to illustrate how these strategies can produce important information. Finally, limitations of the model are addressed.

Cell Culture Methods

Dissociated cell cultures of central nervous system tissue can be prepared from essentially any mammalian species. The majority of cultures used to date, however, are derived from rat and mouse embryos or early postnatal animals. Data derived from the cultured tissue can be compared with comparable data from intact tissue, as rat and mouse brain slices are also commonly employed as experimental preparations. Interestingly, there is very little literature on potential differences between rat and mouse CNS neurons. Comparisons in culture would be extremely useful, given that clear differences between species, and among strains within each species, have been identified in intact systems.

Most forms of epilepsy occur in the archicortex or neocortex. As such, studies of cellular mechanisms most relevant to epilepsy or antiepileptic drugs have been performed on cultures developed from embryonic cortex or hippocampus. Other brain regions can also be studied, as dictated by specific research questions. In addition, many studies involving mechanisms of action of AEDs have utilized dissociated cultures of spinal cord or dorsal root ganglion (DRG); these regions produce large, relatively easily studied neurons with reproducible characteristics.

CNS neurons can be cultured in a variety of formats, depending on the desired experimental paradigm. For most physiological experiments, neurons are cocultured with glial cells, in a relatively enriched medium. For other experiments, neurons can be grown in an almost glial-free environment with carefully controlled and characterized media. Cells can be grown at varying densities, depending

on the nature of the experiment to be performed. For example, for routine physiology and pharmacology, neurons are often grown at relatively high density on top of proliferating astrocytes. These neurons are best studied at a density that allows identification of individual cells (soma and dendrites) in a microscopic field filled with many neurons. Under these conditions, the neurons are healthy, synapse formation is abundant, and small networks develop. For biochemical experiments, cultures such as these can be grown on the surface of large plates or in flasks.

One drawback of the usual 'high density' cultures is that finding pairs of synaptically connected neurons is difficult. In these cultures, all the neurons receive abundant synaptic inputs, but monosynaptic connections among neighboring neurons are rarely found. By growing cultures at very low densities, such that only two or three neurons are visible in a microscopic field, coupled pairs are easily found. An extreme example of this arrangement is found when neurons are cultured in isolation, often seeded at very low densities onto astrocytes or microdots of adherent substrate. Under these conditions, neurons form abundant autapses onto themselves. There are many subtle variations on the main methodological scheme employed to establish CNS cell cultures. These can best be obtained from either original papers or from texts devoted to CNS cell cultures.

Within hours of plating, most viable cells settle on the substrate, adhere, and begin extending processes. Even at this early time point, neurons can appear pyramidal, stellate, and bipolar. Whether these morphologies correspond to what the cells were destined to become in vivo has never been determined. By 3–5 days in vitro (DIV), the neurons have clearly differentiated and developed extensive processes. Synapses begin to appear at about 4–7 DIV and increase in density and complexity for days thereafter. Given the nature of the connectivity in the high density cultures, it is likely that axons extend fairly significant distances before synapses are made. Cultures of this kind can be maintained in a robust state for 4–6 weeks or longer. As the cultures age, there is a gradual decline in the number of viable neurons, although those that remain often are quite large, and have extensive dendritic trees and abundant spines.

When synaptic interactions are to be studied using paired recordings, cultures are often grown at a very low density. These cultures are maintained in lower volumes of media in high potassium (e.g., 20 mM). Under these conditions, glial cells grow very slowly and do not overgrow the cultures. The basic properties of the neurons in low density cultures appear similar or identical to those grown at higher density, although this similarity has not been rigorously studied.

Results – CNS Cell Cultures as a Model to Help Understand the Pathophysiology of Epilepsy and Mechanisms of Action of Antiepileptic Drugs

Neuronal and Synaptic Physiology and Pharmacology

Ionic currents underlying action potentials in cortical neurons

The physiological basis of the action potential (AP) in mammalian CNS is now well understood and was derived from pioneering studies of the squid giant axon and other similar preparations. Early studies in cell culture demonstrated that voltage-dependent Ca currents also contributed to AP generation in some mammalian neurons. These currents were first demonstrated in cultured dorsal root ganglion (DRG) neurons (a preparation that then became very useful for studying mechanisms by which neuromodulators were able to affect Ca currents), and later in mammalian cortical neurons both in slice preparations and in culture. Under baseline conditions, these currents were not apparent because of the rapid depolarization–repolarization sequence initiated by Na and K currents; however, when Na currents were inhibited, the cortical neurons developed slower, powerful Ca-dependent APs. Once the Ca APs were identified, it was noted that some neurons in cortex, hippocampus, and thalamus had quite prominent Ca APs and tended to fire bursts of action potentials when depolarized. These neurons appeared capable of acting as 'pacemaker' neurons for seizurelike discharges, and hypotheses about the origin of rhythmic discharges in cortex, hippocampus, and thalamocortical circuits were developed based on these findings.

Physiology and pharmacology of inhibitory synapses

Early in vivo studies had demonstrated that IPSPs were generated by opening of Cl channels, and were associated with an increase in membrane conductance. Studies in mammalian CNS cell culture demonstrated that GABA was the main, if not exclusive, neurotransmitter responsible for postsynaptic inhibition. These studies also showed that GABAergic inhibition was very labile at most synapses – declining rapidly with repetitive stimulation due mostly to presynaptic factors (i.e., not due to receptor desensitization or changes in Cl gradient). These discoveries in cell culture led to extensive examinations of the pharmacology of GABAergic synapses, including the elucidation of the mechanisms of action of some of the most important AEDs.

From both biochemical (e.g., receptor binding) studies on brain tissue and studies at the cellular level in cell culture, the $GABA_A$ receptor was recognized as a GABA

receptor *complex* (GRC). Studies utilizing cell cultures first described the allosteric modulation of the GRC by benzodiazepines, a group of drugs that was used extensively to treat status epilepticus and other forms of seizures, but whose mechanism of action had been unknown. Similar studies demonstrated that barbiturates, another class of AEDs, could allosterically modulate the GRC at a different site. Further, unlike the benzodiazepines, barbiturates at high concentrations could directly activate the GRC. Studies of membrane patches removed from cultured neurons demonstrated how these drugs worked at the single channel level.

GABA also activates other classes of receptors, most notably $GABA_B$ receptors that are found on both somas and axon terminals. Much of what we know of the physiology of $GABA_B$ receptors is derived from studies in cell culture. These receptors are G-proteins coupled to Ca channels in axon terminals, where they block Ca entry and thereby down-regulate neurotransmitter release; they are also found coupled to K channels on somas and dendrites, where they mediate slow and prolonged inhibition. Both of these sets of events have important implications for epileptiform mechanisms. Activation of $GABA_B$-mediated inhibition likely plays a role in the termination of interictal discharges and possibly in the termination of seizures. Increased activation of $GABA_B$ receptors is thought to contribute to enhanced bursting in thalamocortical relay neurons and the generation of spike-wave discharges in primary generalized epilepsy. Inhibition of GABA release by activation of $GABA_B$ receptors at GABAergic terminals has also been implicated in the reduction of inhibition seen with repetitive activation of inhibitory synapses. In addition, activation of $GABA_B$ receptors is likely to be responsible for the complex effects of two newer AEDs that were designed to increase GABA levels at inhibitory synapses – tiagabine (by blocking GABA uptake) and vigabatrin (by blocking GABA metabolism). Both of these agents are antiseizure drugs, effective against partial seizures. However, both can also enhance primary generalized absence seizures and possibly status epilepticus – presumably by allowing released GABA to act on presynaptic GABA terminals and paradoxically decreasing inhibition, or by enhancing hyperpolarization of thalamocortical neurons and thereby enhancing synchronized spike-wave activity.

Physiology and pharmacology of excitatory synapses

The physiology of excitatory synapses was much less understood than inhibitory synapses before the introduction of neuronal cell cultures. In culture, the response of neurons to applied glutamate was characterized; the odd *I–V* curve was determined to be due to a voltage-dependent block by Mg of a subpopulation of glutamate receptors; different glutamate receptor subtypes (e.g., AMPA, NMDA, kainate) were identified and characterized. Each of the advances in our understanding of the mechanisms of excitatory transmission had direct impact on epilepsy research. The nature of the large depolarizing events seen both during interictal discharges and ictal events was characterized within the framework of different glutamate receptor events. The role of NMDA receptors, especially, was emphasized as an important component of epileptiform activity. And the autoregenerative nature of NMDA-mediated synaptic events (with depolarization reducing the Mg block of NMDA receptors, which in turn promoted further depolarization by glutamate) became a critical part of the explanation for the all-or-nothing development of some forms of epileptic activity.

The characterization of excitatory synaptic transmission was also determined at the single channel level. AMPA receptors were demonstrated to have very rapid and unusual desensitization properties, such that they opened once in response to agonist and then desensitized until agonist was removed. NMDA receptor-coupled channels, however, continued to open and close for as long as glutamate remained present. In addition, it was demonstrated that NMDA receptors were very sensitive to changes in extracellular pH, becoming inhibited at acidic values and enhanced by alkalosis, within pH ranges that were found in the brain during physiological or pathological stimuli. Investigators also demonstrated that most AMPA receptors were permeable to only monovalent cations, whereas NMDA receptors were permeable to Ca as well. As the molecular biology of the receptors was further understood, AMPA receptors that lacked a properly edited version of the GluR2 subunit were found to be permeable to Ca. These channels occurred naturally on some inhibitory interneurons, and were proposed as a contributing component of some disease states. Each of these discoveries had great implications for our understanding of epileptic processes and for current theories of epileptogenesis. For example, the use of hyperventilation to stimulate epileptic changes in the EEG may be due to the brief metabolic alkalosis induced by this procedure. In addition, the vulnerability of subsets of inhibitory interneurons to hypoxia may be related to their Ca permeable AMPA receptors.

In addition to the fast depolarization mediated by ionotrophic glutamate receptors, it was also noted that glutamate could produce a slow and long-lasting change in neuronal properties due to activation of 'metabotropic' receptors. The discovery and characterization of these receptors also added significantly to our understanding of how epileptiform events could become established and propagate throughout the brain.

Synaptic Plasticity

Many studies of synaptic plasticity in the vertebrate CNS were performed using the preparation of in vitro brain slices, most commonly hippocampal slices. A vast literature

on long term potentiation and long term depression is derived from these studies. In general, with a few recent exceptions, such studies rely on pathway stimulation, and on single cell or field recordings to infer synaptic mechanisms. Parallel studies in cell cultures have allowed direct investigation of synaptic plasticity using intracellular or patch clamp recordings from both the pre- and postsynaptic elements of the synaptic pair. This approach provides more precise control over the relevant synaptic events. However, this preparation also has significant drawbacks for such studies. The cell culture tissue appears to be more immature than the tissue usually studied in slices; the nature of the synaptic interactions may be different from those that develop in the more complex CNS; and it may be very difficult or impossible to activate multiple inputs to the same postsynaptic neuron in culture preparations. In addition, some cellular mechanisms 'wash out' during the intracellular dialysis that occurs with whole cell patch clamp recordings (although this problem is not unique to cell culture preparations). The latter issue is particularly important when considering longer term changes in synaptic efficacy. Nevertheless, important observations about short term plasticity have been made utilizing cell culture preparations.

In general, when studied with paired recordings, inhibitory synapses that start out with a high probability of release almost always decrement when repetitively activated. After even a single action potential, the probability of release of GABA declines for as long as several seconds. With brief trains of action potentials, inhibitory synaptic efficacy may decline by more than 50%, all due to presynaptic factors independent of $GABA_B$ autoreceptor feedback. Such decreases in inhibitory efficacy may have profound implications for ictogenesis.

By contrast, excitatory neurons have different response characteristics to repetitive activation. They also often demonstrate a decrease in release of synaptic transmitter after single action potentials, but this decrease is much more variable than at inhibitory synapses. With trains of high frequency stimuli, some excitatory synaptic responses potentiate, some appear to remain stable, and some decrement. The net result is that if both excitatory and inhibitory synapses are part of a recurrent circuit, and if they are simultaneously activated repetitively, net inhibition will decline with respect to net excitation and the circuit will become unstable. This developing instability is likely one of the critical mechanisms underlying the development and spread of seizures through the brain.

Molecular Expression Profiling at the Level of Single Cells

One of the most widely employed techniques in molecular biology is expression profiling of various tissues under various physiological and pathological conditions. Changes in gene expression, as measured at the mRNA level, are often analyzed to determine how a specific group of cells reacts to a change in physiology. For the complicated CNS, such expression profiling is most informatively performed on single identified neurons (or at least on small groups of neurons) to attempt to understand how changes in gene expression produce changes in network behavior. Some of the earliest applications of single cell gene expression profiling were performed on identified neurons in cell culture. These studies were then extended to neurons identified in live brain slices. Subsequently, it was determined that RNA could be relatively faithfully amplified and analyzed even from fixed tissues.

Initial studies focused on molecular differences between identified inhibitory and excitatory neurons. Surprisingly little difference was found in the relative expressions of most channels and receptors. Even more surprising, however, was the finding of relatively large amounts of mRNA for glutamic acid decarboxylase (GAD), the enzyme responsible for the synthesis of the inhibitory neurotransmitter, GABA, in all excitatory neurons in culture. High levels of GAD mRNA were found, despite the fact that these neurons were not synthesizing GAD protein or GABA. Because of a concern for a possible artifact of neurons in culture, these observations were extended to more intact CNS tissues, where it was also found that excitatory pyramidal cells, and hippocampal and cerebellar granule cells contained relatively large amounts of GAD mRNA. These unexpected results led to the formulation of the 'standby neurotransmitter' hypothesis, that proposed that excitatory neurons could become inhibitory. Again unexpectedly, it was subsequently demonstrated that just such a switching on of GABA synthesis could occur during prolonged seizures! Dentate gyrus granule cells, which are normally excitatory, will exhibit dense staining for GAD and GABA, extending all the way into axon terminals, after prolonged seizures. These cells can then exert inhibitory effects on downstream neurons. Obviously, control of inhibitory elements in critical brain circuits may have a profound effect on excitability and seizure development and spread.

The application of single cell molecular profiling has been extended well beyond the early studies in cell culture. Much recent work focuses on changes in gene expression in different cells within the hippocampus after status epilepticus-induced or injury induced epileptogenesis. Changes in receptor numbers and subtypes, and alterations in ion channels, have been implicated in the development of the hyperexcitable state.

Excitotoxicity

The concept of excitotoxicity is so common at present that it is perhaps surprising to recognize that this hypothesis was only developed in the 1970s and 1980s. Although

initial observations by Olney and others were based on in vivo work, much of the subsequent progress in understanding underlying mechanisms proceeded in studies of CNS cell cultures. This understanding included insights into the roles played by the various glutamate receptor subtypes, the cellular mechanisms involved in both the necrotic and apoptotic components of cell death, and the effects of drugs on these processes.

Mechanisms of Action of AEDs

Almost all the currently available AEDs were developed based on animal screening models, and their mechanisms of action were unknown when the drugs were introduced. Studies in neuronal cell cultures have significantly contributed to an understanding of the mechanisms of many AEDs.

Na channel blockers

Several very potent AEDs have, as a primary mechanism of action, the ability to block voltage-dependent Na channels in a voltage- or use-dependent fashion. Using preparations of cultured spinal cord or cortex, several groups demonstrated that phenytoin, carbamazepine, lamotrigine, and zonisamide could block sustained repetitive firing (SRS). Early action potentials in the train were generally minimally affected, but as the neurons remained depolarized, the drugs were increasingly able to block more and more channels. Subsequent voltage clamp experiments demonstrated the voltage-dependent nature of the block.

GRC allosteric modulators

Several of our more commonly employed AEDs work as allosteric modulators of the GRC, mechanisms first elucidated by studies of cell cultures. Benzodiazepines enhance the affinity of GABA at the receptor and allow enhanced channel openings; barbiturates prolong channel openings and can produce channel openings even in the absence of GABA.

GABA uptake inhibitors

Tiagabine was developed as an inhibitor of GABA uptake. It was hypothesized that such inhibition would result in more GABA extracellularly, and thus there would be enhanced inhibition. Studies in cell culture have produced evidence that this assumption may reflect an oversimplification of the processes involved. More GABA at the synapse did not produce stronger inhibition at $GABA_A$ receptors. Paradoxically, the increased GABA primarily affected presynaptic $GABA_B$ receptors on axon terminals and down-regulated GABA release – thereby producing *less* inhibition. This surprising result may account for the complex response of some seizure types to tiagabine; absencelike seizures may be exacerbated and nonconvulsive status epilepticus may occur, even when partial seizures are reduced.

Similarly complex results were obtained when analyzing the effects of glutamate transport inhibitors on excitatory synaptic physiology. Instead of enhancing synaptic excitation, these agents often dampened excitatory synapses by an action of the excess synaptic glutamate on presynaptic mGluRs – which decreased transmitter release from excitatory terminals.

Circuit Behavior and Ictogenesis – 'Seizure-Like' Phenomena in Cell Cultures

Models of epilepsy in cell culture

When neurons from cortex or hippocampus are grown in dissociated cell culture, they form extensive synaptic interactions and local circuits. The nature of the circuitry has not been extensively examined but a few principles have emerged. Neurons in routine, high density culture do *not* tend to form synapses with nearest neighbors in culture, despite extensive overlap of processes. Instead, synapses are formed with more distant targets. Neurons in these cultures tend to fire in bursts at either irregular or occasionally regular intervals. These bursts, which resemble the paroxysmal depolarizing shifts seen in epileptic tissue in vivo, occur despite an *inability* of these neurons to generate endogenous bursts in response to depolarizing pulses (as opposed to some endogenous bursting neurons in intact cortex and hippocampus), and therefore appear to be driven by large EPSPs. When pairs of neurons in culture are recorded, it is very common for the neurons to exhibit synchronous bursting or synchronous activation – presumably driven by synaptic events originating elsewhere in the cultures. Thus, the circuit organization and synaptic interactions among neocortical and hippocampal neurons maintained in dissociated cell cultures spontaneously recreate the kinds of circuits needed to sustain epileptiform types of activity, even in the very simplified environment of a Petri dish and a two-dimensional substrate.

Synchronous bursting in the cultures can be enhanced by many of the same maneuvers that provoke epileptic activity in intact cortex (e.g., GABA receptor antagonists and NMDA receptor activators). Such epileptogenicity occurs in the absence of endogenous bursting cells, electrical interactions between the neurons, or ephaptic interactions among the neurons. This synchronous discharge can also occur when neurons are grown in the virtual absence of astrocytes. Surprisingly, this 'epileptic-type activity' and epileptogenic circuitry have been largely ignored by epilepsy researchers using cell cultures to study other aspects of cellular biophysics, physiology and pharmacology, or mechanisms of action of AEDs.

One prominent exception is the work of Delorenzo and colleagues, who have exploited the enhanced synchrony

produced by exposing the cultures to a low magnesium environment to create a cell culture model of epilepsy. Reducing Mg in the media in which neurons are bathed enhances activity of NMDA receptors and produces an increase in excitability of the cultures. When cells are maintained in this condition for more than 3 h, the cultures remain hyperexcitable for the life of the cells, even when normal Mg is restored. This group has carried out a number of experiments to support their view that this model simulates focal epileptogenesis – dependent on aberrant activation of NMDA receptors, and related to a disruption of calcium homeostasis, likely through alteration in calcium – and calmodulin kinase II activity.

Chronic cultures of dissociated neurons maintained on a chip

Another recent approach to analyzing epileptic-like events in cell culture has developed around an interesting new way of culturing the neurons. Cells can be grown on microchips with multiple exposed electrodes. When a neuron lands on one of these electrodes, an extracellular recording from that cell can be maintained, often for weeks at a time. Since the microchip contains many electrodes, many individual cells or small groups of neurons can be monitored simultaneously. Under these conditions, it has been shown that the neurons tend to fire in bursts, and that there are varying degrees of synchrony occurring in the cultures. Since the neurons can be monitored for weeks, this preparation has the potential for important studies of dynamic circuit behaviors, including the response of these synchronously discharging cells to antiepileptic drugs or other possible antiseizure therapies.

Limitations

Despite the extensive studies performed on dissociated cell cultures since they were first 'invented' in the early 1970s, there are a number of limitations that must be mentioned about 'models' of epilepsy developed with this approach. Many different laboratories now maintain cell cultures, and there is undoubtedly significant variability in cultures (methods of generation and maintenance, cell types, etc) from one lab to the other. For some types of experiments, this variability may be unimportant. However, for some studies that focus on epileptic-like events and conditions, such differences could be quite important – for example, a variation in the percent of inhibitory neurons(from 30 to 40% of the culture to less than 10% have been reported). Similarly, the presence or absence of astrocytes may be important for some kinds of studies.

Another issue that always arises with relation to cell cultures is how faithfully they mimic their in vivo counterparts. Surprisingly, for most properties studied, the resemblance is remarkable. However, for other properties, there may be very significant differences between cultured neurons and neurons in the intact brain. For example, one group of $GABA_B$ receptors (K channel-coupled $GABA_B$ receptors on cell bodies and dendrites) are absent from cultured neurons. Similarly, as we understand more about developmental expression of different ion channels and transmitter receptors, it will be necessary to validate culture studies with in vivo data. It is now clear that different mechanisms of epileptogenesis, seizure generation, and response to AEDs are characteristic of CNS neurons at different stages of maturity. It will therefore be important to determine what maturational level is reproduced in cell cultures before drawing general conclusions from culture data for the intact brain.

Perhaps the most interesting aspect of the comparison between cell cultures and the intact brain relates to the epileptiform activity expressed by neurons in culture. Do the circuits that form after cells are completely dissociated and then plated onto a two dimensional surface (covered with an artificial substrate) recreate the kinds of circuits that are capable of developing and sustaining epileptiform activity? Depending on how one defines 'epileptic,' it appears that cell cultures can do just that. What insights will this preparation provide about the developing brain and the response to injury? How will culture-determined properties of cells and circuits be useful for designing strategies to prevent seizures or the hyperexcitability that underlies human epilepsy?

Summary and Directions for Future Research

Insights into human epilepsy derived from studies of neuronal cell cultures

Studies utilizing the preparation of dissociated cell cultures have already provided significant insights into the basic physiology, pharmacology, and molecular biology of neocortical and hippocampal neurons and synapses, and have led to many advances in our understanding of epileptiform events. Such studies have contributed greatly to our current understanding of the mechanisms of action of AEDs. Further work on receptor (or channel) subtype-specific pharmacology will likely lead to new classes of AEDs that have more focused targets and (hopefully) better side-effect profiles. Other studies focusing on mechanisms underlying short term synaptic plasticity, and on the local circuitry involved in synchronous neuronal activation, may also lead to new strategies for suppressing seizures. Each of these areas may result in new targets for AED screening.

One of the main areas of neglect in epilepsy research is that of 'epileptogenesis.' Studies in cell culture on such issues as axonal sprouting, new synapse formation, and

altered molecular phenotype (e.g., in response to activity or injury) may provide new insights into these processes – and lead to new hypotheses about how to control them.

See also: **Models**: Acutely Dissociated Neurons; Model Characterization in Relationship to Human Disorders; Seizure Models in Acute and Organotypic Slices; **Networks**: Determinants of Neuronal Network Activity in Cultured Hippocampus; **Plasticity**: The Role of Short Term Synaptic Plasticity in Development and Spread of Seizures.

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Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis

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Introduction

Epileptic discharges are synchronized discharges of large populations of cortical neurons in the brain. The in vitro slice model, in particular, the CA3 neuronal circuit in the hippocampal slice has been useful in the elucidation of the mechanisms of synchronization. Studies in this preparation have focused on two major types of synchronized discharges. The first, a shorter-duration discharge (<500 ms) serves as a model for interictal spikes. The second, a more prolonged discharge (in the order of seconds) has been used to study the generation of ictal discharge and interictal-to-ictal transition.

Interictal-like spikes in hippocamapl slices can be elicited by the application of $GABA_A$ receptor antagonist. Ictal-like discharges can be activated in the same preparation by the addition of group I metabotropic glutamate receptor (mGluR) agonist (S3HPG or DHPG). mGluR agonist can elicit ictal-like discharges under two conditions. First, addition of mGluR agonists to hippocampal slices exhibiting interictal-like spikes (i.e., in the presence of $GABA_A$ receptor antagonist) causes a gradual prolongation of interictal spikes into ictal-like events (interictal–ictal transition). Secondly, mGluR agonist, when applied alone (without the presence of $GABA_A$ receptor inhibitor), causes the sequential appearance of short interictal-like discharges followed by prolonged ictal-like discharges. Recent studies reveal that, in addition to mGluR agonist, activation of synaptic mGluRs in the recurrent connections between CA3 pyramidal cells, under at least one pathological condition (that of Fragile X mental retardation syndrome, FXS), causes the generation of ictal-like discharges in the hippocampal CA3 population.

Epilepsy is a disorder of recurrent seizures. Epileptogenesis is the plasticity process by which a normal brain becomes epileptic. In this context, mGluR-induced, synchronized discharges in the CA3 population can be viewed as a model of epileptogenesis, because ictal-like discharges, once induced by agonist, persist for hours – even when the inducing agonist is washed out. Similarly, synaptically induced, mGluR-dependent, ictal-like discharges elicited in Fragile X Syndrome (FXS) model mouse preparations

are long-lasting events which persist for the duration of subsequent recording periods (over several hours). Recent studies suggest that mGluR-dependent epileptogenesis is probably the underlying cause of epilepsy suffered by FXS patients. In vivo studies show that FXS mice have increased propensity to audiogenically induced seizures. MPEP, a mGluR subtype (mGluR5) blocker, is uniquely effective in suppressing the seizure activity.

Methods

In vitro slices have been widely used for physiological studies of isolated brain regions. The hippocampal slice, with its easily identifiable regional demarcations, is a preparation of choice. The preparation was first used to study long-lasting potentiation (LTP) in the CA3–CA1 synapse and epileptiform discharges. The rapid and substantial progress in these two areas serves as a testimonial to the experimental advantages offered by this in vitro preparation.

After an initial developmental period, the hippocampal slice has become a relatively simple and reliable preparation to make. Subregions of the hippocampus, including CA3, CA1 or dentate gyrus areas, can be dissected out via visual guidance and studied in isolation. In addition, because of the largely separated location of the pyramidal cell soma and interneuron soma in the slice, the properties of interneurons can be examined independent of pyramidal cells using intracellular electrode recordings, either "blind" or using infrared, differential interference contrast microscopy (IR-DIC) to visualize isolated somata – including those of interneurons. Particularly for interneuron somata located close to the pyramidal cell body layer, the IR-DIC technique has proved to be particularly useful.

The identifiable and separated location of the dendrites and somata of pyramidal cells in subregions of the hippocampus also makes it possible to carry out detailed studies of pyramidal cell dendritic properties. Dendrites can be examined via intracellular recordings or they can be studied in isolation from the soma, using microdissection to separate dendrite from soma. The latter procedure has been useful in demonstrating the involvement of local dendritic mRNA translation in the induction of LTP.

The properties of the CA3 neuronal circuit have been extensively explored in studies on the basic mechanisms of epilepsy. The most powerful method for examining the recurrent synapses between CA3 pyramidal cells is paired intracellular recordings. Such studies provide data on the strength of recurrent connections between individual CA3 pyramidal cells, and the extent of connectivity within the network. Paired pyramidal cell–interneuron studies also provide opportunities to evaluate plasticity of inhibitory control, an issue of significant and ongoing inquiry relevant to epileptogenesis.

Recent studies have revealed the presence of axon-axonal gap junctions between principal cells in the hippocampus and dentate gyrus. The slice preparation will be useful for exploring the function of such axon-axonal gap junctions. The role of gap junctions in enhancing CA3 pyramidal cell synchrony, within a synchronized episode or in the generation of high frequency population oscillations, requires further attention.

Pharmacological tools are used to elicit epileptiform synchronized discharges in hippocampal slices. Generally, synchronized discharges are elicited in the hippocampal network via the excitatory connections between CA3 pyramidal cells. However, robust, prolonged, epileptiform discharges can also be elicited in the CA1 region by a number of manipulations, including the use of low divalent cation (Ca^{2+} and Mg^{2+}) solution for extracellular perfusion. In addition to pharmacological tools, detailed developmental profiles of synaptic connectivity have been carried out; some of these studies have focused on the prolonged synchronous discharges that are elicited in disinhibited, hippocampal slices from immature animals (compared to tissue from adult animals).

Background and Results

Synchronization Mechanisms for Interictal Spikes – Synaptic Activation of Ionotropic Glutamate Receptors and Network Simulation

Properties of CA3 pyramidal cells

Action potentials in CA3 pyramidal cells are followed by prominent depolarizing afterpotentials. Depolarizing afterpotentials often reach threshold to recruit additional action potentials, causing the firing of a cluster (burst) of 3–4 action potentials. Bursts of action potentials are a common form of spontaneous activity of CA3 pyramidal cells, recorded in vitro and in vivo. Burst firing can be viewed as a signal amplification process in that a single suprathreshold excitatory synaptic potential can trigger multiple action potentials from CA3 pyramidal cells.

In addition to somatic action potential firing, CA3 pyramidal cell dendrites can also generate independent bursts. The combined excitability of the soma-dendritic complex of CA3 pyramidal cell serves to increase the effectiveness of the recurrent synapses (between CA3 pyramidal cells) to synchronize the CA3 neuronal population (see below).

Properties of recurrent synapses between CA3 pyramidal cells

CA3 pyramidal cells are connected to each other via recurrent glutamatergic synapses. Recent in vitro data, using anatomical methods to trace the axon of single dye-filled CA3 pyramidal cells in hippocampal slices,

reveal that the recurrent network is the densest in the CA3a region, where each pyramidal cell axon makes up to 650 terminal contacts onto neighboring pyramidal cells. Data derived from paired recordings between CA3 pyramidal cells indicate that of unitary synaptic potentials have an average amplitude of 1.5 mV and synaptic conductance 1 nS. Recordings from CA3 pyramdical cells have also shown that the synaptic conductance underlying interictal-like synchronized discharges (recorded during disinhibition) is 70–300 nS (see below), indicating that single pyramidal cells receive 70–300 unitary excitatory synaptic inputs during the discharge. Given that each pyramidal cell can fire clusters of at least three action potentials during each interictal-like discharge, and that the recorded interictal-like event results from summation of synaptic potentials generated by these action potentials, a conductance of 70–300 nS for an interictal-like discharge suggests a lower limit of 23–100 presynaptic cell for each pyramidal cell; that is, on average, one pyramidal cell is functionally connected to 23–100 postsynaptic pyramidal cells.

Recurrent connections between CA3 pyramidal cells and interictal discharges

Recordings from pairs of CA3 pyramidal cells show that a burst generated in a single pyramidal cell has a 35% chance of causing action potential firing from its monosynaptically connected postsynaptic elements. Further, experimental and simulation studies show that, through the recurrent connection, single bursts in a pyramidal cell can initiate and pace synchronized discharges of the entire CA3 pyramidal cell population, when GABAergic inhibition is blocked. In addition to glutamatergic synapses between CA3 pyramidal cells, gap junctions, by enhancing cell–cell excitatory interaction and spiking activity, also significantly contribute to epileptic synchrony of the CA3 network.

Normally, the powerful synchronizing mechanism provided by the recurrent CA3 network is tightly controlled by feedforward and feedback inhibition mediated via local GABAergic interneurons. IPSPs are highly effective in stopping the spread of the excitatory wave in the recurrent network for two reasons: First, the conductance underlying unitary IPSPs (6–9 nS) is larger than that underlying the recurrent EPSPs (1 nS). Second, CA3 pyramidal cells are connected to GABAergic interneurons via a powerful monosynaptic excitatory connection; that is, a single action potential in a presynaptic pyramidal cell can elicit action potentials in postsynaptic GABAergic interneurons. An action potential in a single interneuron in turn elicits IPSPs in about 200 postsynaptic pyramidal cells. Thus, under normal circumstances, recurrent or feedforward inhibition serves to control excitatory spread within the CA3 pyramidal cell network and prevents the occurrence of hypersynchronized epileptiform discharges. This inhibitory control, and its variability in the face of a powerful recurrent excitatory network, is often viewed as a potential site of vulnerability that may lead to seizures.

Generation of Ictal-Like Discharges – Activation of Group I Metabotropic Glutamate Receptors (mGluRs)

Effects of mGluR activation on CA3 pyramidal cells

CA3 pyramidal cells normally generate spontaneous periodic bursts of 2–5 action potentials. The duration of a burst is less than 100 ms. Upon stimulation by a mGluR agonist, burst firing in CA3 pyramidal cells is significantly prolonged, reaching periods of 2–10 s. The prolongation in firing is caused by the activation of a voltage-dependent cationic current, $I_{\mathrm{mGluR(V)}}$. $I_{\mathrm{mGLuR(V)}}$ emerges following mGluR agonist exposure in CA3 pyramidal cells – but not in CA1 pyramidal cells. The current has a threshold around the resting potential of CA3 pyramidal cells (−65 mV) and its amplitude increases with depolarization. The current has a reversal potential of about −10 mV and shows no inactivation.

At the population level, mGluR agonist elicits ictal-like synchronized discharges of 2–10 s from the CA3 pyramidal cell population. mGluR-dependent ictal-like discharges have three notable properties. (1) As mentioned above, the discharge is long lasting. Further, once induced, ictal-like discharges persist for hours (invariably for the entire duration of recording time in an experiment) following the wash out of the inducing agonist. (2) Induction of mGluR-dependent ictal-like discharges is dependent on mRNA translation, and (3) $I_{\mathrm{mGluR(V)}}$ is a necessary cellular response underlying the expression of ictal-like discharges.

These data reveal that ictal-like discharges are elicited in CA3 population through mGluR agonist stimulation. The agonist changes the firing pattern of single CA3 pyramidal cells from burst firing of less than 100 ms to periodic prolonged firing of 2–10 s. Synchronization of mGluR activated CA3 pyramidal cells (with their altered mode of prolonged firing) via their powerful recurrent connection gives rise to ictal-like discharges.

Epileptogenesis: Synaptic Activation of mGluR-Dependent Ictal-Like Discharges

Application of DHPG stimulates synaptic and extrasynaptic mGluRs. For the mGluR-mediated epileptogenesis to be functionally relevant and activatable in the brain, the epileptogenesic process should be inducible by stimulation of synaptic mGluRs. Data obtained in wild type preparations show that, although mGluR-mediated epileptogenesis is elicited by DHPG, synaptic stimulation of mGluRs is not effective in inducing epileptogenesis. The reason for this apparent paradox is that, in wild type

preparations, synaptically stimulated mGluR-mediated mRNA translation is suppressed by fragile X mental retardation protein (FMRP), thereby preventing the induction of epileptogeneis. Absence of FMRP function is the cause of fragile X mental retardation syndrome (FXS). In a mouse model of FXS, in which FMRP is not functional, synaptic stimulation of mGluRs becomes effective in inducing epileptogenesis. In vivo studies show that FXS mice exhibit enhanced audiogenic seizure susceptibility that is sustained by exaggerated mGluR responses. The difference in DHPG versus synaptic stimulation of mGluR in wild type preparations, in terms of effectiveness in inducing epileptogenesis, is unclear. There are at least two explanations. First, FMRP may only regulate synaptically located mGluRs and not extrasynaptic mGluRs. Second, prolonged stimulation by DHPG (and not phasic synaptic stimulation) may cause proteolysis of FMRP via an activation of the ubiquitin-proteasome system, thereby abrogating the repressive effect of FMRP on mGluR-mediated mRNA translation (and thus enabling epileptogenesis).

Future Goals

The CA3 neuronal network has proved to be a useful tool for the study of basic mechanisms of epilepsy. Considerable understanding of the synchronization mechanisms underlying interictal discharges, and the process involved in ictal discharge generation, have been derived from in vitro preparations involving the CA3 neuronal network. In addition, the network provides a unique model for the study of signaling and plasticity mechanisms underlying one form of epileptogenesis – that of mGluR-dependent epileptogenesis. This mGluR-dependent process is likely to constitute a cause of epilepsy suffered by FXS patients. Efforts are now directed toward examining whether, and how, the mGluR form of epileptogensis can occur in subjects other than FXS patients. Regulation of the mGluR-stimulated translation process is a point of focus. In particular, vulnerability of the FMRP regulation sites will need to be explored and identified.

Although progress has been made mainly in the study of conventional 'chemical' synapses between CA3 neurons, recent studies suggest the presence of gap junctions between principal cells in hippocampal formation (i.e., between granule cells, and probably also between CA3 pyramidal cells). Both modeling and in vitro experimental data suggested that axonal gap junctions could account for very fast oscillations (>70 Hz), including ~200-Hz ripples, as well as play a critical role in the generation of persistent γ (30–70 Hz) and neocortical $\beta 2$ (20–30 Hz) oscillations. At present the function of gap junctions in neuronal synchronization in epilepsy is not well defined. That gap junctions may be important in epilepsy as has been suggested by EEG records showing high frequency oscillations (exceeding 100 Hz) that occur just before a seizure begins. More details are needed regarding the mechanisms by which gap junctions contribute to the very fast brain oscillations that can presage a seizure. Studies should also focus on whether attenuating or preventing these very fast oscillations can prevent seizures. Toward this end, preliminary in vitro studies have shown that carbenoxolone, a gap junction blocker, suppresses very fast oscillations and prevents subsequent epileptiform discharges.

See also: **Glutamate**: Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; **Imaging**: Optical Monitoring of Circuitry Activity In vitro; **Imaging**: Using Intrinsic Optical Signal Changes to Analyse Epileptic Activity and Seizures; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization.

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Determinants of Neuronal Network Activity in Cultured Hippocampus

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Introduction

Spontaneous network activity, expressed as synchronized, spreading bursts of action potential discharges over extended periods of time, is a typical attribute of all neuronal networks studied to date, both in vivo and in vitro. Intuitively, spontaneous activity is important for the development and refinement of connections among the neurons in the network, and may underlie the ability of the network to respond to afferent input in a dynamic fashion. Still, the mechanisms related to the triggering, spread, and the control of frequency, synchrony, and magnitude of the activity of the network remain elusive. Spontaneous network activity sometime goes astray, leading to the development of synchronized epileptic seizures. Such abnormal activity can last for the lifetime of the affected individual, and interferes with the person's ability to cope with the environment. Thus, understanding the mechanisms that regulate 'normal' network activity, while having its own merit, becomes extremely important as it may lead us to a better understanding of the conversion of network activity into an epileptic one.

Background

Several pertinent issues related to the generation of network activity have been dealt with only fragmentarily in recent years. One issue of major interest concerns the process of development and maturation of effective networks of synchronized activity. Among those issues, the questions related to network development are as follows: (1) Which are the crucial participants in network maturation? (2) Is activity in and of itself important for the organization of the network, or can the network develop in a situation where activity is blocked chronically? (3) Do excitatory or inhibitory synaptic activity speed up or slow down the development and activity of the network? (4) Since every neuronal network is composed of different cell types that are either intermingled (as is the case of the cerebral cortex) or are relatively clustered (as is the case of the hippocampus), what is the contribution of different cell types to the network activity? Finally and perhaps most importantly, is the network burst a collective behavior of many neurons, or are there 'master' neurons that entrain the network? In the latter case, who are these master neurons and what make them so unique?

A great deal of information has been gathered to characterize network activity in the intact brain. Cortical and hippocampal neuronal assemblies express different patterns of activity, associated with different 'states' of the organism, from slow delta rhythm (0.5–4 Hz), through theta (4–8 Hz), alpha (8–12 Hz) to fast gamma (12–80 Hz) oscillations. While it is quite clear that these rhythms involve synchronized activity of interneurons, the main issues that are pertinent to the understanding of the mechanisms of generation of these patterns remain elusive. A slow rhythmic bursting activity has been initially described in the developing hippocampus and later found in other brain areas, and this activity has been assumed to play a major role in the developmental organization of these structures. This rhythm is also present in the isolated hippocampus, indicating that the rhythm is genuinely intrinsic; however, the rules governing its generation are still elusive. It is virtually impossible to analyze these rules in the intact tissue, since it is hard to isolate

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systematically the parameters that may contribute to the network activity and to study them later in isolation. For example, it is difficult to chronically expose intact tissue to drugs that block action potential discharges (e.g., tetrodotoxin), and study the effect on the maturation of the network. The few studies that employed such a method reported interesting observations on the outcome of a chronic blockade of cortical activity on hyperexcitability of the tissue. Also, it is impossible to vary systematically the proportion of excitatory and inhibitory neurons in a network in vivo.

The dissociated culture of cortical structures provides a convenient test system for the examination of the rules that govern the emergence, generation, and spread of network bursting in populations of densely interconnected neurons. Network activity can be recorded for extended periods of time in a noninvasive manner and with infinite time resolution using multielectrode arrays. Using such a method, spike activity of dozens of cells can be recorded simultaneously. However, even in this case, the recording is made of a select subpopulation of neurons, leaving the majority of neurons in the dish unseen. An alternative method involves imaging of action potential (spike)-associated changes in intracellular calcium concentrations of an entire network. This recording method, now used also in vivo, is limited to several hours after loading of the fluorophore; it can be used to detect changes in every cell and even specific compartments of the cell, but is limited by the sensitivity of the dye, the field of view of the objective, and the temporal resolution of the detection system (confocal microscope, high speed CCCD camera, etc). Needless to say, the dissociated culture is only an approximation of the network in vivo. It is raised in artificial growth medium, lacks blood supply and afferent innervation, and the cells may produce aberrant connections not found in vivo. Thus, whatever rules established in the dish need to be reexamined in vivo. Nonetheless, the ability to control the connectivity pattern of the cells and the growth conditions are unmatched in any in vivo experimental situation, allowing one to examine fundamental issues related to formation and operation of a small neuronal network. The following are illustrations of some of the issues relevant to neuron network study that can be tackled in cultured neurons.

Methods

Experiments are conducted with hippocampal or cortical neurons, dissociated from the brain of 1–3 day old pups and grown on glass coverslips, which were precoated with a glial bed (as detailed elsewhere). Recording of spontaneous electrical activity of individual neurons is made under an inverted, phase contrast microscopy, with a patch pipette, while imaging of population neuronal activity is made using the calcium sensitive dye Fluo-4AM. For these latter experiments, a high spatial and temporal resolution CCD camera is used to facilitate the detection and correlation of activities among several adjacent neurons in a spontaneously active neuronal network. Individual neurons can be recorded and stimulated while imaging all adjacent neurons at high resolution to detect presence and strength of connectivity between them.

Recent Results

The Development of Network Activity

When plated in a dish, most young neurons begin to express spontaneous action potential discharges almost as soon as they get attached to the surface and begin to send out processes. Following plating, cells develop morphological connections rather rapidly, and within 2 days in culture they already express networks of synaptic markers. However, at this early age, electrical activity is rather sporadic and little synchronicity among neurons can be found (**Figure 1**). It takes another day in culture before synchronized activity can be seen. Over the first week in culture, more neurons are recruited into the network, resulting in fully synchronized activity across neurons, likely to span the entire culture plate and endure for the entire life of the culture.

In assessing the importance of different neurotransmitter systems in the emergence of network activity and its maintenance, cultures were exposed to drugs that block excitatory synapses (DNQX), inhibitory synapses (bicuculline), or total action potential discharges (TTX). As expected, TTX and DNQX block ongoing activity. The GABAergic receptor antagonist has a peculiar effect; at early culture stages (up to one week in culture), blockade of GABA receptors causes silencing of any detectable spontaneous network activity, indicating that GABA possesses an excitatory drive, as suggested by previous work in acute slices. However at one week in culture, blockade of GABAergic receptors causes an enhancement of the burst discharge, indicating that GABA receptors are becoming inhibitory at this stage in culture.

A related issue concerns the role of excitatory and inhibitory neurotransmitters in the development of network activity. As indicated already, spontaneous network activity emerges between 3 and 7 days in culture, and matures over the following week. Will the blockade of all activity, or of only GABAergic or glutamatergic receptors, disrupt the emergence or maturation of the synchronized activity? To address this question, we exposed the cultures to a chronic (3 days) blockade of activity/receptors (from the day of plating), and found that neither blockade of specific receptors nor of action potential

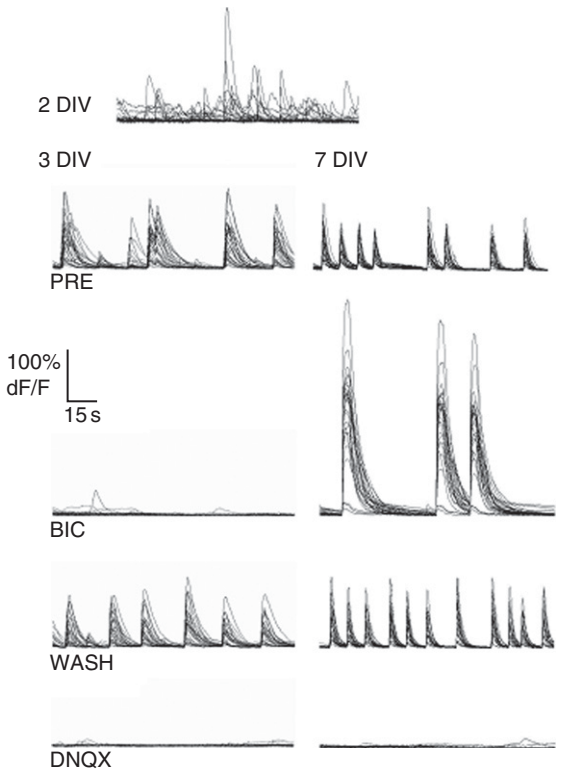


Figure 1 Development of network activity in culture. Cells were taken from the hippocampus of P1 pups, dissociated and grown in culture for 2–7 days. Activity of neurons in a 40× field of view of an inverted microscope was recorded using Fluo-4AM loaded neurons. Individual neurons were identified, and the variations in intracellular calcium concentrations, associated with action potential discharges, were recorded. In this and the following figures each line represents the calcium fluorescence in a single neuron, and the number of overlapping lines represent the number of cells in the field of view. At 2 days in vitro (2DIV) there is some spontaneous activity, but it is not synchronized. At 3 days in vitro, the activity is already partially synchronized, and each burst is long-lasting. At 7DIV more cells participate in a burst and each event is rather fast. Bicuculline, a GABA antagonist, blocks activity of a 3DIV culture, but enhances activity of a 7DIV culture. DNQX, a glutamate antagonist blocks network activity in both ages. Adapted from Cohen and Segal, in preparation.

generation prevented (or even delayed) the emergence of network activity. This result indicates that the initial growth of the dendrites/axons and the formation of functional connections are not dependent on ongoing activity of the neurons. The general issue of maturation of the activity is somewhat more complex, as the blockade of activity with TTX changes the morphology of the neurons, converts dendritic spines into long filopoda, and causes shrinkage of dendrites and eventual cell death. However, removal of TTX after a week of continuous blockade of activity allows spontaneous activity to reappear, as well as synchronization of the network (see later).

GABAergic interneurons play an important role in the synchronization of network activity and the timing of the individual bursts, as seen in vivo. The blockade of GABA receptors has been used traditionally, in many preparations, as a means for generating epileptic seizures, and indeed, blockade of GABAergic receptors produces a marked change in the behavior of the culture, in that bursts of activity are much larger and long-lasting than those of control cultures. However, synchronization persists in GABA-blocked cultures; in fact, the silent intervals between bursts become longer and more persistent. This observation suggests that other cellular mechanisms may assume control over the synchronization of activity when GABAergic transmission is blocked.

Generation and Spread of Activity

Two pertinent issues, important for the understanding the rules that govern network activity, concern the velocity of spread of the activity and the likelihood that activity is generated in only a small subset of neurons, To address these issues, we resorted to a one-dimensional culture that grows along a line in the culture dish. The advantage of such a network is that one can trace the origin of a burst of activity as well as the speed at which it spreads along the line, simplifying the more complex situation of a two-dimentional network where one cannot easily trace neither the origin of activity nor how it spreads. Imaging such network activity with Fluo-4 calcium sensitive dye (**Figure 2**), we were able to reach two important conclusions: First, network activity seems to originate in small, discrete regions along the length of the culture; these regions have a high probability of initiating bursts of activity. Although it is not yet clear what makes these 'trigger zones' so unique, it appears that the density of neurons in such a zone, as well as a high proportion of excitatory to inhibitory neurons, determine the likelihood that a burst will be initiated there. Second, the wave of activity spreads along the line of neurons in two modes: The first is a slow mode, which corresponds to slow synaptic connection, while the second is evident once the burst passes a local 'threshold,' after which it propagates rather rapidly. The speed of such a wave reflects both axonal and synaptic transmission along the line of neurons. The fast mode is within the range of the speed of transmission in typical unmyelinated fibers (**Figure 2**). The transmission of activity along the line of neurons in culture is also affected by local GABAergic inhibition such that GABA blockade speeds up the spread of the burst of activity.

Two more related experiments address the issue of how the network is 'driven' to produce spontaneous activity. The first study examines how many neurons in a network need to be interconnected in order to enable

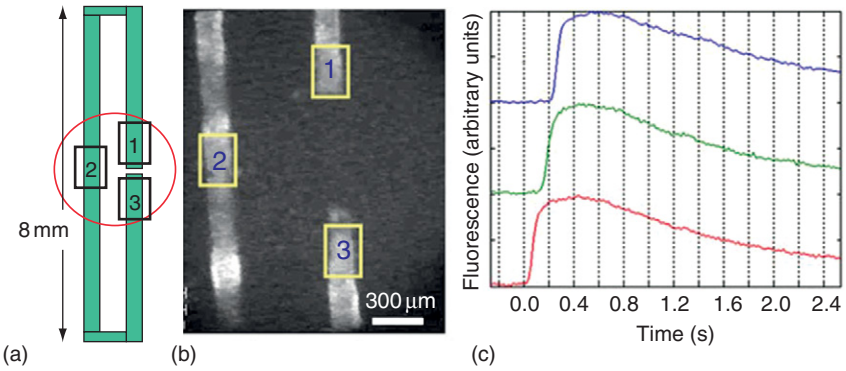


Figure 2 One-dimensional cultures used for the study of the origin and spread of network activity. (a). Illustration of a line of supportive material used to plate neurons, and the three regions of interest where activity, in the form of variations of calcium fluorescence, is recorded. (b) A close-up of these regions of interest. (c) A calcium transient recorded in regions 1–3, showing a distinct difference in latency to the event. The difference in threshold is used to calculate both the origin of the event and its spread. Adapted from Feinerman *et al*. (2007) Identification and dynamics of spontaneous burst initiation zones in unidimensional neuronal cultures. *Journal of Neurophysiology* 97: 2937–2948.

the expression of spontaneous synchronized activity. In other words, is there a 'critical mass' below which spontaneous activity will not be seen. The second study focuses on the question of whether a single neuron (rather than a mass of adjacent ones) can trigger a network burst? If so, what are the critical parameters needed for a neuron to be a 'master cell'? Furthermore, will the removal of this neuron affect the spontaneous activity of the rest of the network? These and other related questions are currently analyzed using small networks of neurons. Neurons are plated on islands of a glial bed, where the entire network can be visualized in the field of view of the microscope. The number of neurons in a small network varies between single cells and several hundreds of them. Spontaneous activity, as well as responses to spikes generated by depolarizing patch-clamped neurons, were visualized at high imaging frame rate of Fluo-4 dye loaded cells to enable the tracing of synaptic delays among the different cells in the field (**Figure 3**). The results can be tentatively summarized as follows: First, the larger the population of interconnected neurons, the more likely they are to express spontaneous network activity. The 'quality' of this network activity, expressed in the frequency of the burst activity and the magnitude of synchronicity among adjacent neurons, varies among the cultures – with the less dense cultures being more synchronized. Second, triggering a network event by stimulation of a single cell was negatively correlated with the number of neurons in the network. When the culture contains few neurons (10–100), the synaptic currents among them are large. In fact the largest synaptic currents are generated by autoptic synapses, in a single neuron culture (up to several µA, compared with 10–50 pA in a dense (10^5– 10^6 cells) culture. Thus, with few cells in a cluster, stimulation of one of them could evoke a large network burst, even if the network did not produce spontaneous events. With a larger network, synaptic currents between adjacent neurons are much smaller, and consequently, stimulation of one neuron could not trigger the generation of a network event.

The present series of studies indicate that spontaneous burst activity is an attribute of networks of neurons; that this, synchronous activity is an emergent property of the network that can appear even without 'training' of the network (i.e., in networks that were chronically silenced for several days after plating of dissociated neurons). Furthermore, the quality of the activity is correlated with the number of participating elements. We could not identify individual 'master' neurons that trigger activity to spread across the network; however, such a possibility cannot be ruled out. It is evident that small clusters of cells within the network can generate bursts of activity, which then spread across the entire network. Since our imaging method is not sensitive to synchronized subthreshold oscillations, we cannot rule out the possibility that such oscillations are responsible for the triggering of network discharge. This is subject for further explorations.

Network Activity – Kills or Heals

The dissociated culture can be used to address a fundamental question regarding the functional significance of network activity. On the one hand, neurons spend a great deal of energy to stay active in the absence of extrinsic afferent input. Given this situation, one would intuitively guess that spontaneous intrinsic activity has an instrumental role in maintaining survival of the neurons. On the other hand, small networks or even single neurons that do not express spontaneous network activity can be maintained for long periods of time in culture – so the significance of activity is not self evident. Enhancement of network activity – e.g., seizure activity that take place following blockade of

GABAergic synapses – leads to cell death both in vivo and in cultured hippocampal slices. Will enhanced activity of cells in dissociated culture result in neuronal death, and will cessation of activity salvage the hyperactive neurons?

To address these issues we exposed cultures to TTX for 1–2 weeks, and found that blockade of activity caused massive degeneration of the cultured neurons (**Figure 4**). In contrast, enhanced activity caused by blockade of

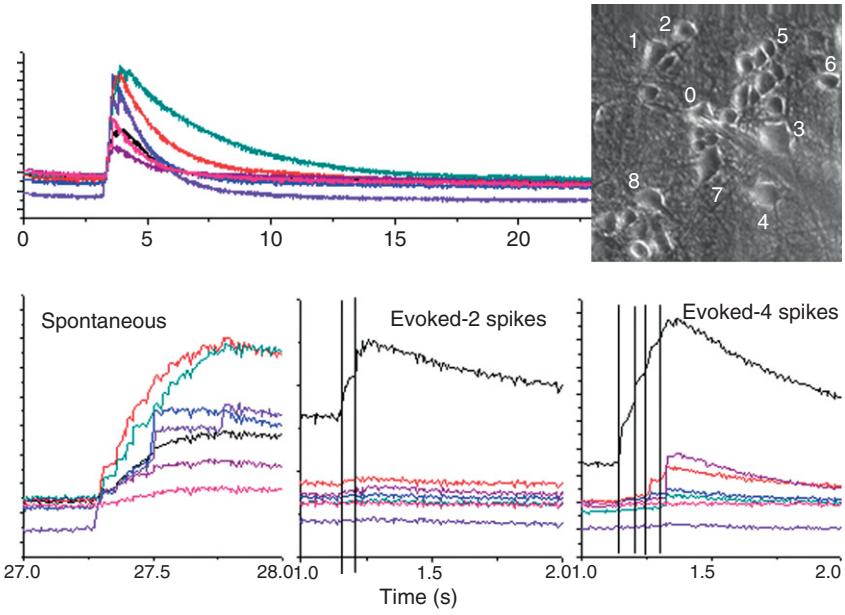


Figure 3 Calcium transients recorded at high resolution (200 Hz) of a population of neurons, in an attempt to identify threshold differences and to outline the spread of signals from one cell to another. Top image is a sample illustration of such a burst of activity, and a picture on the right illustrates the cells imaged and their sequence of firing. Below are one spontaneous event (left) and two events where one cell was recorded (illustrated above, patch in cell0) and stimulated to produce an action potential. Two action potentials did not evoke synaptic responses in the adjacent neurons, and 4 spikes (bottom right) produced a small response, but not a large synchronized burst, as seen in the spontaneous case (left). Adapted from Segal, unpublished observations.

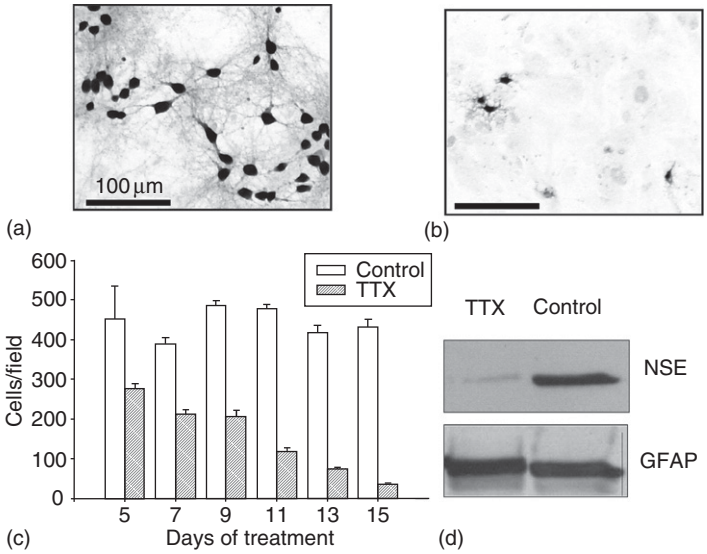


Figure 4 Tetrodotoxin kills neurons in culture, in a slow process. Cultured cortical neurons in control (a) and in TTX-treated culture (b). (c) The number of viable neurons is going down over 2 weeks of exposure to TTX, compared with controls. (d) Western blots to show that neuron specific enolase is drastically reduced, while GFAP, a glia marker, does not change. Adapted from Fishbein and Segal (2007) Miniature synaptic currents become neurotoxic to chronically silenced neurons. *Cerebral Cortex* 17: 1292–1306.

GABAergic receptors did not cause a significant cell loss. In an analysis of the causes of the cell death following secession of activity, we found that exposure to TTX caused a twofold increase in magnitude of miniature synaptic currents. Surprisingly, TTX-induced cell death was reversed by the addition of the AMPA receptor antagonist, DNQX, indicating that residual nonsynchronized activity (i.e., glutamate release in the absence of action potential discharge) is responsible for the cell death. Although we still analyze this unique action, it is obvious that network activity protects neurons from massive cell death, in the absence of supplementary growth factors and/or conditioning media.

Why is it that dissociated cultured neurons do not die during enhanced synchronized activity (epileptiform bursting discharge) caused by blockade of GABAergic receptors? There is no clear answer to this question. In fact, it is not entirely clear why neurons do die in other situations (e.g., via 'excitotoxicity'). After all, enhanced activity is supposed to cause a rather fast reduction in excitatory synaptic strength through a homeostatic mechanism. It is, of course, possible that this homeostatic plasticity is not sufficient to prevent continuous depolarization and influx of calcium in vivo, as hypothesized by some to underlie neuronal cell death. Other investigators have demonstrated that enhanced spontaneous activity in culture can last for hours, without the 'accommodation' expected from a homeostatic reduction in excitatory drive. One possibility is that neurons do not die as an immediate consequence of an increase in firing, or as a consequence of the depolarization leading to influx of calcium, but rather due to a downstream effect – e.g., an increase in reactive oxygen species. That latter change may not take place in cultured neurons because of the large extracellular space surrounding the neurons, which prevents the accumulation of hazardous diffusible chemicals in the neurons' immediate vicinity – unlike the neurons in vivo or in a slice, where the cells are densely packed and diffusion is slow.

Summary and Future Goals

Regardless of the possible causes of cell death in epilepsy, it is quite obvious that network activity per se supports survival of neurons, and that such activity can be maintained at high rates for months in dissociated cultured neurons that are deprived of extrinsic afferents. The rules that govern the generation and spread of network activity are being unveiled in these simple systems and are expected to shed light on similar, more complex systems of the intact brain. Future goals of this research include the identification of possible 'leader neurons' and the characterization of their biophysical, biochemical, and morphological properties. The use of more advanced molecular and imaging tools will allow investigators to activate and/or suppress selective neurons in a network, and to examine in more detail their effects on network properties. The role of trophic factors in the development and maintenance of network activity, and the balance of their effects on electrical activity and cell survival and elimination, should also be clarified. Finally, the sequence of molecular events leading to a slow, activity-regulated cell death, as well as the means for protecting cells from death, should be clarified.

See also: **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; **Glia/Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters; **Glutamate**: Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain; **Imaging**: Optical Monitoring of Circuitry Activity In vitro; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures.

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Interactions between Cortico-Thalamo-Cortical and Limbic Seizures

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Introduction

The co-existence of temporal lobe seizures in a patient with juvenile or childhood absence epilepsy is uncommon. This co-existence was reported to occur in <1% of the population with idiopathic generalized epilepsy. These clinical observations raise questions about how absence epilepsy and temporal lobe epilepsy might interact with each other so as to be mutually exclusive.

In line with these clinical observations, pharmacotherapy of given forms of epilepsy also suggests that cellular mechanisms of absence seizures differ from those of limbic focal seizures. Phenytoin, carbamazepin, oxcarbazepin, vigabatrin, and tiagabin are highly effective anticonvulsant agents in partial and/or secondary generalized focal seizures, whereas these agents may worsen typical absence and myoclonic seizures, and may trigger status epilepticus in patients with absence epilepsy.

Background

Increased basal levels of GABA have been demonstrated in the primary motor area of the cortex and in the ventrolateral thalamus of genetic absence epilepsy rats from Strasbourg (GAERS) when compared with those of non-epileptic Wistar controls. Further, the injection of $GABA_A$ or $GABA_B$ receptor agonists, and GABAergic drugs such as γ-vinyl GABA (an inhibitor of GABA transaminase) has been shown to increase the duration of spike-and-wave discharges in animal models of absence epilepsy as do drugs that increase GABAergic tonus in human absence patients. The exacerbation of absence seizures by excessive GABA-mediated activity is consistent with the hypothesis that this type of seizure is due to an imbalance between inhibition and excitation, with an abnormally strong inhibitory component. In contrast, neuropathological alterations in mesial temporal lobe epilepsy are associated with a relative increase in excitation, with an increased excitatory synaptic drive onto neurons and hyperexcitability of hippocampal cells. Enhanced glutamatergic activity is a main component of the hyperexcitability characteristic of epileptic discharge; cellular hyperexcitability of the hippocampal formation is one of the main processes underlying the progression of epileptogenesis in mesial temporal lobe epilepsy. For example, investigators have demonstrated that following a kindled seizure, there is a prolongation of *N*-methyl-D-aspartate (NMDA) channel openings in granule cells and enhanced NMDA receptor-mediated synaptic excitability for at least 60 days.

Taken together, the clinical, pharmacological and pathophysiological data suggest a critical difference in underlying mechanisms between mesial temporal lobe epilepsy and absence epilepsy. To show if or how these different seizure types might co-exist (or be mutually exclusive) it would be useful to have an animal paradigm. Studying such a paradigm could help elucidate interactions of different types of seizures in many clinical phenotypes, and help us understand the distinct mechanistic differences between focal secondary generalized convulsive and generalized non-convulsive seizures. Amygdala

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kindling (with repetitive electrical stimulations) provides an animal model of focal epilepsy with secondary generalized limbic seizures; it is a well studied model of clinical mesial temporal lob seizures. Genetic absence epilepsy rat models, such as GAERS and Wistar Albino Glaxo Rats from Rijswijk (WAG/Rij), are among the best representatives of the human condition of typical absence epilepsy. We have investigated the course of kindling – an experimental model of temporal lobe epilepsy – in two well-validated genetic models of absence epilepsy: the GAERS, and the WAG/Rij. In addition, the effect of kindling on spike-and-wave discharges in these rat models was studied, the age-related effects of the kindling paradigm was investigated (conducting kindling in 30-day-old GAERS), and the effect of ethosuximide (a specific anti-absence agent) on kindling resistance was assessed in adult GAERS.

Methods

In the first group of experiments, we used adult non-epileptic control Wistar, GAERS, and WAG/Rij animals. Animals were equipped with stimulating/recording electrodes in the basolateral nucleus of the amygdala and recording electrodes in the fronto-parietal cortex bilaterally. After a recovery period of one week, animals were stimulated at their afterdischarge thresholds two times a day. The kindling stimulus, a rectangular current of 1 ms duration, at 60–80 Hz, was applied for 1–2 s. The EEG from amygdala and cortex were recorded continuously before and after the each stimulus. Racine's 5-stage scale was used for rating the behavioral development of kindling: stage 1, facial movements; stage 2, rhythmic head movements, head nodding; stage 3, unilateral forelimb clonus; stage 4, bilateral forelimb clonus and rearing; stage 5, bilateral forelimb clonus, loss of balance, and falling. Stages 1 and 2 are associated with focal limbic seizures, and stage 3, 4, and 5 are viewed as manifestations of generalized motor seizures. Animals showing two stage 5 seizures were accepted as kindled. If the animal did not reach stage 5 following the maximum number of 30 stimulations determined by the experimental protocol, electrical stimulation was terminated and the rat was labeled as 'kindling-resistant.'

In the second group of kindling experiments, adult GAERS were pretreated with intraperitoneal administration of ethosuximide (50 mg per kg, i.p.), which is known as a pure anti-absence drug and is not effective against kindling. The ethosuximide application was started after the determination of initial afterdischarge threshold on the first day of the experiment, and was then given 1 h before the electrical kindling stimulation. The effect of ethosuximide on the suppression of spike-and-wave activity was monitored from the cortical EEG.

In the third group, we applied kindling stimulations to postnatal 30-day-old GAERS. Spike-and-wave discharges in GAERS are first detected at about 30-days of age, and increased gradually with age to reach 100% at 3 months. The number and frequency of spike-and-wave discharges, and the duration of each complex, increased with age. Hence, we hypothesized that the immature animals – with immature spike-and-wave EEG activity – would provide an intriguing tool for investigating the effects on limbic kindling seizures on the development of thalamo-cortical activity (i.e., for the development of spike-and-wave generation). In these experiments, we used a kindling stimulus of 400 microA, 80 Hz alternate current, 1 ms pulse duration, 2 s train duration. The kindling stimulations were carried out at 90-min intervals (a maximum of 6 stimulations per day) in 30-day-old animals so that the experiments could be completed within a few days and thus avoid technical problems due to the ongoing head growth.

At the end of all three experiments, animals were killed and the electrode placements were checked for correct localization in the basolateral amygdala.

Recent Results

Kindling in Adult GAERS and WAG/Rij Animals

The afterdischarge thresholds of the groups were: 129 ± 7.1 μA in GAERS, 125 ± 17.1 μA in WAG/Rij, and 102 ± 14.2 μA in Wistar rats; there were no statistical differences among the groups ($P > 0.05$). The numbers of stimulations to reach stage 2 were comparable across groups, suggesting that the induction of the kindling process and of the limbic seizures was not different for the genetic absence epileptic and non-epileptic control animals. Interestingly, however, all GAERS and 3 of 12 WAG/Rij animals showed a resistance to the development of full kindling; they did not progress beyond stage 2 (**Figure 1**). Since they did not show any stage 3, 4, or 5 seizures when they were stimulated at their afterdischarge threshold, they were referred to as the kindling-resistant group; since all Wistar control animals and a group of WAG/Rij animals reached stage 5 and became kindled, they were referred to as kindling-prone. Importantly, once a WAG/Rij rat showed a stage 3 or 4 seizure, it quickly progressed to stage 5.

Our observations indicate that there is a resistance to generalization of limbic seizures in the absence epilepsy groups. This resistance was negatively correlated with the intensity (duration and number) of spike-and-wave discharges present on the baseline EEG of the GAERS and WAG/Rij animals. That is, GAERS and WAG/Rij rats having the highest number of spike-and-wave discharge in baseline EEG (before the kindling experiment started) were the least susceptible to kindling (**Figure 2**).

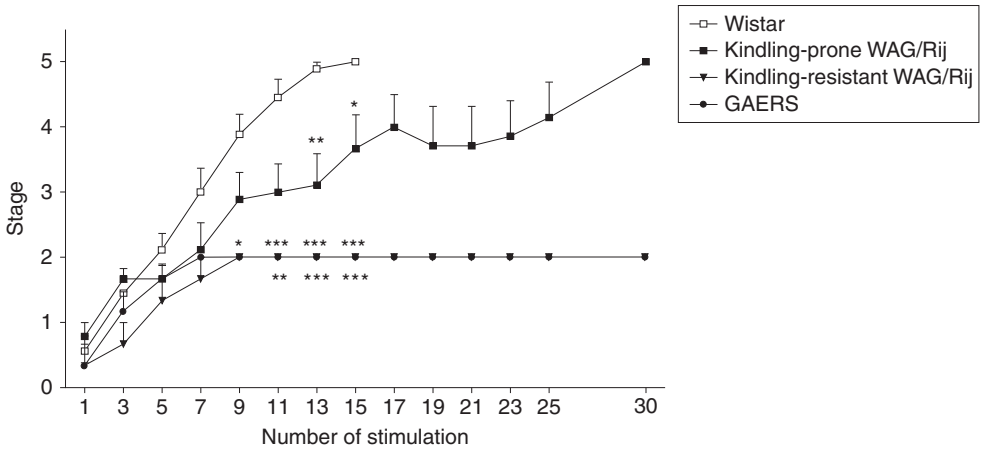


Figure 1 Seizure stages in non-epileptic Wistar control (n = 9), kindling-prone WAG/Rij (n = 9), kindling-resistant WAG/Rij (n = 3), and GAERS (n = 6) rats. Data are expressed as mean ± SEM. Two-way analysis of variance was performed for the first 15 stimulations of the groups because all the animals in the control Wistar group reached stage 5 seizures and were not stimulated beyond the 15th stimulation. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, significant differences between Wistar control and other groups.

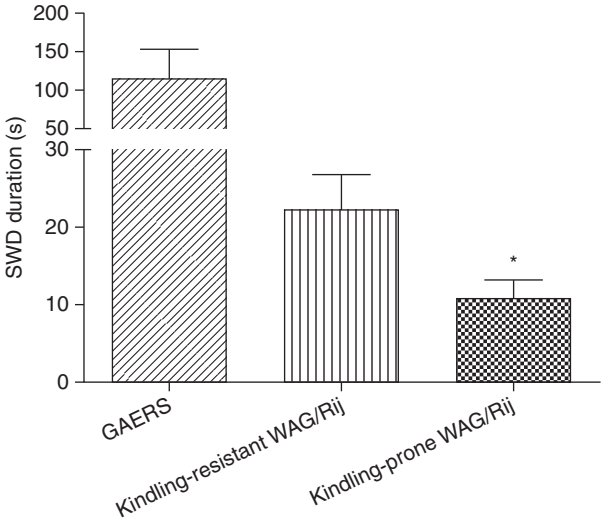


Figure 2 Basal spike-and-wave discharge (SWD) duration of GAERS (n = 6), kindling-resistant WAG/Rij (n = 3), kindling-prone WAG/Rij (n = 9) rats. Data are expressed as mean ± SEM of first five stimulations. *, $p < 0.05$, significant difference between GAERS and kindling-prone WAG/Rij. Modified from Onat *et al.* (2007) *Epilepsia* 48 (Suppl. 5): 150–156, with permission.

Other findings, such as decreased density of glutamate immunolabeling in mossy fiber terminals in the hilar region of GAERS compared to control rats, and reduced number of spike-and-wave discharges after intrahippocampal administration of tiagabine (an inhibitor of GABA reuptake) in the CA1–CA3 region of the dorsal hippocampus, also suggest that there is some limbic involvement in absence epilepsy. These results are consistent with imaging studies in these genetic models of absence, showing hippocampal activation associated with spike-and-wave discharge.

Kindling in Adult GAERS Pretreated with Ethosuximide

Since the resistance to kindling in absence epileptic animals showed a significant negative correlation ($r = -0.789$; $p < 0.001$) with spike-and-wave activity, we treated GAERS with ethosuximide (50 mg per kg, i.p.), an anti-absence drug, to see if this resistance could be broken-down by the pharmacological suppression of spike-and-wave activity. Ethosuximide effectively suppressed spike-and-wave activity in the EEG. Further, GAERS pretreated with ethosuximide progressed to stage 3 or 4, showing a partial drug effect on kindling resistance. Ethosuximide also delayed the development of kindling in Wistar control animals (**Figure 3**).

Kindling in 30-Day Old GAERS

We compared the resistance to amygdala kindling in 30-day-old non-epileptic Wistar rats vs. 30-day-old GAERS (an age when the first spike-and-wave discharges are observed) (**Figure 4**). All of the non-epileptic 30-day-old Wistar rats reached the stage 5 seizure state by the 18th stimulation. Of nine 30-day-old GAERS rats, seven animals reached stage 5 seizures by the 33th stimulation; two failed to reach stage 3, 4, or 5 seizures and stayed at stage 2, even after the application of 36 stimulations (**Figure 5(a)**). Statistical analysis showed a significant difference between the kindling rates of non-epileptic Wistar and the kindling-prone subgroup of GAERS ($p < 0.001$). The mean number of stimulations needed for the development of first stage 5 generalized seizure was 14.9 ± 1 for the non-epileptic Wistars and 29.6 ± 1.1 for the kindling-prone subgroup of GAERS. Statistical analysis revealed a significant difference in the mean number of stimulations for the development of

the first stage 3, 4, and 5 seizures between the non-epileptic control and the kindling-prone subgroup of GAERS rat groups ($p < 0.001$) (Figure 5(b)). No significant difference was found between the afterdischarge durations of 30-day-old GAERS and non-epileptic Wistars.

These findings suggest that resistance to kindling may be related to the existence of absence epilepsy and changes in the circuits that are responsible for the production of spike-and-wave activity. Taken together, the evidence indicates that pre-existing absence activity inhibits secondary generalization of the kindling progress in GAERS and the kindling-resistant WAG/Rij, and significantly retards kindling in the kindling-prone subpopulation of WAG/Rij animals. Absence activity appear to have an antagonistic effect on generalization of limbic seizures, suggesting an opposition between the mechanisms underlying limbic epilepsy and absence epilepsy (at least in our animal models). Interestingly, bilateral synchronization of the hippocampi during spike-and-wave discharges has recently been demonstrated in chemical absence epilepsy model in rat, showing a potential interplay between hippocampal (TLE-associated) circuits and those involved in absence seizure generation.

We have also shown that the spike-and-wave activity increases immediately after a kindling stimulation (i.e., during the 10 min poststimulation period) in kindling-prone WAG/Rij animals (Figure 6). This observation suggests that the kindling stimulations cause an activation of cortico-thalamo-cortical circuitry that is responsible for the production and maintenance of spike-and-wave activity. These results also suggest a reciprocal interaction between the secondary generalization of limbic seizures (e.g., during stage 3 kindling) and absence epilepsy.

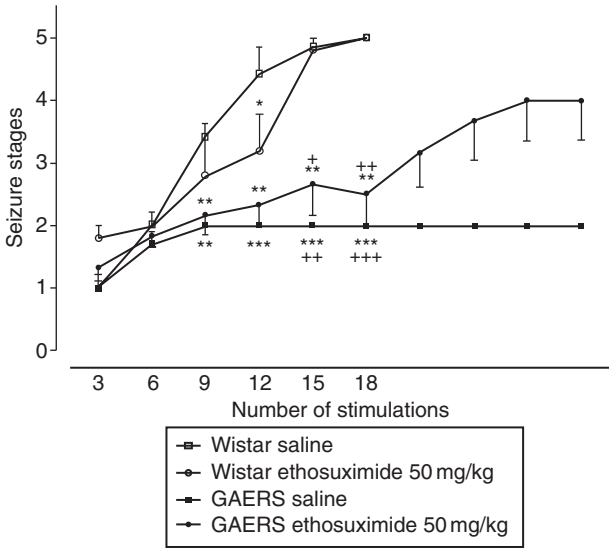


Figure 3 The effect of repeated kindling stimulations on seizure stage in Wistar and GAERS pretreated with saline or 50 mg per kg ethosuximide i.p. before each stimulation. Data are expressed as mean ± SEM. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) indicate significant differences when compared with Wistar saline group; +($p < 0.05$), ++($p < 0.01$), +++($p < 0.001$) indicate significant differences when compared with Wistar ethosuximide 50 mg per kg pretreatment group with two-way analysis of variance fallowed by post hoc Bonferroni test.

Future Goals

These findings all point to interactions between the cortico-thalamo-cortical and the limbic circuits, but do not show how these interactions occur or in which parts of the brain the kindling resistance acts. Thus, there is a need to investigate further the links between absence epilepsy and limbic circuitry. One possibility link deserving attention is connection between the amygdala and the limbic nuclei of the thalamus (such as the mediodorsal, centromedial, and anterior nuclei). These nuclei have two-way connections to the rostral part of the thalamic reticular nucleus and to the limbic part of the reticular nucleus. It has recently been shown that this part of the thalamic reticular nucleus also receives hippocampal input. Thus, the thalamic reticular nucleus can receive excitatory input through its limbic connections from the amygdala, enthorhinal, and piriform cortices during kindling, and in turn might influence thalamo-cortical activity. These connections of the rostral thalamic reticular nucleus with limbic structures, through the limbic thalamic nuclei, may be particularly relevant for understanding the resistance to secondary generalized convulsive seizures in the genetic absence epilepsy models, or

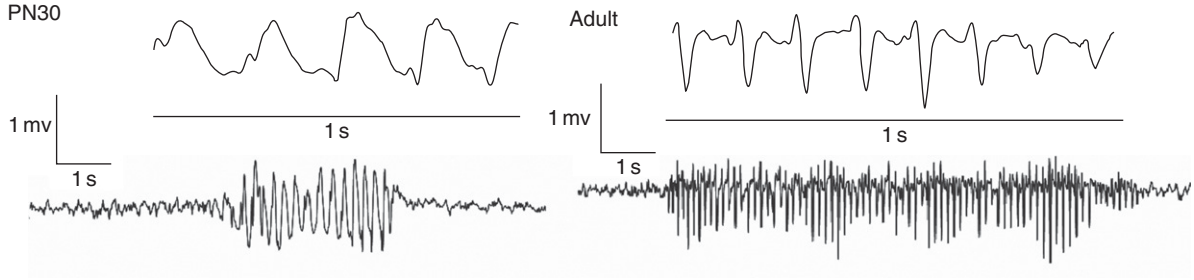


Figure 4 Ontogeny of spike-and-wave discharges (SWD) in GAERS. Representative examples of basal EEG of 30 day-old (PN30) and adult GAERS showing pattern of SWD.

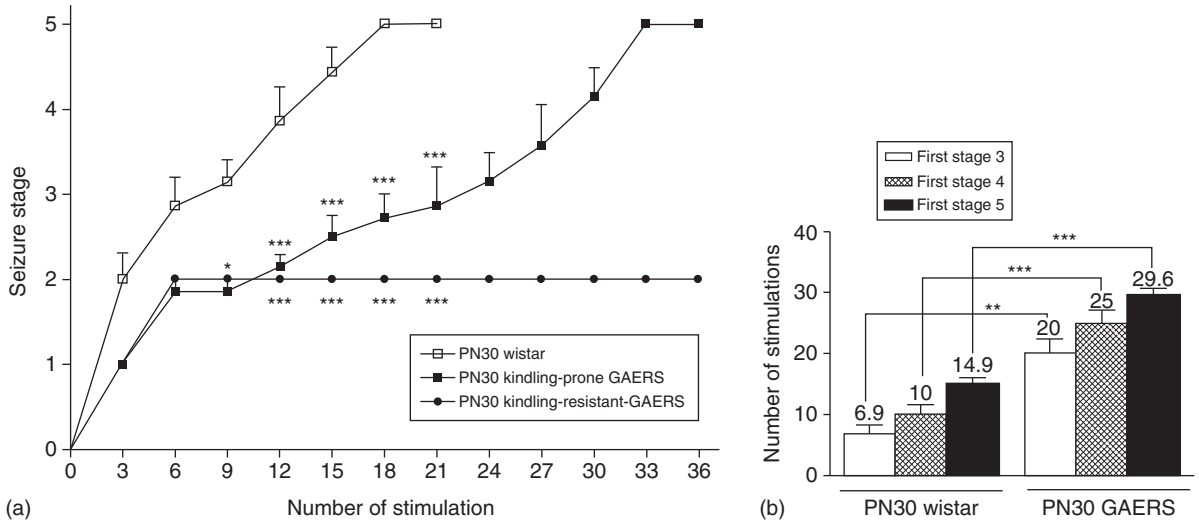


Figure 5 Seizure stage (a) and the mean number of stimulations for the development of first 3, 4, and 5 seizure stage (b) in 30-day-old (PN30) non-epileptic Wistar control (n = 7), PN30 kindling-prone GAERS (n = 7), PN30 kindling-resistant GAERS (n = 2). Data are expressed as mean ± SEM. Two-way analysis of variance was performed for the first 21 stimulations. $p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significant differences between Wistar and GAERS groups.

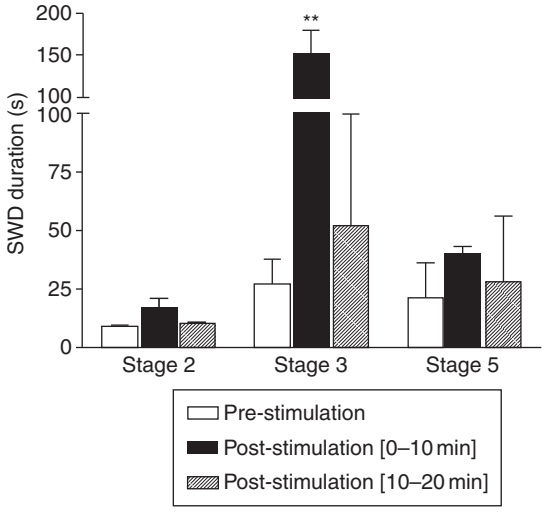


Figure 6 The cumulative total duration of spike-and-wave discharges (SWD) at the first stage 2, 3, and 5 in the prestimulation and poststimulation periods in kindling-resistant groups. Data are expressed as mean ± SEM. $^{**}p < 0.01$, significant difference between prestimulation and poststimulation periods within the same stimulation interval compared with repeated measures of one-way ANOVA followed by post hoc Dunnett test. Modified from Onat *et al*. (2007) *Epilepsia* 48(Suppl. 5): 150–156, with permission.

elucidating the increases in spike-and-wave discharges induced by kindling.

Another possibility to explain this kindling resistance is the propagation of limbic seizures to cortical structures such as the parietal cortex. The spread of seizure activity to the somatosensory part of the parietal cortex would trigger cortico-thalamo-cortical activity, leading to an increase spike-and-wave activity in the poststimulation period. This cortical activation then could limit motor manifestations of limbic seizures through activation of the basal ganglia. A study of the role played by the limbic nuclei of the thalamus or by the basal ganglia in the kindling-resistance of genetic absence epilepsy models may prove to be an important study for the future.

See also: **Absence Seizures**: Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Network Interactions in the Limbic System and Epileptiform Synchronization; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures.

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Network Interactions in the Limbic System and Epileptiform Synchronization

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Introduction

Limbic neuronal circuits are involved in normal brain functions (e.g., memory and learning) and in pathological processes such as the seizures occurring in TLE, a common form of partial epilepsy. Temporal lobe epilepsy is associated with a pattern of brain damage known as mesial temporal (also termed *Ammon's horn*) sclerosis that may significantly alter the modes of interaction among limbic areas. The sclerotic hippocampus presents with almost complete loss of CA1 neurons (the Sommer sector), especially at the boundary with the subiculum, as well as disappearance of hilar cells in the dentate gyrus (endfolium sclerosis). Neuronal cell loss is also found in the medial EC (especially in layer III) and in the amygdala. Recent evidence obtained from studies performed in animal models mimicking TLE suggests that the pattern of neuronal damage found in Ammon's horn sclerosis cuts off the normal physiological route of hippocampal network activity (the trisynaptic hippocampal pathway), thus reinforcing the activity of direct connections between EC-CA1 (when spared) and EC-subiculum. These findings have challenged the more traditional view that has emphasized the role played by dentate gyrus damage and consequent mossy fiber sprouting in epileptogenesis. Understanding the pathophysiogenesis of TLE remains an important challenge in epilepsy research.

Background

The parahippocampal region conveys information of neocortical origin into the hippocampus and redistributes back projections from the hippocampus into the temporal neocortex. The parahippocampal region includes the perirhinal/postrhinal cortex (PC), which borders the temporal neocortex, the EC interposed between the PC and the hippocampus proper (that is composed by the CA1, CA2, and CA3 subfields), and the subicular complex (in turn, consisting of pre- and parasubiculum and subiculum – although some authors consider the subiculum to be part of the hippocampus proper since it represents the most lateral extension of the CA1 region). The reciprocal connections between parahippocampal cortices, hippocampus, and temporal neocortex are highly preserved phylogenetically.

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proper have been analyzed with electrophysiological techniques both in vivo and in vitro. Neurons in layers II and III of the EC target different subfields of the hippocampus, with different species specificity: In the rat and monkey, layer II connects the EC to the dentate gyrus and CA3, while layer III projects to CA1 and subiculum; however, in the mouse, EC layer II sends terminals to dentate gyrus only and the hippocampus is innervated by layer III. PC and EC are strictly and reciprocally interconnected.

Depth electroencephalographic (EEG) recordings in TLE patients have shown that epileptic discharges are recorded from the hippocampus and parahippocampal areas. Two different types of interaction between the hippocampus and EC have been identified. The first type is characterized by coupling between the EC and hippocampus, in which the latter is the 'leading' (i.e., seizure-initiating) structure. In this pattern of epileptic activity, seizures are initiated by low-frequency (<2 Hz) spikes – generated in the hippocampus, not in the EC – that are subsequently followed by fast activity. The second type of interaction consists of tonic discharges without preictal spiking, which appears simultaneously in the hippocampus and EC; in this type of activity, the EC more frequently takes the lead. Studies in animal models have demonstrated that preictal (defined as *interictal* in these studies) spiking activity can precede the ictal discharge. However, in these models, low-frequency stimulation that induces spiking activity was found to control, rather than to trigger, tonic (i.e., ictal) discharges. We will consider here these findings by addressing three main issues: (i) the contribution and the interaction of different limbic neuronal networks to epileptiform synchronization, (ii) the relationship between interictal and ictal activities, and (iii) the roles of subiculum and PC in controlling the spread of epileptiform discharges.

Methods

For studies on the whole isolated guinea pig brain, adult Hartley guinea pigs (150–200 g) were used according to standard procedures (see DeCurtis, this volume). Following anesthesia with sodium thiopental (125 mg kg^{-1} i.p.) and transcardial perfusion with a cold (10 °C) carboxygenated (95% O_2, 5% CO_2) saline solution (composition: 126 mM NaCl, 3 mM KCl, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, 2.4 mM $CaCl_2$, 26 mM $NaHCO_3$, 15 mM glucose, 2.1 mM HEPES, and 3% dextran M.W. 70 000, pH = 7.1), brains were dissected out, transferred into the recording chamber, and perfused with the saline solution (15 °C, pH = 7.3) via a cannula inserted into the basilar artery. Brain temperature was slowly raised to 32 °C to perform electrophysiological recordings that were obtained from different hippocampal–parahippocampal subfields with micropipettes or 16-channel silicon probes, positioned under direct visual control. Experiments in brain slices maintained in vitro were made according to standard procedures. Epileptiform activity was induced by drugs such as bicuculline or 4-aminopyridine, dissolved in the perfusate and applied via the resident arterial system (whole brain experiments) or to the bath (slices experiments).

Results

Region-Specific Propensity to Epileptiform Synchronization

Field recordings obtained from extended brain slice preparations (which include interconnected limbic structures, **Figure 1(a)**) have demonstrated the occurrence of several types of synchronous events during application of artificial cerebrospinal fluid containing convulsant drugs (such as 4-aminopyridine), nominally zero Mg^{2+}, or cholinergic agents (such as pilocarpine). The most impressive pattern (**Figure 1**, continuous lines in (a) and (b)) consists of a prolonged discharge – lasting several seconds – that is reminiscent of the EEG activity seen during a seizure in vivo (therefore, referred to as 'ictal discharge' in this article). This discharge is initiated by EC, PC, or amygdala networks. Other types of spontaneous epileptiform events have durations shorter than few seconds, thus resembling the interictal discharges occurring in epileptic patients between seizures. In these in vitro models of epilepsy, one type of interictal discharge (arrows in **Figure 1(a)** and **1(b)**) occurs at short intervals and is often confined to the hippocampus proper, where it originates in the CA3 area; the other type of interictal activity (asterisks in **Figure 1(a)** and **1(b)**) has a slower rate of occurrence, is recorded from all limbic areas, and often initiates outside the hippocampus. These two types of discharges will be referred to as 'CA3-driven' and 'slow' interictal discharge, respectively.

Ionotropic glutamatergic receptor-mediated mechanisms contribute to the interictal and ictal activity recorded from limbic networks in vitro. In addition, the generation of ictal events usually depends on NMDA receptor activation; in the EC, ictal generation appears to reside in the deep layers, in which a significant loss of interneurons occurs. Paradoxically, $GABA_A$ receptor-mediated conductances may also be required for the occurrence of ictal discharges (as shown in the 4-aminopyridine as well as in other models of epileptiform synchronization), suggesting that interneuronal networks actively participate in the initiation and the spreading of electrographic seizures.

Ictal–Interictal Relationships

Evidence emerging from in vitro studies indicates that interictal discharges can have both anti- and proseizure

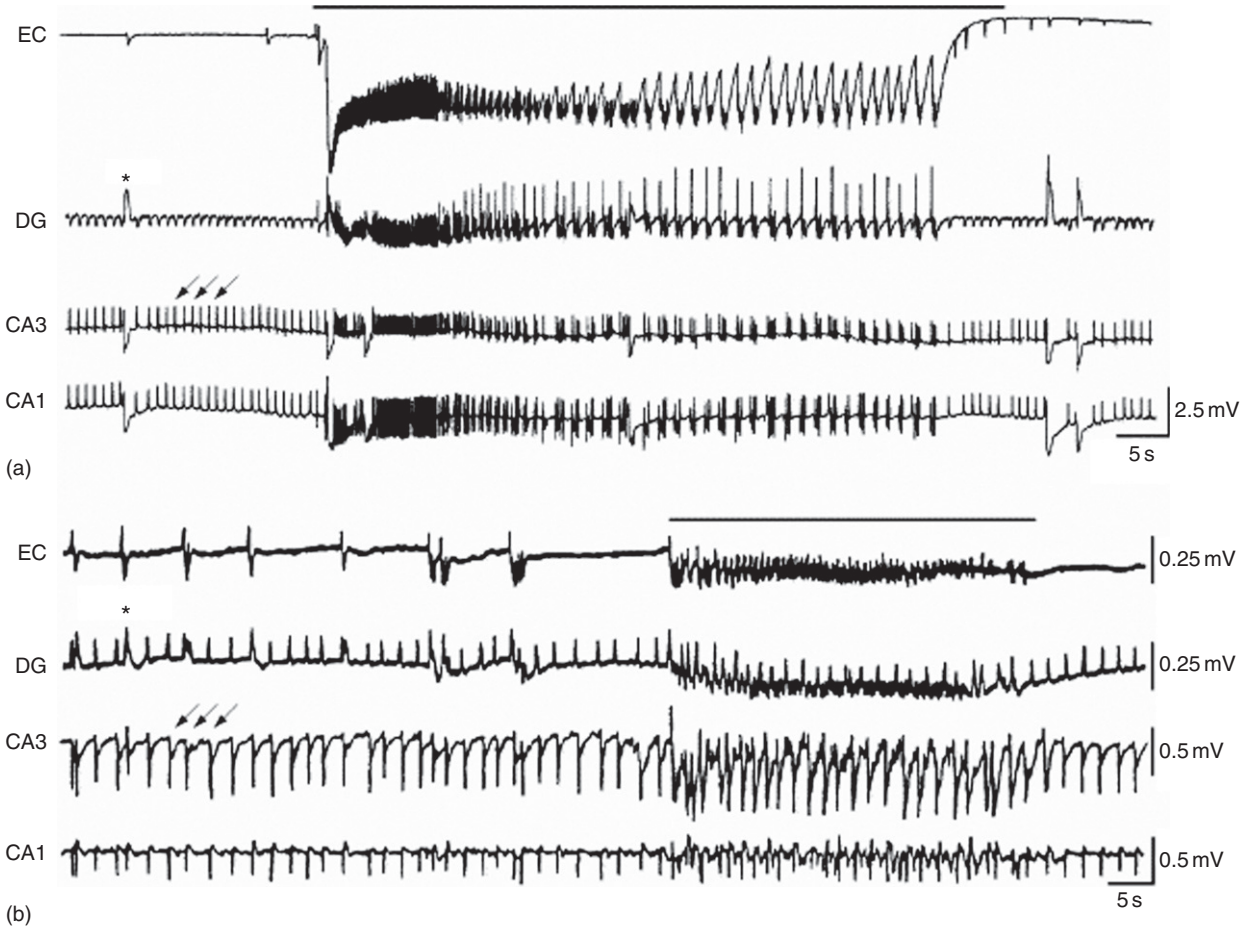


Figure 1 Synchronous epileptiform activities induced in hippocampus–entorhinal cortex slices that were obtained from adult rats during bath application of 4-aminopyridine (4-AP; 50 μM, (a)) or pilocarpine (10 μM, (b)). Note that in both cases three different types of discharges are evident. First, a continuous pattern of interictal discharges (arrows) that are confined to the hippocampus proper (note that their presence in the dentate gyrus reflects volume conduction of the signal generated by the CA3 network). Second, a slower type of interictal discharge that is seen in entorhinal cortex, dentate gyrus, and CA3 and CA1 subfields (asterisks). Third, a long-lasting discharge that resembles an ictal event and is recorded in all areas of the slice as well (continuous lines). EC, entorhinal cortex; DG, dentate gyrus. Reproduced from Avoli M, D'Antuono M, Louvel J, *et al*. (2002) Network and pharmacological mechanisms leading to epileptiform synchronization in the limbic system in vitro. *Progress in Neurobiology* 68: 167–207.

actions. As shown in **Figure 2(a)**, ictal discharges disappear in these brain slices within 1–2 h of treatment with proepileptic agents, while the CA3-driven interictal activity occurs throughout the experiment. Moreover, ictal discharges can be reestablished in these slices following a cut of the Schaffer collaterals, which connect CA3 to CA1. Transection of the Schaffer collaterals abolishes the propagation of CA3-driven interictal events to the EC (where ictal events are initiated) (**Figure 2(b)**). These experiments therefore demonstrate that interictal activity originating from the hippocampus proper can control, rather than sustain, the ability of EC networks to generate ictal events. Similar evidence has been obtained from brain slices that included the PC, the amygdala, or the insular cortex; in these experiments, CA3-driven interictal spikes prevented ictal generation in these other brain areas.

Further support for the view that CA3-driven interictal activity exerts antiseizure actions stems from experiments in which ictal discharges recorded in parahippocampal areas, following Schaffer collateral cut or surgical separation from the hippocampus, could be blocked by electrical stimuli at rates that are similar to those of the CA3-driven interictal activity (i.e., ~1 Hz). In addition, an inverse relationship between ictal and interictal discharges is seen in hippocampal slices when using pharmacological procedures (such as baclofen application) that depress interictal events. Epileptic rats and mice present with a significantly decreased frequency of interictal spikes. Finally, it has been reported in the in vitro isolated guinea pig brain that periodic interictal spiking in the piriform cortex prevents the involvement of this region in seizure-like (ictal) activity generated in the EC-hippocampus.

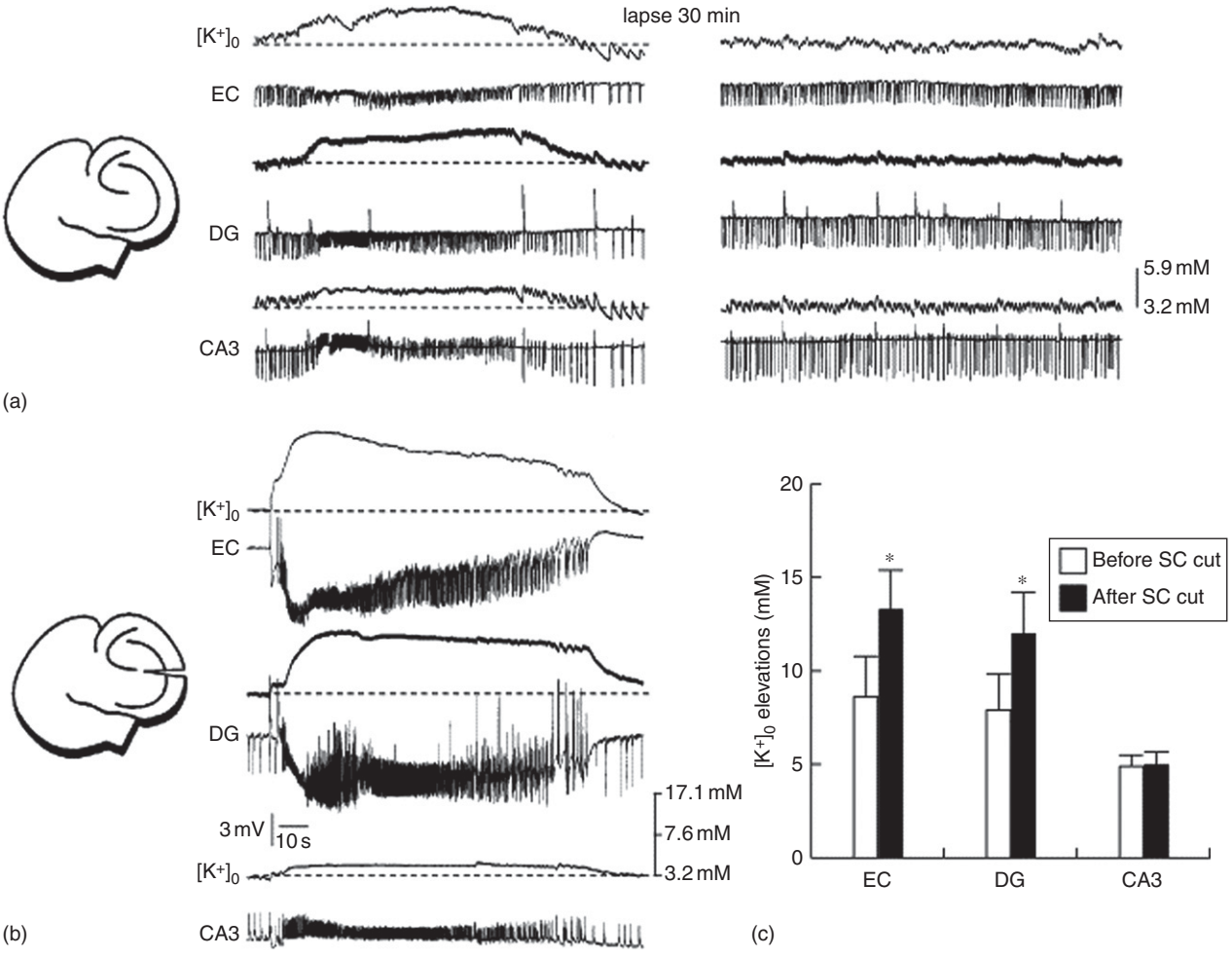


Figure 2 Effects induced by cutting the Schaffer collaterals on the 4-AP-induced epileptiform discharges and the associated $[K^+]_o$ rises in the EC, dentate, and CA3 areas. (a) Field and $[K^+]_o$ measurements of 4-AP-induced epileptiform activity in the intact hippocampus-EC slice before (left panel) and after (right panel) ictal discharges stop occurring. (b) After Schaffer collateral cut, ictal discharges are disclosed and their associated rises in $[K^+]_o$ are larger than prior to Schaffer collateral cut in the dentate gyrus and EC. Note the different calibrations of the $[K^+]_o$ before and after the cut. (c) Summary of effects of Schaffer collateral cut on $[K^+]_o$ rises associated with ictal activity in different limbic areas. Schaffer collateral cut results in both dentate and EC sectors in significant ($P < 0.05$, asterisks) increases in $[K^+]_o$ elevations associated with ictal discharges as compared with the intact slice preparation. Note also that the $[K^+]_o$ increases associated with ictal activity are comparable in the dentate and entorhinal cortex both before and after Schaffer collateral cut, and that they are significantly lower in CA3. Note also that Schaffer collateral cut does not affect the changes in $[K^+]_o$ associated with CA3 ictal discharge. Measurements were obtained from seven slices for dentate and EC and from five slices for CA3. Modified from Barbarosie M, Louvel J, Kurcewicz I and Avoli M (2000) CA3-released entorhinal seizures disclose dentate gyrus epileptogenicity and unmask a temporoammonic pathway. *Journal of Neurophysiology* 83(3): 1115–1124, with permission of the American Physiological Society.

The mechanisms responsible for the antiseizure action exerted by the fast CA3-driven interictal activity are unclear. Studies in animal models of epileptiform activity and in patients with neocortical epilepsy indicate that interictal spikes are followed by a prolonged period of inhibition during which the threshold for the generation of an epileptic discharge is increased; therefore, it is plausible that ongoing interictal activity can support a state of inhibition that is not favorable for the initiation or maintenance of ictal synchronization. Alternatively, a decrease in ictal discharge occurrence may result from activity-dependent (i.e., driven by the CA3-driven interictal events) changes in excitability that lead to depression of slow interictal discharge. Indeed, the slow interictal events are associated with transient elevations in $[K^+]_o$, and are often seen immediately preceding ictal onset; they are, therefore, hypothesized to play a critical role in initiation of electrographic seizure activity. Consistent with this view, data obtained in the 4-aminopyridine model suggest that the elevations in $[K^+]_o$ associated with the slow interictal discharges increase in amplitude once CA3-driven interictal events stop entering the EC

network (i.e., after cutting the Schaffer collaterals); in addition, increases in $[K^+]_o$ characterize the initial component of the ictal event (**Figure 2**). It is well established that extracellular K^+ can modulate neuronal excitability. Ictal discharge onset recorded intracellularly from EC neurons during 4-aminopyridine application is characterized by a long-lasting depolarizing event resembling the depolarization seen during a slow interictal event.

The Subiculum as a Gate of Hippocampal Input–Output Activity

GABAergic circuits within the subiculum control the spread of CA3-driven interictal events to the EC and to other parahippocampal areas. Hence, the subiculum does not represent a simple anatomical exit relay for hippocampal output activity, but rather a structure that can dynamically process the hippocampal outputs directed to downstream structures. This characteristic of the subiculum is relevant to understanding its role in the spread of seizures. For example, in patients where the epileptogenic focus has been localized to the hippocampus, any reduction in subicular inhibitory networks could provide a means for hippocampal seizures to generalize. However, if CA3-driven activity has the ability to reduce seizure occurrence (as discussed earlier), then changing levels of inhibition at the level of the subiculum will modulate the efficacy with which these CA3 discharges control ictal generation in parahippocampal areas.

It has also been shown that in combined hippocampus-EC slices obtained from chronically epileptic rodents, the subiculum supports EC ictogenesis, and can also provide a monosynaptic bypass for seizure activity to enter the hippocampus proper via the temporoammonic pathway. Although loss of principal cells in TLE brains is minimal in the subiculum, cellular and network reorganization within the epileptic subiculum may confer this structure with the propensity to sustain abnormal synchrony and hyperexcitability. Accordingly, in vitro studies in human epileptic tissue have demonstrated the presence of spontaneous interictal activity originating within the subiculum and spreading to adjacent hippocampal regions.

The Role of Perirhinal Cortex in Limbic Seizure Propagation

Seizure activity has been recorded in the parahippocampal region of the guinea pig isolated brain maintained in vitro, either using multiple extracellular electrodes or by analyzing optical signals measured after brain impregnation with voltage-sensitive dyes. These recordings have shown that reciprocal propagation of excitation between the PC and the EC is under the control of feed-forward inhibitory circuits. Stimulation of the neocortex or the PC, both in the in vitro isolated brain and in PC tangential rat slices, induced small-amplitude responses in the EC characterized by a prominent inhibition. The reciprocal propagation of activity from the EC to the PC is also highly regulated by intrinsic inhibitory connections. In vivo studies, in which spontaneous fast activities in EC and PC have been correlated, support the conclusion that these two regions show a low degree of functional interaction under conditions of normal excitability, even though they are directly connected.

To explore the effects of reduced inhibition on the interactions between EC and PC, experiments were carried out in which we injected $GABA_A$ receptor antagonists locally into these parahippocampal structures. $GABA_A$ receptor blockade resulted in enhanced propagation of activity from the PC to the EC. These studies revealed that the area responsible for the functional control of the interaction between these two regions resides in the lateral part of the EC and in area 35 of the PC. Local injections of bicuculline into these regions promoted propagation of synaptic responses evoked by local PC stimulation across the rhinal sulcus (Biella *et al.*, 2008; cf. citation at the end of this chapter). These findings strongly support the notion of a gating function of feed-forward inhibitory projections, in controlling PC-to-EC connectivity.

Notably, seizures induced by systemic application of bicuculline were initiated in the EC-hippocampus and did not show a tendency to propagate into the PC. The latter regions were recruited into the epileptiform discharge only after several seconds, when excitability built up to generate large-amplitude afterdischarges correlated with a highly synchronous activity throughout the hippocampus. Moreover, PC was progressively recruited when seizures were reiterated by repeated bicuculline applications, suggesting that an activity-dependent release/blockade of the inhibitory control between EC and PC developed over time, promoting the propagation of epileptiform discharges from the hippocampal-EC region to the PC (and from there to the neocortex) (**Figure 3**). That is, these findings suggest that an activity-dependent disinhibition may occur in the PC, as a consequence of complex dynamic changes in excitability supported by ictal discharges in the PC. The increasing involvement of PC during repetitive ictal discharges supports this hypothesis.

In vivo studies in the rat have shown that the rostral part of the PC, at the border of the insular cortex, is critical for the development of kindling, suggesting that this region is involved in the early stages of the establishment of an experimental temporal lobe epilepsy. In vitro recordings from the same rostral PC area support a distinct role for this region in ictal discharge initiation that is independent of activity generated in the hippocampus. Taken together, these experimental results lead to the hypothesis that a breakdown in the inhibition that characterizes the interactions between the PC and the EC may initiate hyperexcitability within these circuits, thus promoting limbic epileptogenesis.

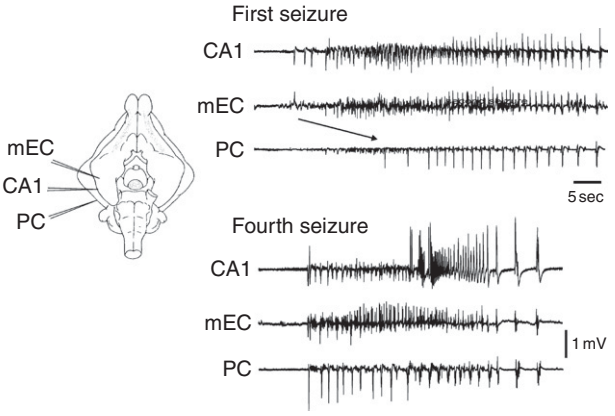


Figure 3 Simultaneous recordings from the hippocampus (area CA1), medial entorhinal cortex (mEC), and perirhinal cortex (PC) of the in vitro isolated guinea pig preparation during seizures induced by a single arterial perfusion of 50-μM bicuculline methiodide. Note that PC recruitment is stronger and occurs earlier when seizures are repeated within the same experiment (bottom panel).

Conclusions and Directions for Future Research

Evidence obtained to date indicates that neuronal networks in the hippocampus proper and in parahippocampal regions interact to produce synchronous patterns of epileptiform activity. Studies performed in in vitro brain slice preparations highlight a protective role played by hippocampal output with respect to seizure generation in parahippocampal areas. Such a mechanism may be relevant in TLE, a neurological condition where damage of hippocampal areas is a consistent finding. In this context, a pivotal role may be played by the subiculum, which appears to be the gate of hippocampal output activity. Moreover, it is increasingly clear that seizure generalization from parahippocampal structures to other regions of the brain requires the breakdown of inhibitory mechanisms controlled by the PC in the lateral EC.

These findings encourage the renewal of efforts to develop restorative approaches in the treatment of TLE associated with neuronal damage. One rationally guided goal would be the recovery of the inhibitory activity associated with hippocampal network synchronization at ~1 Hz. This pattern of neuronal activation is impaired in epileptic animals, and would be expected to be affected also in TLE patients who frequently present with a damaged CA3. Brain stimulation at low frequency, via implanted electrodes aimed at activating the subicular-EC networks, could be effective in suppressing ictal activity in patients, as demonstrated in animal models. This approach could represent an alternative to treating refractoriness to drug with purely surgical interventions.

Another alternative approach is to restore inhibitory mechanisms in parahippocampal regions involved in filtering the outgoing ictal activity generated in the limbic system. As epileptic foci are found in the hippocampus, EC, and amygdala of TLE patients, the PC appears to be a particularly likely brain region in which such restoration of inhibition could be effective. In fact, this area is interconnected with all the ictogenic regions discussed here, and could be targeted by specific interventions. However, only few data are available about changes affecting the PC in TLE patients and animal models. There does not appear to be extensive damage in PC, but there are no systematic studies to address the changes in specific cell populations – and in particular, in interneuron populations – in PC. These cells, especially parvalbumin-positive interneurons, are highly sensitive to seizure-induced damage and could be lost in areas extending beyond the hippocampal boundaries, as seen in the subiculum. Interneuron replacement in rat brains damaged after status epilepticus represents a novel and promising approach to reinforcing inhibition in neuronal networks in which inhibition has been compromised.

See also: **Absence Seizures**: Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; Thalamocortical Synchronization and Absence Epilepsy; **GABA**: $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; **Models**: Computer Models of Seizures and Epilepsy: Understanding Epileptogenesis using Complex Large-Scale Models; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Oscillatory Activity**: Cellular and Network Oscillations in Epilepsy; Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; Neuronal Networks Generating Pathological High Frequency Oscillations.

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Recurrent Synaptic Actions and the Genesis of Epileptiform activity

C Le Duigou, G Huberfeld, and R Miles, INSERM U739, Paris, France

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Introduction

Intracellular recordings were crucial in unraveling the circuits underlying epileptiform activities. The first records of epileptiform activity were made by Spencer and Kandel from the hippocampus of cats during focal application of penicillin. Penicillin suppresses γ-aminobutyric acid (GABA) receptor signaling and acts as a convulsant, provoking repetitive interictal EEG spikes. Pyramidal cells near the focus were found to fire in high-frequency burst discharges synchronous between most cells in a local region. During these synchronous bursts, neurons depolarize nearly simultaneously in a paroxysmal shift associated with EEG spikes. Some years later, Schwartzkroin and Prince showed that penicillin produces similar synchronous depolarizing shifts in the then-new hippocampal slice.

The cellular or synaptic origin of the paroxysmal depolarizing shift (PDS) was pursued in the slice. The discovery of calcium-mediated action potentials with similar, slow kinetics to those of an EEG spike and records from dendrites where these calcium spikes were initiated supported a cellular origin. But why should large numbers of pyramidal cells generate such events simultaneously? Maybe synaptic transmission was also needed. Evidence that the PDS reflected synaptic activity came from the voltage clamp records of Johnston and Brown, which revealed large synaptic currents associated with depolarizing shifts in CA3 pyramidal cells. These currents occurred rhythmically and were independent of cellular membrane potential, suggesting that they depended on synaptic signaling.

Background and Experimental Methods

If interictal activity corresponds, in part, to a giant excitatory postsynaptic potential (EPSP), where does the EPSP come from? Interictal events in disinhibited hippocampal slices were clearly generated in the CA3 region (**Figure 1**), but they could be initiated by stimulating any pathway afferent to the region, or could occur spontaneously in the absence of any stimulus. Computer modeling helped to identify the origin of the EPSP. Traub and Wong made a model of an isolated network of 100 cells, with a sparse connectivity. They argued that if each cell excited just a few of its neighbors strongly enough that they fired, the size of the group of firing cells would increase with time and so generate a local population synchrony. Sparse but strong connections could also explain a delay of several 10 s of milliseconds that was sometimes seen between afferent stimulation and an interictal event.

A delay of several 10 s of milliseconds does not reflect monosynaptic input, consistent with the observation that the giant EPSP was not specific to any afferent fiber system. The architecture of the Wong and Traub model consisted of synaptically connected pyramidal cells in a single array. The biological correlate of this connectivity was suggested to be recurrent excitatory synapses

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between pyramidal cells of the CA3 region. The model predicted such recurrent excitatory connections, but no data were available on whether pyramidal cell axon terminals contacted other pyramidal cells in the CA3 region. And the technique of stimulating afferent pathways while recording from a single cell, transferred to the slice from work on the spinal cord, gave no insights into local recurrent synaptic circuits.

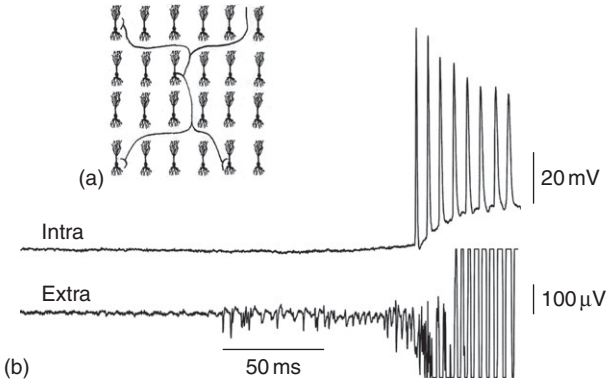


Figure 1 Interictal-like activity in the CA3 region of disinhibited slices. (a) Sparse but strong recurrent connectivity within a single pyramidal cell population is suggested to underlie interictal-like bursts in disinhibited slices. (b) Extracellular records show burst initiation is associated with an acceleration of the frequency of multiunit activity. It is correlated intracellularly with a barrage of temporally summating EPSPs that lead to action potential firing together with the extracellular field potential.

A different technique and a lucky experiment supported the idea that recurrent circuits were crucial in the genesis of epileptiform synchrony in the disinhibited CA3 region. The approach was borrowed from work in invertebrate preparations on the entrainment of rhythmic motor activities by single-cell firing. It consisted in showing that firing induced in one cell could, in some cases, affect the timing of spontaneously generated population events. These experiments showed that the CA3 recurrent excitatory connectivity was high enough that action potential discharge of a single cell could entrain or initiate population discharges. A delay of several 10 s of milliseconds between single-cell firing and an epileptiform burst was a striking vindication of the modeling work.

While it seemed possible that interictal-like population activities might depend on local synaptic interactions in the CA3 region, the existence of recurrent connections between CA3 pyramidal cells was not proven, and it was not clear why suppressing inhibition favored epileptiform bursting. Simultaneous records from CA3 cell pairs were needed to resolve these points.

Simultaneous recordings from pairs of pyramidal cells confirmed the existence of recurrent excitatory synapses in the CA3 region (**Figure 2**). Morphological data show that CA3 pyramidal cell axons arborize extensively, mostly within the CA3 stratum oriens and radiatum,

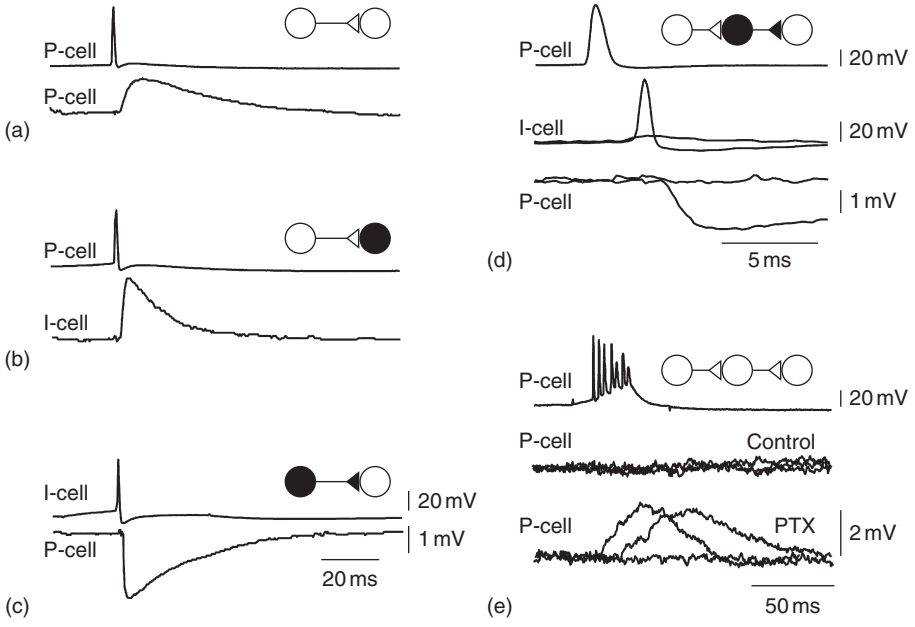


Figure 2 Synaptic circuits for interictal activity in the CA3 region of the hippocampus. (a) Monosynaptic recurrent excitatory interaction between two pyramidal cells. (b) Excitation of interneurons occurs via unitary events that are faster and larger than at connections with pyramidal cells. (c) Inhibitory interaction between an interneuron and a pyramidal cell. (d) Interactions underlying a short-latency disynaptic inhibition. Single pyramidal cell EPSPs can make single interneurons fire and so initiate a disynaptic IPSP. (e) Loss of inhibitory control induced by blocking $GABA_A$ receptors with their antagonist picrotxin, PTX, reveals latent polysynaptic excitatory connections between previously uncoupled pyramidal cells.

with an interterminal distance of 5–10 μm. The probability that two cells in a slice are connected via synapses established by these axon collaterals is about 2–5%. A connection between two CA3 pyramidal cells may be mediated by few – maybe only one or two – terminals. Unitary synaptic events at recurrent synapses have amplitudes at resting potential of 0.1–1.2 mV and a time to peak in the range 5–10 ms. These synaptic events decay with time constants on the order of 15–30 ms, and may be prolonged by the activation of inward postsynaptic currents at near-threshold potentials. Some synaptic events fail – that is, some presynaptic action potentials evoke no postsynaptic response – probably due to the stochastic nature of transmitter liberation. With these synaptic properties, firing in a single pyramidal cell can sometimes induce firing in a postsynaptic partner – as predicted by the simulation studies.

Characterization of unitary inhibitory postsynaptic events, in simultaneous records from a GABAergic interneuron and a pyramidal cell, illuminated how suppressing inhibition might favor epileptiform activity. Inhibitory synaptic connectivity can be very high in a local region, and inhibitory connections may comprise multiple synaptic contacts. Unitary inhibitory synaptic events have mean amplitudes in the range 0.3–3 mV at resting potential. Their time to peak is in the range 3–8 ms and decay in the range 20–40 ms. Inhibitory synaptic events rarely fail. There is an exquisite precision and great variability in sites of inhibitory synapses established between different interneurons and the pyramidal cells that they contact. Some interneurons inhibit dendritic sites, others synapse with postsynaptic somata, and still others contact the initial parts of pyramidal cell axons. These differences presumably underlie different functions. For example, perisomatically terminating interneurons might control pyramidal cell output, whereas inhibitory cells that target dendrites might counter specific excitatory inputs or dendritic electrogenesis. Suppression of both these functions probably contributes to the disinhibition-induced synchrony.

Dual records from interneurons and pyramidal cells revealed that synapses that excite inhibitory cells have distinct properties from those that excite pyramidal cells. Synaptic events are larger, with mean amplitudes in the range 0.5–3 mV at resting potential. Their rise time is faster (2–5 ms) and EPSPs also decay faster, with a time constant of 10–15 ms. Two differences between interneurons and CA3 pyramidal cells may be responsible for these striking differences in the excitatory events recorded from the respective cell types. The subunit composition of AMPA receptors expressed by interneurons tends not to include the GluR2 subunit, so giving rise to synaptic currents that are faster and larger than those of pyramidal cells. Furthermore, excitatory synaptic depolarizations activate fast, intrinsic K-currents that curtail EPSPs in interneurons but not pyramid cells. Functionally, these differences, together with the relatively depolarized interneuron resting potential, assure that pyramidal cell firing can synaptically initiate firing in an interneuron at latencies of 2–8 ms. In contrast, latencies for synaptic transmission of firing between monosynaptically connected pyramidal cells are in the range 5–25 ms.

This difference in latencies for synaptically induced firing at recurrent excitatory connections with interneurons and with pyramidal cells helps explain how the suppression of fast synaptic inhibition leads to the initiation of interictal synchrony. Firing of a CA3 pyramidal cell can spread if postsynaptic cells can be made to fire, and in turn induce firing in further generations of postsynaptic cells to generate an interictal spike. As fast inhibition acts to suppress the transmission of firing between pyramidal cells, it prevents the development of interictal population activities. Inhibitory circuits are more efficient than excitatory circuits (1) at the synaptic level, with less failures and larger conductance events; (2) within the network because of more densely arborizing axons; and (3) functionally, with a more efficient and shorter latency transmission of firing. Subsequent experiments proved that inhibition controls the spread of activity in recurrent circuits by showing that suppressing inhibition uncovered 'latent' polysynaptic pathways between previously uncoupled pyramidal cells.

Recent Results

If the initiation of interictal bursts depends on the spread of neuronal discharge mediated via recurrent excitatory synapses between CA3 pyramidal cells, the properties of these connections may influence population events in other ways. The duration of interictal events has been suggested to depend on the depletion of transmitter at recurrent synapses following high-frequency release. The replenishment of release-ready vesicles takes 5–15 s and this process, occurring across the population of CA3 recurrent excitatory synapses, seems to control the interval between interictal bursts.

How does epileptiform bursting emerge? Single CA3 cells normally discharge spontaneously in bursts that are not synchronous with bursts in other cells. Suppressing inhibition enables transmission in polysynaptic, excitatory pathways. New traffic in these latent polysynaptic circuits is responsible for the synchronization of CA3 pyramidal cell firing. Recent multiunit studies on the transition between asynchronous firing and interictal synchrony have shown that rhythmic burst discharges emerge, and grow in amplitude and coherence, before fully synchronous firing is established. Multiunit activity during an emerging interictal event consists of increased firing of some but not all cells in a local region; this emerging interictal event stops before developing into a fully epileptiform

event. The interictal field potential corresponding to an interictal event has a spike-and-wave shape, with the wave component corresponding to the cessation of unit-firing; intracellular records suggest that this cessation of firing is due to a $GABA_B$-mediated IPSP. Thus, interictal rhythmicity emerges from events mediated by sequences of glutamatergic and $GABA_B$-mediated signals.

What determines the timing of an interictal burst? While the replenishment of synaptic vesicles controls the interval between population events, distinct active processes may be involved in the precise timing of burst initiation. Multiunit recordings revealed a distinct acceleration in activity before interictal firing. The observation that firing frequency is higher just before a burst than at any other point in the interburst cycle suggests that firing frequency must exceed a certain threshold to initiate a burst. This idea might explain how single pyramidal cell firing can sometimes initiate firing in the larger CA3 population. If population activity induced via polysynaptic pathways activated by the stimulated pyramidal cell exceeded a threshold level, a population burst should be initiated. Reducing either neuronal excitability or excitatory synaptic strength reduces population firing and increases the time to reach burst threshold. The frequency of population events is reduced and eventually spontaneous events are suppressed. Small reductions in excitatory synaptic efficacy reveal partial population events curtailed by intracellular $GABA_B$-mediated hyperpolarizations, similar to the 'partial' events seen during the emergence of synchrony. These results show that two synaptic processes are engaged during interictal-like population bursts: one excitatory process, mediated by glutamatergic receptors, acts to recruit cells into the synchronous discharge; the other inhibitory process, mediated via $GABA_B$ receptors, tends to stop recruitment into these bursts. The opposition of two such processes, like the inward and outward currents engaged during action potential generation in a single neuron, may be an essential feature of systems with a threshold.

Interictal Synchrony in Slices from the Temporal Lobe of Epileptic Patients

It is essential, at some point, to confront models of disease with the human pathology that they model. Since pharmacotherapies for focal temporal lobe epilepsies may be ineffective, the best therapeutic option for some patients is to excise the damaged tissue. Epilepsies of the temporal lobe are one of the rare neurological diseases where human tissue can be available for physiological comparison with tissue from model systems. Recordings made from slices of human tissue (obtained after careful surgical excision of epileptic tissue from the temporal lobe) have sometimes – although not always – revealed a spontaneous interictal-like activity. Interestingly, GABAergic synaptic mechanisms seem to contribute to this population activity, which consists of recurring spontaneous field potentials of similar frequency, amplitude, and duration to those generated by disinhibition in the CA3 region of guinea-pig slices.

Human temporal lobe epilepsies are often associated with a hippocampal sclerosis consisting of a mild to severe cell loss in the CA3 and CA1 regions. In tissue from such patients, we have found that an interictal-like activity, similar in frequency to that observed in intracranial EEG records from the same patients, occurs spontaneously at frequencies in the range 0.3–2 Hz. It is generated in the subiculum, an output region of the hippocampus that has suffered little cell loss but that is presumably severely deafferented because of sclerosis in the CA1 region. The interictal activity is suppressed by antagonists at either glutamate or GABA receptors.

The unexpected involvement of GABAergic signaling seems to depend on a depolarized reversal potential for GABAergic events in a minority (about 20%) of subicular pyramidal cells. A depolarized reversal potential for normally inhibitory GABAergic events points to a defect in homeostatic mechanisms that control intracellular chloride. Such responses are rare in the normal adult cortex, but do occur during early development before full expression of the K-Cl cotransporter, KCC2, which acts to extrude chloride. Possibly the denervation of the subiculum due to CA1 cell death induces a replay of developmental mechanisms with proepileptic consequences. Combining biocytin staining of recorded cells with immunostaining showed that KCC2 expression was perturbed in some of the subicular cells with depolarizing responses to GABA.

Comparison of Human Interictal-Like Synchrony and Disinhibition Induced Synchrony

Slices of human subiculum that generate interictal activity are larger (about 3×5 mm) than those of the guinea-pig CA3 region (about 1.5×2 mm) that generate an interictal-like activity upon disinhibition (**Figure 3**). Perhaps because of the larger cell population involved, attempts to affect the interictal rhythm in human tissue, by single-cell stimulation, are unsuccessful. Comparison of the timing of events recorded by spatially segregated electrodes suggests that multiple pacemaker zones often exist in human slices. In contrast, interictal-like events are always initiated in the CA3a region of disinhibited guinea-pig slices. The interval between population events in human subiculum (0.3–2 s) is notably shorter than that (4–12 s) between interictal-like firing in disinhibited guinea-pig CA3 region. The form and duration of interictal field potentials, filtered in the same way, is very similar across preparations.

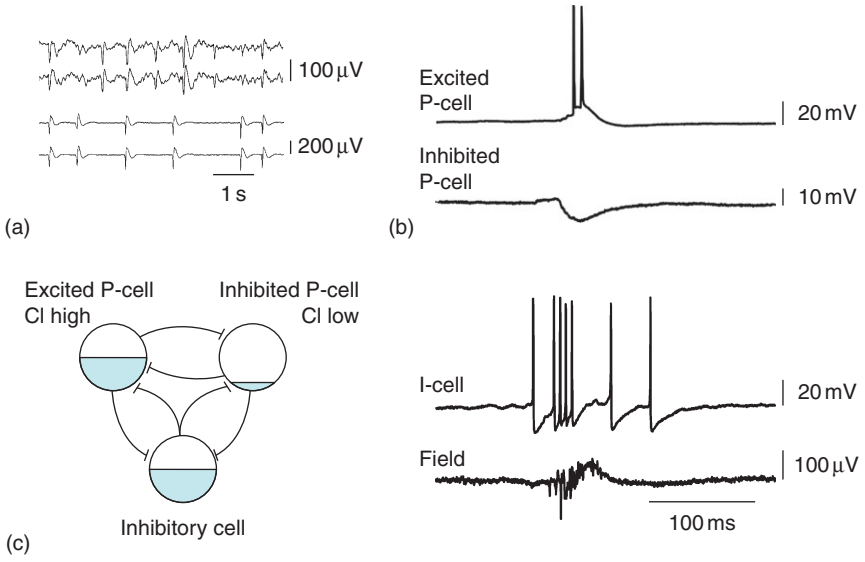


Figure 3 Interictal-like activity generated by the human subiculum. (a) Comparison of interictal activity recorded in situ, with intracranial electrodes, and in vitro, with extracellular electrodes. (b) Distinct patterns of activity simultaneous with interictal-like activity in vitro, recorded from an excited pyramidal cell, an inhibited pyramidal cell and an interneuron. (c) Possible circuit diagram for human subicular interictal activity. The two types of pyramidal cells and interneurons are all connected to each other. Internal Cl, indicated by a horizontal line in each cell symbol, is high in excited pyramidal cells and interneurons.

Some comparison of the mechanisms involved may be useful. While interictal bursts in disinhibited guinea-pig slices seem to be initiated by a local acceleration in pyramidal cell firing, in human subiculum interneurons consistently discharge before interictal events. While neither mechanism has been confirmed, early-firing subicular interneurons could be synchronized by electrical coupling or perhaps by GABAergic signaling, which is not strikingly inhibitory in human tissue. With GABAergic reversal potentials distributed between positive values for a minority of pyramidal cells, and values hyperpolarizing from resting potential in the majority, the same interneuron should excite some and inhibit other postsynaptic cells. These depolarizing GABAergic signals might fulfill the same role as recurrent excitation between pyramidal cells in the guinea-pig CA3 region: to transmit firing within an interconnected population. However, some points argue against such a role. The driving force for depolarizing GABAergic events is small, not more than about 15 mV, and shunting effects may oppose action potential generation in target cells. If interneurons do not initiate human interictal synchrony, subicular pyramidal cells that possess a respectable recurrent connectivity may fill this role. In the human subiculum, recurrent synaptic excitation would have to generate synchrony in the presence of a partial disinhibition due to the perturbation of Cl-homeostasis and the resulting effects on GABAergic signaling. In contrast, in the disinhibited CA3 region of guinea-pig slices, recurrent excitatory systems generate synchrony in the absence of resistance from $GABA_A$ receptor-mediated signals.

Future Goals

The shape and kinetics of interictal-like field potentials, in slices from both human and animal tissue, are not very different from those of field potentials that have been called sharp-waves in EEG records from non-anaesthetized animals. In the sleeping or resting rodent, sharp-waves have been associated with processes of memory consolidation, and are generated in the CA3 region, possibly by recurrent synaptic mechanisms similar to those involved in generating the synchrony observed in disinhibited slices. Clinically however the term sharp-wave is used to describe an anomalous epileptiform event recorded from the temporal cortex during interictal periods. Since intrahippocampal EEG records are rarely made from healthy humans, it is not clear whether sharp-wave-like events are generated in more physiological conditions. The lack of a clear nomenclature should be clarified. Possibly a reasonable criterion to distinguish between such population events could be made from estimates of the proportion of simultaneously discharging pyramidal cells. In the disinhibited slices from the guinea-pig, probably 100% of pyramidal cells fire synchronously. Approximately 20% of pyramidal cells seem to suffice to generate the interictal-like activity in slices of subiculum from epileptic patients. Estimates from in vivo records with tetrodes suggest that 5–10% of the CA3 population may discharge quasisimultaneously in a sharp-wave of a sleeping animal.

Another question to be resolved is the origin of the perturbed chloride homeostasis, which appears to

contribute to human interictal-like activity. The molecule or molecules involved in this perturbation must be identified, as must the signaling pathways involved in pathological changes to their function. The discovery of other cellular modifications correlated with depolarizing GABAergic signaling in a fraction of subicular cells might help to clarify this point. More work is also needed to understand how these and other reactive changes contribute to the initiation of seizure-like activity in human temporal lobe tissue.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; **Metabolic Changes**: Glucose Metabolism and $GABA_A$ Receptor Dysfunction in Human Partial Epilepsy; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization; **Transporters**: Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures

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Introduction

Epilepsy is a chronic disease characterized by the regular alternation of short synchronous oscillations and long-lasting interictal periods (with or without EEG abnormalities). These periods without seizures can vary from a few hours to several months. This alternation of ictal and interictal conditions suggests the existence of both passive conditions and active processes that control the occurrence of seizures. Control mechanisms of epileptic seizures may involve existing circuits or processes that are capable of controlling physiological oscillations in the brain. Such mechanisms may also develop in parallel with epileptogenesis, and may constitute an adaptative response to the development of epileptic circuits.

Our knowledge about the mechanisms that control epileptogenesis and/or epileptic seizures has improved during the last decades, but remains very partial.

contribute to human interictal-like activity. The molecule or molecules involved in this perturbation must be identified, as must the signaling pathways involved in pathological changes to their function. The discovery of other cellular modifications correlated with depolarizing GABAergic signaling in a fraction of subicular cells might help to clarify this point. More work is also needed to understand how these and other reactive changes contribute to the initiation of seizure-like activity in human temporal lobe tissue.

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The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures

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Introduction

Epilepsy is a chronic disease characterized by the regular alternation of short synchronous oscillations and long-lasting interictal periods (with or without EEG abnormalities). These periods without seizures can vary from a few hours to several months. This alternation of ictal and interictal conditions suggests the existence of both passive conditions and active processes that control the occurrence of seizures. Control mechanisms of epileptic seizures may involve existing circuits or processes that are capable of controlling physiological oscillations in the brain. Such mechanisms may also develop in parallel with epileptogenesis, and may constitute an adaptative response to the development of epileptic circuits.

Our knowledge about the mechanisms that control epileptogenesis and/or epileptic seizures has improved during the last decades, but remains very partial.

The study of such mechanisms is critical to better understand not only the development of the epileptic state (epileptogenesis), but also (once the seizures are established) the transitions between normal and paroxysmal activities of the epileptic networks. Insight into these processes should offer the possibility to develop innovative therapeutical strategies, and also the opportunity to better understand brain mechanisms that control synchronized oscillations in general (whether physiological or pathological).

Several lines of evidence from experimental studies in animal models have suggested that different structures of the BG are critical in the control of epilepsies. Indeed, in addition to their strong involvement in the control of movement, the BG also contribute to a wider set of functions, including cognitive and motivational aspects of behavior. In particular, the BG have a critical position in the CNS, with connections to a large set of forebrain structures known to be involved in the generation of seizures and, more generally, in the emergence of large-scale synchronized normal and pathological oscillations. Although the BG do not by themselves generate epileptic discharges, they may well provide a subcortical system that controls the alternation between interictal and ictal cortical activity.

Background

Functional Anatomy of the BG Circuits

The BG have a privileged position in the brain to control circuits involved in the generation of seizures, as they are connected to the cerebral cortex, limbic areas, and thalamic relay nuclei through several multisynaptic loop circuits. Through glutamatergic afferent projections, the striatum – one of the BG input nuclei – processes information from all areas of the cerebral cortex, the midline and intralaminar thalamic nuclei, the amygdala, and the hippocampus. The glutamatergic inputs to the subthalamic nucleus (STN) arise from a more restricted part of the cortex, involving the motor, premotor, and prefrontal areas and from the parafascicular thalamic nucleus. In turn, the two outputs of the BG, the substantia nigra pars reticulata (SNR) and the internal segment of the globus pallidus (or the entopeduncular nucleus in the rodent), project to a diversity of thalamic and brain stem nuclei. Through the thalamic nuclei, the BG influence mainly the frontal cortex. They also affect, via nigral GABAergic projections, the intermediate and deep layers of the superior colliculus and the pedunculo-pontine nucleus. Via the ascending projections of these brain stem structures, the BG can also indirectly influence widespread areas of the cerebral cortex. A conceptual model of BG circuits and their relationships with the cerebral cortex has been proposed, where by cortical information is transmitted via three main pathways: (i) a direct striatonigral pathway, (ii) a direct subthalamo-nigral pathway, and (iii) an indirect striato-pallido-subthalamo-nigral pathway (see Further Reading). Since the striatal projection neurons are GABAergic whereas STN neurons are glutamatergic, the trans-striatal and trans-subthalamic pathways exert opposite effects, respectively inhibitory and excitatory, on output neurons (i.e., the GABAergic nigral projection cells). According to this model, electrical stimulation of the cortex induces in these BG neurons a sequence of synaptic events consisting of an inhibition, preceded or not by an early excitation, and followed or not by a late excitatory event. Since the BG output neurons are GABAergic and tonically active, their inhibition by the direct striatal pathway leads to a *disinhibition* of the BG targets like the thalamic or the brain stem nuclei. Conversely, the trans-STN circuits reinforce the inhibitory influence that BG exerts on their targets (see **Figure 1**, middle panel).

The BG as a Generator of Seizures?

Although the BG circuits are known to generate spontaneous oscillatory activities in several physiological as well as pathophysiological conditions, that they will initiate epileptic seizures appears quite unlikely. Indeed, oscillatory activities observed in BG circuits have very low frequencies; when recorded during seizures, activity in BG appears to result from propagated influences. Furthermore, electrical or chemical stimulations of the BG structures rarely initiate seizures, and their lesions never abolish the occurrence of seizures (see Further Reading). On the contrary, changes in neuronal activity revealed by EEG, measures of glucose metabolism, or expression of immediate early genes studies suggest that the BG circuits rather «detect» the beginning, the localization, and/or the intensity of a seizure. It is thus conceivable that changes of activity of these BG circuits may have some consequences on the development of ongoing seizures.

Early Evidence of a Gating System for the Control of Epileptic Seizures

In 1980, Gale and Iadarola were the first to associate an increase of GABA in the substantia reticulata (SNR) with antiepileptic effects. Since then, several studies showed that the potentiation of the GABAergic neurotransmission within the SNR, by bilateral microinjections of GABAmimetic drugs, suppressed seizures in a large variety of models of generalized seizures in the rat. Inhibition of the SNR, obtained by blockade of the glutamatergic input from the STN, also suppressed seizures. More recently, it was shown that 60–130 Hz stimulation applied to the SNR can interrupt seizures in several models. The critical nigral projection(s) involved in these antiepileptic effects remain(s) to be determined. Disinhibition of neurons of the superior colliculus, one of the main targets of

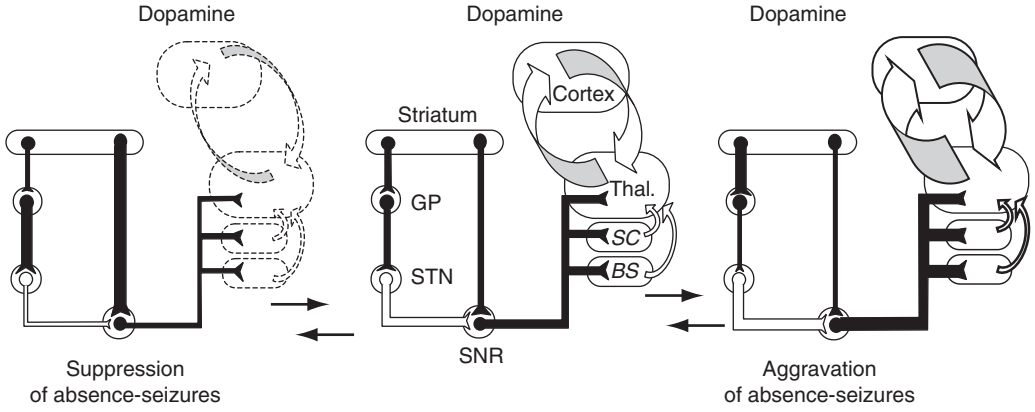


Figure 1 Effects of pharmacological manipulations of the basal ganglia structure. Schematic representations of the connectivity within the basal ganglia system thought to be involved in the control of absence-seizures in GAERS. Thickness of neuronal outputs is proportional to the activity of the projection, as modified following intracerebral pharmacological manipulations. An increased activity in the striatal dopaminergic tone has antiepileptic effects (left panel), whereas a decreased activity in the dopaminergic tone has proepileptic effects (right panel). For instance, dopamine at the striatal level is thought to simultaneously potentiate the inhibitory influence of the trans-striatal direct pathway and decrease the excitatory influence of the trans-striatal indirect pathway through D1 and D2 dopaminergic receptors, respectively. BS: brain stem; GP: globus pallidus; SC: superior colliculus; SNR: substantia nigra pars reticulata; STN: subthalamic nucleus; Thal: thalamus.

the SNR, appears to play a significant role in their mediation, at least in some forms of seizures. The projection towards thalamic nuclei could also be involved in this control circuit.

Methods

Most of the data presented below were collected from in vivo experimental studies performed in rodent models of epilepsy. They were mainly obtained using intracerebral pharmacological manipulations and electrophysiological recordings, with subsequent histological analyses.

Results

Role of the Different BG Pathways in the Control of Seizures (see Figure 1)

Involvement of the direct striatonigral pathway

The possibility that the direct GABAergic striatonigral pathway could modulate seizures – through inhibition of the SNR – was first suggested by experiments showing that intranigral injection of GABA agonists have antiepileptic effects in most models of seizure in the rat. Later, activation of neurons in the striatum, by glutamate or D1 agonists, which results in release of GABA in the SNR and decrease of SNR neuronal activity, was also shown to suppress seizures. This effect could be reversed by a subsequent injection of a GABA antagonist into the SNR. These effects, however, appear to vary according to the model used and type of seizures. In addition, these antiepileptic effects appear to be region-dependent within the striatum, suggesting that different circuits are involved depending on the type of seizures.

Involvement of the direct subthalamo-nigral pathway

The glutamatergic input from the STN is critical in the maintenance of the SNR neuronal activity; bilateral injections of a GABA agonist into the STN, or high-frequency stimulation of this structure, decreases the activity of nigral neurons. In the genetic model of absence epilepsy in the rat (GAERS), we showed that these manipulations suppress seizures without behavioral side-effects. This observation is consistent with our previous findings showing that blockade of NMDA receptors in the SNR has antiepileptic effects. The antiepileptic effects of pharmacological STN inhibition have been confirmed in different models of generalized convulsive seizures. Similarly, 130 Hz stimulation of the STN was recently reported to protect against different forms of convulsive seizures.

Involvement of the indirect striato-pallido-subthalamo-nigral pathway

The involvement of the GABAergic input from the pallidum to the STN has been investigated in only a few models of convulsive epilepsy, but these studies support the view that this projection could be part of a circuit involved in propagation of seizure activity during secondary generalization. In the GAERS, we showed that disinhibition of the globus pallidus or the ventral pallidum, by $GABA_A$ antagonists, suppresses absence seizures, an effect which was correlated with a decrease in glutamate levels in the SNR measured by microdialysis. These results suggest that the antiepileptic effect is mediated, at least in part, through a reduction in activity of the STN. Conversely, an increase in the occurrence of absence-seizures was observed following inhibition of the ventral pallidum.

The involvement of the pallidum in the control circuit of seizures is consistent with data obtained following intrastriatal injection of dopaminergic agonists of the D2 receptors (see below). Although still debated, several sets of results have suggested that these receptors are preferentially located on neurons projecting to the globus pallidus, and that their activation leads to disinhibition of pallidal neurons. Injections of D2 agonists into the striatum block convulsive or nonconvulsive seizures, whereas injections of their antagonists have aggravating effects. An additive suppression of absence-seizures was obtained in the GAERS with combined injections of low doses of a D1 and a D2 agonists into the striatum, and more precisely into the core of the accumbens. This result is similar to the potentiation of antiepileptic effects observed after combined injection of a $GABA_A$ agonist and an NMDA antagonist into the SNR, at doses shown to have no effect by themselves. These data suggest that the trans-striatal and trans-subthalamic pathways could cooperate in the suppression of absence seizures, and that striatal dopaminergic transmission could play a key role in the modulation of epileptic seizures by the BG (see below).

Evidence for an Endogenous Control System by the Basal Ganglia Circuits

Electrophysiological recordings of the BG circuits

The data reported in the different models of generalized epilepsy have lead to the hypothesis that some BG circuits may act as an endogenous control system capable of interrupting epileptic seizures and/or of modulating their occurrence. Accordingly, changes of neuronal activity within the BG should be associated with the occurrence and/or interruption of seizures. Indeed, changes in basal activity is always observed in the BG concomitantly with an epileptic discharge, in both human patients and animal models. In animal models, data were mainly collected during pharmacologically or electrically induced seizures, and the «endogenous» nature of the observed changes of activity was thus difficult to determine. To investigate the possible relationships between unit activities in the BG and the cortical EEG during ictal and interictal periods, the genetic models with spontaneous seizures are preferred. In particular, single unit recordings in immobilized GAERS (maintained under neuroleptanalgesia) indicated that SNR and STN neurons increase their firing rate concomitantly with the occurrence of spike-and-wave discharges, whereas dopaminergic neurons of the substantia nigra pars compacta (SNC) tend to decrease their activity. Conversely, at the end of the seizure, SNR and STN neurons decrease their firing rate, whereas dopaminergic neurons of the SNC display a transient overactive state. Furthermore, multiunit recordings with a chronically implanted movable tetrode, in the freely moving GAERS, revealed that the frequency of discharge of the SNR neurons increased and action potentials became organized into bursts during an epileptic spike–wave discharge (see **Figure 2**). These SNR bursts always followed the spike component (of each spike-and-wave element) by a few milliseconds. In addition, a few seconds before the end of the EEG discharge, a decrease in SNR neuronal activity was often observed. The delay observed between the occurrence of the cortical EEG spikes and the burst of action potentials suggests that the SNR is involved in the propagation rather than the generation of the seizure. In addition, the decrease of unit activity observed before the end of the seizure, suggests that inhibition of SNR neurons could participate in the blockade of an ongoing seizure. The nature and the temporal pattern of these changes are in agreement with what could be predicted from the pharmacological data, and suggest that these events could contribute to the antiepileptic effects resulting from a physiological triggering of the control system.

The involvement of the BG was further supported by recent in vivo extracellular and intracellular recordings, performed in GAERS, at different levels of the circuit. The propagation of the electrical discharges in corticostriatal and corticosubthalamic neurons induced a feed-forward inhibition of the GABAergic striatonigral neurons and synchronized rhythmic bursting activity in the glutamatergic subthalamo-nigral neurons, respectively. This rhythmic activity in subthalamo-nigral neurons, together with an imbalance between excitation and inhibition within the SNR, lead in turn to synchronized burst firing in GABAergic nigrothalamic neurons, in phase with the spike-and-wave complexes in the EEG. This circuit activation pattern is likely to reflect an endogenous mechanism controlling (positively) the maintenance and the duration of the seizure. The rebound of excitation observed in most of striatal output neurons at the end of a spike-and-wave discharge, and in parallel with the decrease of SNR activity, may have a negative modulatory effect on spike-and-waves and thus contribute to the interruption of an ongoing absence-seizure.

As compared to the other thalamic nuclei, neurons of the ventromedial thalamus – the main thalamic output target of the SNR – display an increase in rhythmic bursts of action potentials during seizures. Pharmacological blockade of excitatory inputs to nigrothalamic neurons led to a transient interruption of spike–wave discharges, correlated with a change in the activity of thalamic cells, which was increased in frequency and converted into a sustained arrhythmic firing pattern. Simultaneously, cortical neurons exhibit a decrease in their firing rate that was associated with an increase in membrane polarization and a decrease in input resistance. These findings demonstrate that an inhibition of SNR neurons changes the activity of their thalamic targets, which in turn could

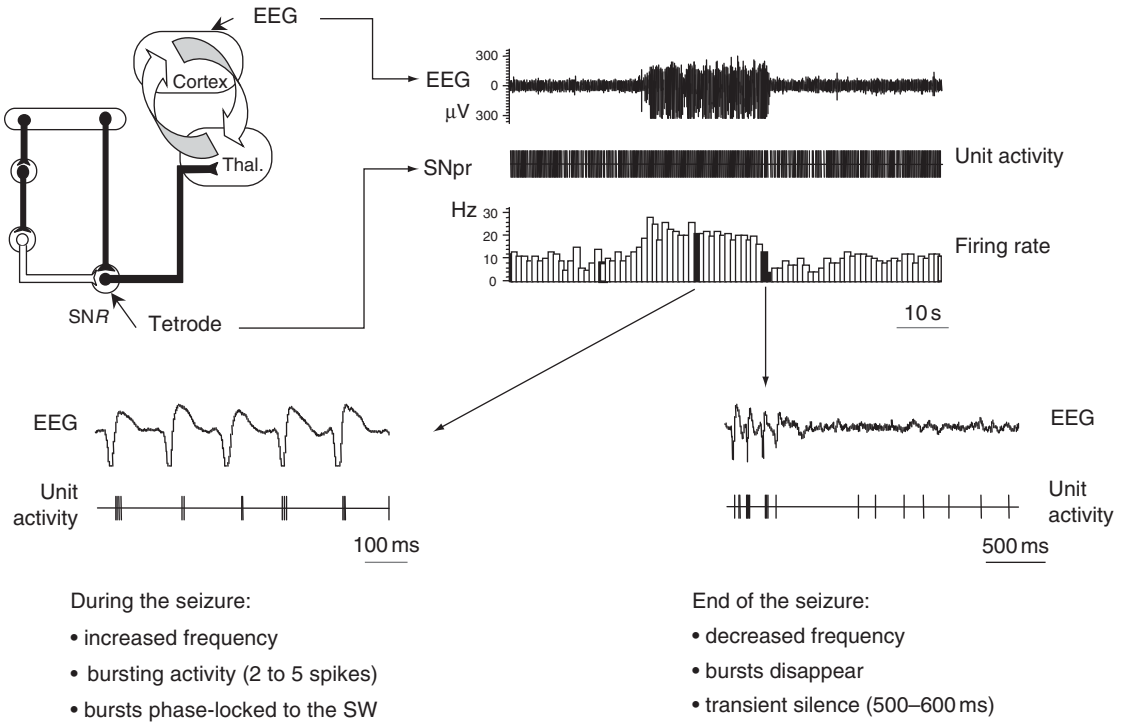


Figure 2 Changes in extracellular unit recordings in the SNR in a freely behaving GAERS. Cortical EEG and unit activity obtained from a freely behaving rat equipped with a movable tetrode chronically implanted within the substantia nigra *pars reticulata*. The lower panels display an enlargement of spike-and-wave complexes with simultaneous bursts of action potentials (*left panel*) and changes occurring at the end of an absence-seizure (*right panel*).

affect cortical neuron excitability, and consequently, the generation of cortical epileptic discharges. Thus, the nigro-thalamo-cortical pathway may provide an online system control of absence seizures.

Role of the dopaminergic neurotransmission

As mentioned above, pharmacological and electrophysiological studies have revealed a critical role of the striatal dopaminergic neurotransmission in the control of seizures. It is well known that dopamimetic drugs have antiepileptic effects, in general, in both human and in animals. In addition, changes in dopaminergic markers have been reported in animal models as well as in some human patients. These changes may result from the recurrent involvement of dopaminergic neurotransmission to control seizures, and may represent compensatory processes. Investigation of the expression of different dopaminergic transcripts in the striatum of GAERS revealed an increased level of D3 mRNA expression in neurons of the nucleus accumbens in adult GAERS (as compared to nonepileptic control rats). This change was specific to D3 receptors (there was no change in the expression of mRNAs coding for other neuronal dopaminergic markers, such as tyrosine hydroxylase, membraneous and vesicular dopamine transporters, or D1, D2 or D5 receptors). Because D3 transcripts were not increased in 21-days juvenile GAERS (i.e., at a time when no absence seizures had yet occurred), these data suggest that this transcriptional change represents either a part of the epileptogenesis process or an adaptive response to repeated seizures. Although the functional consequences of an upregulation of D3 transcripts on neurons of the n. accumbens are still unknown, it is likely to be related to the overactivity of the dopaminergic neurons observed at the end of the absence seizure (see above). Since changes in dopaminergic markers occur in chronic epileptic animals, and since dopamine is thought to be involved in the fine tuning of seizure interruption, changes in dopaminergic tone are likely to occur and further influence the epileptogenesis process and how seizure recurrence develops.

Future Goals

The experimental data reviewed in this article support the idea that changes in the activity of BG circuits influence the occurrence and/or cessation of epileptic seizures. In addition, the dynamic changes observed in neurons of these circuits suggest that the BG stand at a pivotal point, not only to detect but also to interfere with an ongoing seizure activity. Yet, further studies are required to complete the characterization of these

circuits. In particular, the projections and the mechanisms involved in the cessation of seizures remain to be specified. From the few data reported, it appears very likely that these mechanisms differ according to seizures type, and therefore with the circuits that generate the seizure. The possibility that the BG interrupt seizures or control their occurrence via a change in cortical excitability and/or synchronization capacities appears to be the most likely hypothesis, and our laboratory is currently testing this hypothesis. The role of dopaminergic transmission, and its possible plasticity throughout the course of epileptogenesis, are other important issues for further study, using models of epilepsy with spontaneous seizures as well as investigating epilepsy patients with dopaminergic deficits. Finally, experimental studies with different animal models have led to initial clinical trials using high-frequency stimulation of the STN in epileptic patients. Clinical studies are under way to better determine whether this type of stimulation can be used as a possible treatment in patients with drug-resistant seizures, who cannot undergo resective surgery. The precise targets, as well as the stimulation parameters and protocol of stimulations – currently derived from studies in patients with Parkinson disease – will certainly need to be adapted for control of epileptic seizures, and possibly customized to each epileptic patient.

See also: **Metabolic Changes**: Role of Metabolic Substrates and Acid-Base Balance in Seizures; **Networks**: The Substantia Nigra in the Control of Seizures; **Treatments (non-Antiepileptic Drugs)**: Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy; Electrical Stimulation.

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The Substantia Nigra in the Control of Seizures

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Introduction

Most seizures are self-limited events as they stop within minutes. Status epilepticus (SE) is relatively rare. These observations suggest that within the brain, there are intrinsic networks that prevent seizures from continuing indefinitely. Clinical and experimental studies have identified several possible networks, remote to the seizure focus, which may help suppress their initiation, abort the spread of seizures, or even lead to their termination. These intrinsic seizure-controlling networks appear to be operant in many seizure syndromes, suggestive of a commonality in the endogenous systems involved in seizure control. By determining the degree of functionality of the seizure suppressing systems in various epileptic conditions, targeted strategies can be designed to stop

circuits. In particular, the projections and the mechanisms involved in the cessation of seizures remain to be specified. From the few data reported, it appears very likely that these mechanisms differ according to seizures type, and therefore with the circuits that generate the seizure. The possibility that the BG interrupt seizures or control their occurrence via a change in cortical excitability and/or synchronization capacities appears to be the most likely hypothesis, and our laboratory is currently testing this hypothesis. The role of dopaminergic transmission, and its possible plasticity throughout the course of epileptogenesis, are other important issues for further study, using models of epilepsy with spontaneous seizures as well as investigating epilepsy patients with dopaminergic deficits. Finally, experimental studies with different animal models have led to initial clinical trials using high-frequency stimulation of the STN in epileptic patients. Clinical studies are under way to better determine whether this type of stimulation can be used as a possible treatment in patients with drug-resistant seizures, who cannot undergo resective surgery. The precise targets, as well as the stimulation parameters and protocol of stimulations – currently derived from studies in patients with Parkinson disease – will certainly need to be adapted for control of epileptic seizures, and possibly customized to each epileptic patient.

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Introduction

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seizures acutely and to prevent seizure recurrences. Indeed, the idea that there may be specific networks that are involved in the control of seizures is very exciting, since genetic or epigenetic factors or damage to these networks may explain why some patients respond to anticonvulsant therapy whereas others do not (despite having the same seizure syndrome). Furthermore, the ability of the endogenous systems to effectively influence seizure severity or duration may be age-dependent and sex-dependent, and may be influenced by prior experience to seizures. In this laboratory, we have focused our investigations on the identification of seizure controlling circuits that involve the basal ganglia and especially the substantia nigra pars reticulata (SNR), as a function of age and sex.

Background

The notion that the basal ganglia may participate in the expression and control of seizures is long standing. Initial studies focused on striatal–thalamocortical interactions. Subsequent experimental studies suggested that basal ganglia networks that involve the subthalamic nucleus and the SNR are critically involved in the control of seizures. Further, in humans, there is emerging evidence that stimulation of the subthalamic nucleus may have beneficial effects in some patients with intractable epilepsy. The effects of subthalamic nucleus stimulation are mediated through the SNR which is one of its major output targets.

To fully appreciate how the SN may act as a seizure controller, we must first detail some of the important aspects of its functional anatomy. The SN is composed of two distinct parts, the pars compacta (SNC) comprised of dopaminergic neurons, and the SNR, which is the larger subdivision and is predominantly populated by GABAergic neurons with high baseline firing rates. The SN is part of the basal ganglia, which is composed of the striatum (caudate/putamen complex), the globus pallidus, the entopeduncular nucleus, and the subthalamic nucleus. These nuclei have widespread connections with the cerebral cortex, thalamus, and brainstem (**Figure 1(a)**), and are associated with a variety of functions including motor control, cognition, emotions, and learning. The striatum integrates inputs originating from the cortex with those from limbic and extrapyramidal circuits, and conveys this information to the subthalamic nucleus and the SNR through two different routes, the 'direct' (striatonigral) and 'indirect' (striato-pallido-subthalamo-nigral) pathways. The SNR then directs information either to the thalamus, superior colliculus, brainstem, or back to the striatum. Indeed, the SNR has been proposed to 'influence widespread cerebral mechanisms in synchronization and excitability.'

The experimental evidence that the SN may be a critical site in the control of seizures has emerged over the last 35 years, initially from studies in adult male

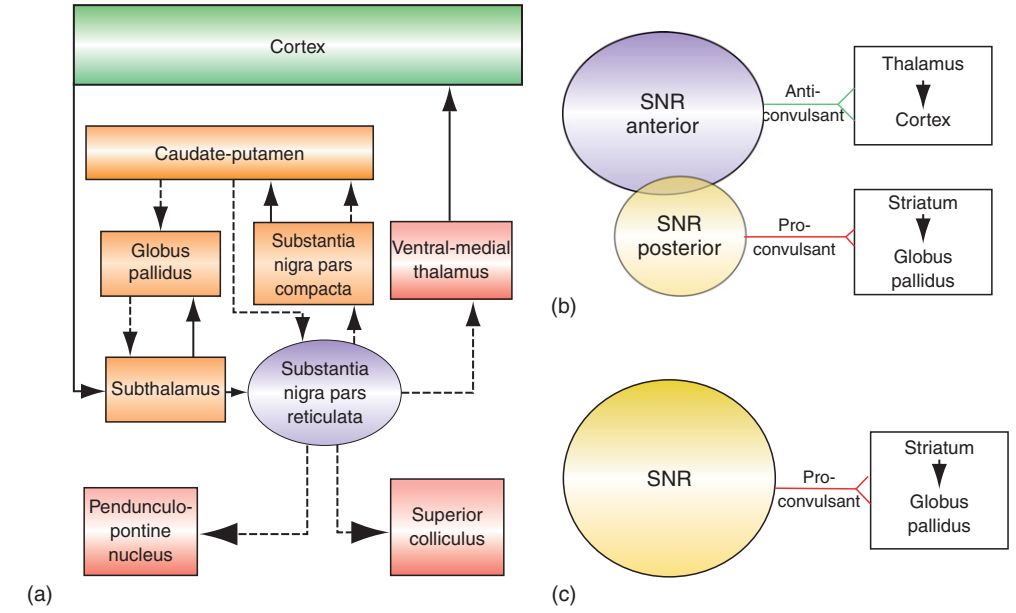


Figure 1 (a) Connections of the substantia nigra pars reticulata (SNR). Solid arrows = excitatory connections, broken arrows = inhibitory connections. (b) and (c) are a representation of the proposed $GABA_A$-sensitive SNR networks involved in seizures as a function of age. Panel (b) shows the adult SNR and the efferent networks whereas panel (c) shows the SNR efferent networks of a P15 male pup. Note that in the P15 male pup there is no topographic segregation of the effects of muscimol infusions in the SNR. This is also the case in P15 females, but in this case muscimol infusions do alter the seizure thresholds. In older female rats, the $SNR_{anterior}$ mediates $GABA_A$ sensitive anticonvulsant effects while the $SNR_{posterior}$ is ineffective.

animals. One pivotal experimental study, using the kindling model of epilepsy, reported that during a kindled seizure, the electrical activity propagated to involve the substantia nigra bilaterally; this observation supported the view that the SNR is involved in the generalization of limbic seizures, either as a facilitator or a suppressor. This conclusion was also supported by a subsequent study, using the [^{14}C]-2-deoxyglucose (2DG) technique, which showed striking increases in the metabolic activity of the SNR bilaterally during generalized kindled seizures. Increased metabolic activity of the SNR was similarly reported in other animal models of epilepsy, especially (but not exclusively) when the animal experienced generalized seizures. Subsequently, in a series of elegant studies, Iadarola and Gale showed that pharmacological treatments that increase GABAergic transmission in the SNR bilaterally suppressed a variety of experimentally-induced seizures in adult rats. At about that time, we showed in this laboratory that the metabolic changes that occur in the SN during seizures were age-dependent. Furthermore, the enhancement of GABAergic transmission in the SNR in postnatal day (P) 15 male rats facilitates the development of seizures – in contrast to its seizure suppressing effects in adult male rats. Over the years, we have provided further evidence that the ability of the SNR to control the expression of seizures in rats is age-dependent and sex-dependent, and influenced by prior history of seizures, especially the occurrence of SE early in life.

Methods

In our laboratory, we have used several methodological approaches to investigate the role of the SNR in the control of seizures as a function of age and sex which are summarized below.

Flurothyl-Induced Seizures

Flurothyl is a volatile convulsant agent which has been used in this laboratory for more than 20 years to establish the age-specific and sex-specific effects of the SNR on seizures. Rats are placed in an airtight 9.2 l chamber, and the liquid flurothyl is delivered via a Harvard pump at the constant rate of 20 μl min^{-1} onto a filter pad from which it vaporizes. An advantage of this model is that flurothyl-induced seizures always occur, in all animals. As flurothyl is constantly infused, changes in latency mirror the amount of flurothyl administered. Therefore, the clonic and tonic seizure thresholds are determined by calculating the amount of flurothyl used to induce seizures (latency x rate of infusion). Changes in the threshold and seizure severity can readily distinguish whether any given pharmacological manipulation produce a facilitation or partial suppression (delay) of convulsions.

Intracranial Drug Administration

Rats are anesthetized with a combination of ketamine and xylazine i.p., and implanted with cannulae that target the $SNR_{anterior}$ or $SNR_{posterior}$. Rats recover from the surgery for 2 days. For all infusions made via these cannulae, the injected volume is 0.25 μl; the injection time is 2 min per site to minimize drug spread.

[^{14}C]-2-Deoxyglucose (2DG) Technique

We determine glucose uptake (GU) from autoradiograms of brain sections, using a modification of Sokoloff's method. GU reflects general neural activity, with likely contributions from several neural elements. The method uses a radiolabeled glucose analog, [^{14}C] 2-deoxy-D-glucose, as a marker for GU and phosphorylation in the brain.

In Situ Hybridization

Both radioactive and nonradioactive in situ hybridization methods are used to determine changes in $GABA_A$ receptor subunit and NKCC1 and KCC2 mRNAs in the SNR. Signal densitometry with the Scion Image 1.62c software (NIH) is used to compare expression across individual cells.

In Vitro Electrophysiology

To study synaptic $GABA_A$ergic PSCs, we use gramicidin-perforated patch clamp recordings of synaptically stimulated SNR neurons, in the presence of glutamate receptor antagonists (AP5 50 μM, CNQX 10 μM). This method does not perturb the chloride gradient during the recordings. Studies also include characterization of the SNR GABAergic neurons and calcium imaging.

Intracranial Single Neuron Recordings in Freely-Moving Rats

A homemade microelectrode/cannula system is advanced slowly to detect action potentials in the $SNR_{anterior}$. Extracellular signals are amplified 10 000× and filtered between 300 Hz and 10 kHz, displayed both on an oscilloscope and digitized on the computer screen. Digitized signals are acquired, processed, and stored using SciWorks 4.0 (Datawave Technologies). After a stable firing baseline is established, muscimol or other $GABA_A$ergic agents are infused into the SNR via the cannula. The recording is resumed 15 min after the cessation of the microinfusion (to allow for resolution of any changes that are directly produced by the infused volume – e.g., change in position of neurons relatively to the recording microwires – that might affect apparent neuronal activity due to mechanical/topography reasons). The recording continues for the next 45 min to determine the drug effects on neuronal activity in $SNR_{anterior}$. Individual action potentials from different

neurons are distinguished based on several parameters, including action potential amplitudes, relative latencies of the negative and positive component peaks, and interspike interval. We create firing rate histograms for the cells over time following $GABA_A$ergic drug infusions.

Quantitative Real-Time-PCR

qRT-PCR reactions are carried out using specific primers for the $GABA_A$ receptor subunits (α1–α6, β1–β3, γ1, γ2l, γ3, δ, ε, π, θ), the $GABA_C$ receptor subunits ρ1–ρ3, the chloride cotransporters and channels NKCC1, KCC1, KCC2, and ClC-2, and the carbonic anhydrase VII (Car-7). We use the ABI Prism 7000 real-time PCR and gene-specific qRT-PCR expression assays developed and standardized by Applied Biosystems.

Immunocytochemistry

Immunochemistry for a variety of $GABA_A$ receptor subunits, parvalbumin, tyrosine hydroxylase, and chloride cotransporters are performed with established protocols to determine the expression of these proteins. Cell counts are performed and signal densitometries are done using the Scion Image NIH 2.62c software.

Results

Studies in Adult Rats

In adult male rats, the involvement of the SNR in seizures was recognized from metabolic mapping studies using 2DG. Among brain structures, the increase in GU in the SNR during different types of seizures is especially striking. While the regional patterns of 2DG uptake vary in different seizure models, the SNR is always activated in adult rats, especially during generalized seizures. Studies using localized drug applications into the SN in rodents have been the bedrock for gaining an understanding of the subcortical mechanisms regulating seizures. Many agents have been infused into the SNR and the results are summarized in Table 1.

In the adult SNR, bilateral microinfusions of the $GABA_A$ agonist muscimol into the SNR revealed the presence of two functionally distinct regions, $SNR_{anterior}$ and $SNR_{posterior}$, that may have different roles in seizure control that are gender-specific (Figure 2). In adult male rats, microinfusions of muscimol into the $SNR_{anterior}$ produce anticonvulsant effects, whereas microinfusions into the $SNR_{posterior}$ are proconvulsant. It should be emphasized that the infusions must be administered bilaterally into the SNR; unilateral infusions are without any effect. Indeed, in adult male rats, a 2DG metabolic study showed that during the evolution of a generalized seizure, the posterior part of the SNR ($SNR_{posterior}$) becomes involved

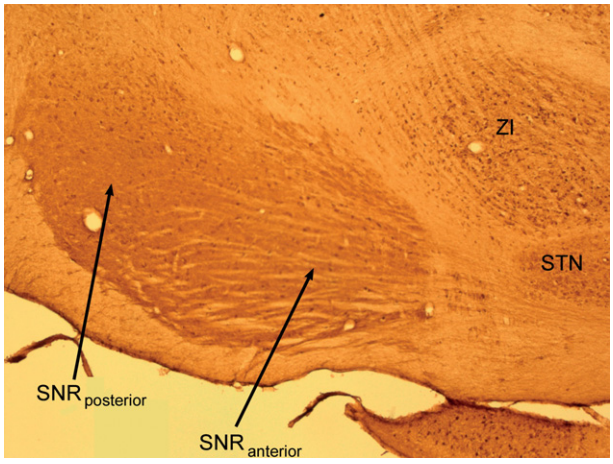


Figure 2 The two SNR regions ($SNR_{anterior}$ and $SNR_{posterior}$) are depicted on a sagittal section stained with GAD67. ZI = zona incerta; STN = subthalamic nucleus.

Table 1 Site-specific effects of various treatments in SNR on flurothyl seizures comparing the effects of various agents on seizures in adult and immature male rats

Agent	*Adult $SNR_{anterior}$*	*Adult $SNR_{posterior}$*	*PN 15 SNR*
Muscimol	Anticonvulsant	Proconvulsant	Proconvulsant
ZAPA	Anticonvulsant	Proconvulsant	Biphasic effects
Bicuculline	Proconvulsant	No effect	Proconvulsant
Zolpidem	Anticonvulsant	No effect	Anticonvulsant
GVG	Anticonvulsant	Proconvulsant	Anticonvulsant
Baclofen	No effect	Not tested	Anticonvulsant
CGP 35 348	No effect	Not tested	Proconvulsant
AP7	Anticonvulsant	Proconvulsant	No effect
Electrical stimulation	Anticonvulsant	No effect	Anticonvulsant

Muscimol, $GABA_A$ receptor agonist at high-affinity and low-affinity sites; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, $GABA_A$ receptor agonist at high-affinity site; Bicuculline, $GABA_A$ receptor antagonist at low-affinity site; ZAPA ((*Z*)-3-[(aminoiminomethyl)thio] prop-2-enoic acid), $GABA_A$ receptor agonist predominantly at low-affinity site and weakly at high-affinity site; Baclofen, $GABA_B$ receptor agonist; CGP 35348, $GABA_B$ receptor antagonist; GVG (γ vinyl-GABA), increases GABA levels by inhibiting GABA transaminase; Zolpidem, agonist at the benzodiazepine receptor site, linked to the low-affinity site; AP-7 (DL-2-amino-7-phosphonoheptanoic acid).

just before the expression of the seizure, while the anterior part ($SNR_{anterior}$) is activated during the seizure – perhaps signaling the imminent termination of the seizure. 2DG autoradiography shows that the pathways by which the two SNR regions mediate their effects are different (**Figure 1(b)**). In the $SNR_{anterior}$, muscimol infusions decrease 2DG uptake in the striatum, sensorimotor cortex, and ventromedial thalamus. In contrast, when muscimol is infused into the $SNR_{posterior}$, glucose utilization in the dorsal striatum and globus pallidus is increased and glucose utilization in thalamus decreases. In female rats, muscimol produces anticonvulsant effects when infused in $SNR_{anterior}$ but does not change the seizure threshold when infused in the $SNR_{posterior}$.

The prevailing hypothesis to explain the effects of $SNR_{anterior}$ on seizures is that during a clonic seizure there is increased GABA release from striatonigral terminals which silences SNR neurons and leads to disinhibition of SNR output systems, resulting in suppression of the ongoing seizure. This hypothesis is consistent with anticonvulsant effects of bilateral, strictly local injections of $GABA_A$ receptor agonists in the $SNR_{anterior}$, and with the results of nigral lesions. Suppression of the SNR glutamatergic input from the subthalamic nucleus, by local microinfusions of glutamate receptor antagonists (e.g., AP7, an *N*-methyl-D-aspartate [NMDA] receptor antagonist) into the SNR, decreases the firing rate of SNR neurons and induces anticonvulsant effects.

Disinhibition of the nigral output systems, such as the superior colliculus and the pedunculopontine tegmental nucleus, is essential for the muscimol induced $SNR_{anterior}$ anticonvulsant effect. Accordingly, bicuculline infusions in the superior colliculus or pedunculopontine nucleus are anticonvulsant but localized muscimol infusions or lesions are proconvulsant.

Age and Sex Specific Effects

The functional difference (with respect to seizure control) between the $SNR_{anterior}$ and $SNR_{posterior}$ is not seen early in life. Studies have shown that the SNR goes through many anatomical and functional changes throughout development, leading to different age-related and sex-related effects on seizure control. For example, in P15 male rats the SNR does not show any increase in 2DG uptake during seizures; these animals have a high propensity to develop SE, suggesting that the 'adult-like' homeostatic mechanisms involved in seizure control are not properly functioning. Indeed, in P15 and P21 rats of both sexes, there is only one $GABA_A$ sensitive functional region. The functionality of the SNR on seizure control at these ages is sex-specific; infusions of muscimol are proconvulsant in males and have no effect on seizure in females in the flurothyl model. The functional separation occurs at about P25 in both sexes. The adult patterns of the SNR effects on seizures are already present at P25 in females and emerge at P30 in males.

Our findings also support the view that the dynamic changes occurring in the SNR throughout development are sex-specific. In our laboratory we have further evaluated the possible involvement of sex hormones in these sex-specific developmental changes that affect the SNR-mediated seizure control. Testosterone has been shown to play a key role in the development of the male SNR phenotype at distinct developmental stages. It is well recognized that in rats there is a surge of testosterone perinatally, followed by relatively stable levels up to the end of third postnatal week when the testosterone levels drop. From P25, testosterone levels start rising again continuously up to adulthood. The role of perinatal testosterone in the development of the SNR proconvulsant region is supported by experiments that prevent the effect of testosterone by neonatal castration in males, or by blocking endogenous androgen receptor (AR) activation by the AR antagonist flutamide in newborn male rats. Male rats that are castrated on the day of birth (to avoid the postnatal surge of testosterone) or treated with flutamide at P0–2 develop the female phenotype (no muscimol mediated effect of the SNR on seizure thresholds at P15). In females or neonatally castrated males, exogenous treatment with testosterone, or its metabolites dihydrotestosterone and estrogen, during the first postnatal week (and especially during the first 3 days after birth) results in the development of the male SNR phenotype. Importantly, the effects of testosterone change with maturation and are biphasic. Testosterone administration in male rats between P18 and P25 (during the period when natural testosterone levels drop) accelerates the onset of the anticonvulsant SNR region, suggesting that at later developmental stages, testosterone regulates the normal, later appearing maturational pattern of the $SNR_{anterior}$.

$GABA_A$ Receptor Changes Early in Life

It is likely that the sex-related effects of muscimol may be influenced by the specific subunit composition of SNR $GABA_A$ receptors during development. In situ hybridization studies found distinct developmental and spatial patterns of $\alpha 1$ and $\gamma 2L$ $GABA_A$ receptor subunit mRNA expression in the SNR in male rats. In adult male rats, neurons located in the $SNR_{anterior}$ contain higher amounts of $\alpha 1$ and $\gamma 2L$ mRNA hybridization grains and have a higher GABA content per neuron than neurons in the $SNR_{posterior}$. At P15, the labeling of neurons is relatively more diffused throughout the anterior and posterior SNR regions, with cells showing moderate numbers of hybridization grains for both subunits. The finding of age-related, differential distribution of $\alpha 1$ and $\gamma 2L$

subunit mRNAs in the SNR suggests that $GABA_A$ receptor heterogeneity may play a role in the age-specific and site-specific effects of $GABA_A$ergic agents in the SNR on seizures. Subsequent studies showed that in P15 and P30 rats, the $GABA_A$ α1 subunit mRNA expression and GABA immunoreactivity (IR) in the SNR were higher in female rats than in male rats. A regional difference in the $GABA_A$ α1 subunit mRNA expression and GABA IR with regards to $SNR_{anterior}$ versus $SNR_{posterior}$ was not observed in P15 rats, but at P30 the $GABA_A$α1 subunit mRNA expression and GABA IR was higher in the $SNR_{anterior}$ in both male and female rats.

Another important factor in the development of the GABAergic system is the influence of the potassium chloride cotransporters (**Figure 3**). The potassium chloride cotransporter type 2 (KCC2) is known to play a pivotal role in the developmental switch of GABA chloride channel conductance. In the immature brain, GABA has a depolarizing action, which results from the efflux of chloride from the intracellular compartment upon activation of $GABA_A$ receptors. This depolarization results in the activation of voltage-sensitive calcium channels (VSCCs) leading to a large influx of calcium into the cell – which in turn activates calcium-sensitive intracellular events that promote differentiation and regulate gene transcription. During development, the amount of KCC2 present on neuronal cells increases. The developmental increase in KCC2 expression decreases intracellular chloride, via outward cotransport of K^+ and Cl^-, and switches the $GABA_A$ receptor response from depolarizing to hyperpolarizing. In addition to the developmental changes in $GABA_A$ receptor makeup, the accompanying changes in KCC2 expression can also influence the responsiveness of SNR neurons to $GABA_A$ergic agents.

Recently the expression profile of KCC2 in the SNR has been detailed. Several variables, including age, sex, SNR region, and hormonal milieu, were shown to affect the expression of KCC2. Using in situ hybridization, it has been shown that KCC2 mRNA increased between P15 and P30 in both males and females. In males there were no regional SNR differences at P15, but by P30 and in adulthood there was a consistently greater amount of KCC2 mRNA in the $SNR_{anterior}$ than in the $SNR_{posterior}$. Females exhibited similar developmental change as the males, with no anterior–posterior difference at P15, but a greater amount of KCC2 mRNA in the $SNR_{anterior}$ compared to the $SNR_{posterior}$ by P30. Overall, females had greater KCC2 mRNA (both regions of the SNR) compared to males. To determine the source of this gender difference, additional studies examined the role of sex hormones on the developmental profile of KCC2 expression. In P15 rats, KCC2 expression was examined after systemic treatment (4 or 52 h) with testosterone, dihydrotestosterone, and 17β-estradiol. Both testosterone and dihydrotestosterone increased KCC2 expression in male and female rats at both time points. The effects of 17β-estradiol were sex-specific. In males, it decreased KCC2 expression whereas in females there was no effect on

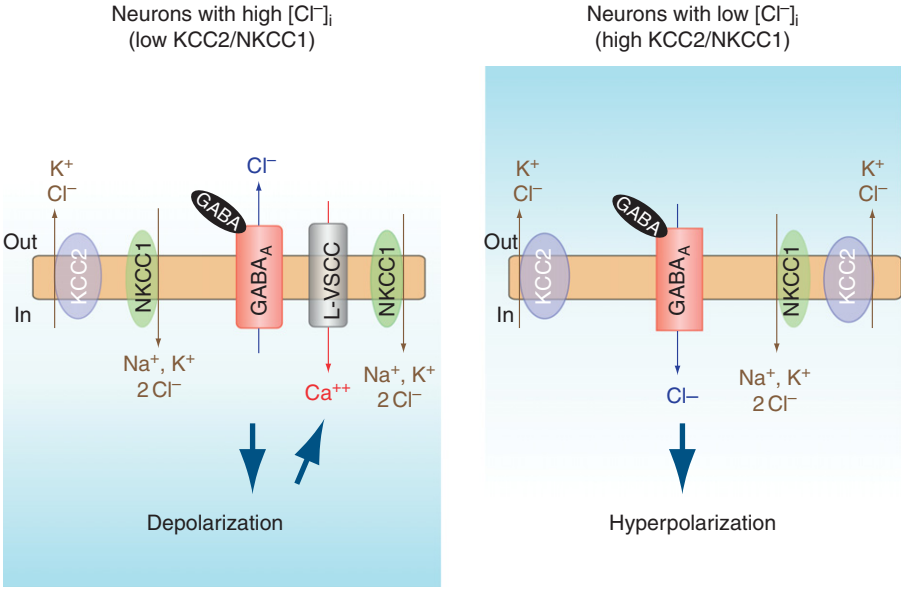


Figure 3 The developmental switch in $GABA_A$ signaling is driven by shifts in the balance of the activity of potassium chloride cotransporters, KCC2 and NKCC1. *Left panel:* In immature neurons, there is high relative expression of NKCC1 over KCC2 which allows the intracellular accumulation of Cl^-. As a result, $GABA_A$ receptor activation leads to depolarizing neuronal responses and activation of L-type VSCCs (L-VSCC) increasing intracellular calcium and activating calcium-signaling processes. *Right panel*: In more mature neurons, KCC2 expression increases and overrides NKCC1 effects, decreasing intracellular $[Cl^-]_i$. As a result, $GABA_A$ receptor activation hyperpolarizes neurons, but cannot activate L-VSCCs or calcium signaling processes.

KCC2 expression. Pretreating SNR neurons with specific inhibitors for either $GABA_A$ receptors or L-VSCCs, it has been shown that the sex-specific patterns of KCC2 regulation by estradiol were linked to the sexually dimorphic $GABA_A$ receptor signaling pathway. Specifically, estradiol decreased KCC2 mRNA only in neurons in which $GABA_A$ receptor activation caused depolarization and activation of L-VSCC, but not in those cells that were hyperpolarized by $GABA_A$ receptor agonists. This differential expression of KCC2 may play a role in the functional compartmentalization of the developing SNR.

Considering the dynamic changes in sex steroid receptors, $GABA_A$ receptors, and KCC2 expression during development, it is apparent that the SNR is a sexually dimorphic brain region. These changes in part reflect the in vivo differences in seizure threshold and susceptibility found between the sexes and during development. Although these differences are readily seen in the flurothyl seizure threshold testing, it is important to further examine the functional effects of these changes at the cellular level. To determine if these sex-related changes are indeed causing functional aberrations in the developing SNR, cellular electrophysiological and calcium imaging experiments have been used. In the SNR, GABAergic neurons of both sexes show spontaneous repetitive firing. These cells also do not show any time-dependent inward rectification to hyperpolarizing current steps, and may in fact also show a sag and inward current of lower amplitude compared to dopaminergic cells. At P14–17, when the male SNR has proconvulsant effects on seizure threshold and females have no effect, SNR neurons were recorded with the gramicidin-perforated patch clamp technique (**Figure 4**). Both males and females had similar resting membrane potentials, spontaneous firing rates, and responses to depolarizing and hyperpolarizing current injections. When the SNR was exposed to muscimol, male SNR cells showed a depolarizing response while female SNR neurons became hyperpolarized. In addition, calcium-imaging data showed that male SNR neurons have an associated increase in intracellular calcium when they depolarize. These results match well with the lower KCC2 expression in males at P15.

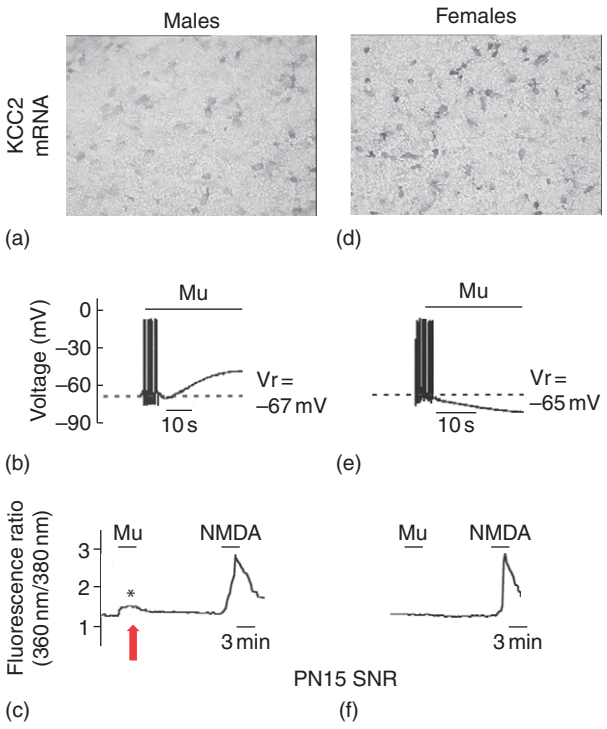


Figure 4 Sex differences in $GABA_A$ signaling in GABAergic neurons of the PN15 rat SNR. The low expression of KCC2 in rat P15 male SNR (a: in situ hybridization) leads to depolarizing responses to muscimol (Mu) (b: gramicidin-perforated patch clamp) and increments in intracellular calcium (red arrow in c: fura-2AM imaging). Female P15 rat GABAergic SNR neurons have higher KCC2 expression (d: in situ hybridization), which triggers muscimol-induced hyperpolarizations (e: gramicidin-perforated patch clamp), without altering calcium intracellularly (f: fura-2AM imaging). In the fura-2AM experiments, NMDA application triggered calcium rises in both male and female neurons. Reproduced from Galanopoulou AS, Kyrozis A, Claudio OI, Stanton PK, and Moshe SL (2003) Sex-specific KCC2 expression and GABA(A) receptor function in rat substantia nigra. *Experimental Neurology* 183: 628–637, with permission from Elsevier.

Future Directions

Given that the SNR is a principal player in the endogenous mechanisms for seizure control, it follows that a deviation from normal developmental processes of the SNR may have profound influences on long-term outcomes on seizure control. Epidemiological studies suggest that early in life (particularly in the neonatal period), the brain is highly susceptible to seizures and especially SE. The increased propensity of the developing brain to experience SE may be related to immaturity of the networks (such as the SNR-based network) that can suppress recurrent seizures in adults. Understanding the spectrum and progressive nature of SE-induced changes in brain function may have important implications in the design of treatments aimed at disease modification. These treatments can potentially be used over relative short periods, thus avoiding the potentially detrimental effects of long-term drug administration.

However, the mechanisms available to terminate seizures in otherwise normal healthy brain may not be available to terminate seizures in a brain that has already experienced seizures. The effectiveness of the SNR to influence seizure duration and recurrences appears to be dependent on previous seizure history. In adult male rats, amygdala kindling modifies the firing pattern of SNR neurons, switching them into a burst firing mode. Also, in adult male rats, SE induced by various

means (flurothyl, pentylenetetrazol, lithium–pilocarpine) results in acute SNR lesions. Long-term studies in the lithium–pilocarpine model show that, during the latent phase prior to the emergence of spontaneous seizures, there are marked, chronic increases in SNR metabolic activity. This observation suggests that the SNR may be involved in the chronic reorganization of brain circuits leading to the development of spontaneous seizures.

Preliminary data from our laboratory indicate that the development of the $SNR_{anterior}$ is distorted if infant rats experiencing three episodes of kainic acid induced SE (3SE) prior to P6 (irrespective of sex) by preventing the emergence of the $SNR_{anterior}$ $GABA_A$-sensitive anticonvulsant region at P30, as revealed by localized microinfusions of muscimol or zolpidem. Three SE are not associated with acute neuronal injury in SNR. However, in the SNR, 3SE rats increase the expression of the chloride cotransporter KCC2 mRNA; accelerate the sex-specific switch of synaptic $GABA_A$-ergic responses from depolarizing to hyperpolarizing (referred to here as E_{GABA} switch); induce changes in $GABA_A$ receptor subunit expression, with downregulation of the expression of $\alpha 1$ and $\gamma 1$ subunits at P30; and attenuate the responsiveness of $SNR_{anterior}$ neurons to $GABA_A$ agents in vivo and in vitro at P30. If 3SE are induced in P14-16 male rats (a period during which the E_{GABA} switch naturally occurs), the $SNR_{anterior}$ $GABA_A$-sensitive anticonvulsant region develops normally. The depolarizing effects of GABA are important for neuronal differentiation. The data suggest that, during a specific developmental window, the 3SE-induced increases in KCC2 expression and the resultant early appearance of E_{GABA} switch may shorten the period during which the normal GABA-mediated differentiation of SNR occurs. Further, the data imply that the detrimental effects of 3SE occur within days after SE and provide a possible means for intervention based on short-duration treatments that may prevent or revert the 3SE-induced increases in KCC2 expression.

Thus, the challenge for the future will be to identify early life insults that can potentially alter the normal development of SNR seizure controlling networks. Accomplishing this goal would constitute a major advance in the study of the critical role of the SNR as an endogenous seizure controller, and would permit us to address some of the mechanisms by which remote early life events can lead to reduced seizure thresholds and chronic epilepsy – issues that are currently poorly understood. To translate the experimental data to clinical practice, it is important to perform studies to identify the contribution of the SN in seizures in humans, especially early in life. To achieve this goal, imaging techniques with better than currently available resolution are needed to determine the role of the SN before, during, or after seizures. To date, PET imaging and NMR spectroscopy are two techniques that have been used to study the role of the basal ganglia networks in the control of generalized seizures in adults only; there are many limitations in carrying out such studies in infants and children, including issues related to sedation. Progress in technology is a much needed step to better understand the role of the diffuse systems involved in the control of seizures (such as the SN) and thus institute age-specific and possibly sex-specific interventions.

Our current research has provided mechanistic explanations for the age-specific and possibly sex-specific effects of neuroprotective/disease modifying agents that can be used to prevent or restore some of the detrimental effects of early life SE on an endogenous system involved in seizure control. These studies, to understand the function of the SNR during development in the male and female brain, should also offer significant insights to the field of developmental biology as it pertains to epigenetic influences on the development of the SNR, a sexually dimorphic structure. We hope that our studies will lead to a better understanding of the role of the SNR in the expression of comorbidities associated with epilepsy. Indeed, the SN may play a significant role in depression, integration of cognition, and expression of movement disorders such as stereotypies. The latter is a prominent feature of autism, a condition in which seizures frequently coexist.

Acknowledgements

Supported by NIH NINDS research grants NS20253, NS36238, NS45243, NS 62947, NS056093 and grants from the Rett Syndrome Research Foundation, P. A. C. E. and the Heffer Family Foundation.

See also: **Hormones and Gender**: Sex Differences in Seizure Sensitivity; **Models**: Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation.

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NEUROGENESIS

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Adult Neurogenesis and Mesial Temporal Lobe Epilepsy

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> In adult centers the nerve paths are something fixed, ended, immutable. Everything may die; nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree.
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Introduction

It was long believed that no new cells were born in the adult brain, and as a result, injury and neurodegeneration were irremediable. Evidence slowly accumulating over the past 40 years has overturned this dogma and suggests a role for adult-born cells both in normal cognition and after brain injury. Germinative zones persist in the adult mammalian forebrain subventricular zone (SVZ) and hippocampal dentate gyrus (DG). These areas contain neural progenitors that continue to generate neurons, as well as glia, throughout life. Adult-born neurons migrate long distances from the SVZ to the otherwise mature olfactory bulb, or a short distance within the DG, and integrate into neural circuits probably involved in olfactory function, and learning and memory, respectively. Normal aging decreases but does not extinguish this process, and decreased neurogenesis has been implicated as a potential mechanism involved in depression and dementia. Further, brain injuries as diverse as stroke, trauma, and seizure stimulate neuronal production and subsequent migration to sites of damage. Adult neurogenesis thus represents a novel mechanism for brain plasticity which not only modifies neural activity but also may rejuvenate or repair the injured brain.

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NEUROGENESIS

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Adult Neurogenesis and Mesial Temporal Lobe Epilepsy

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The causes and pathophysiology of mesial temporal lobe epilepsy (mTLE) are poorly understood. Because this disorder involves a region of persistent neuronal birth (the DG), study of mTLE provides a unique opportunity to examine not only how injury influences adult neurogenesis but also the resulting consequences for neural plasticity, disease pathophysiology, and functional recovery. mTLE is a progressive disorder that is associated with intractable complex partial seizures and disturbed short-term memory. In many cases, the epileptic focus is presumed to be located in the hippocampus and consequently may involve the population of neural progenitors in the DG. The epileptic focus, further, is commonly marked by mesial temporal sclerosis, with loss of neurons in the Cornu Ammonis (CA) and dentate hilus. Seizures are known to acutely stimulate adult neurogenesis, so neural proliferation has been implicated in mTLE – but whether its role is causative, reactive, or compensatory is unclear. Seizures may trigger aberrant adult neurogenesis, recruiting new/immature cells to epileptogenic neural pathways. Alternatively, increased adult neurogenesis may be merely reactive (or even vestigial), responding ineffectively to cell damage. Also possible, and indeed the best of alternatives, is that adult neurogenesis may represent an attempt by the brain to replenish damaged populations and improve recovery. Elucidating how neurogenesis and epilepsy are related, therefore, has the potential to elucidate, counteract, and even prevent epileptogenesis, as well as to clarify how epilepsy impairs learning and memory.

Background

Adult Neurogenesis in the SVZ and DG

The earliest report of adult neurogenesis dates to the early twentieth century, when mitotic figures in the adult rodent brain were identified as evidence of proliferating neural progenitors. In the mid-1960s, mitotic labeling with tritiated thymidine suggested cells in the SVZ and DG continue to proliferate in the adult rodent, and electron microscopy later confirmed these newborn cells as neurons. Since then, adult neurogenesis has been found in the DG and SVZ of every mammalian species examined, including humans, but most data are based on rodent studies. In these DG and SVZ regions, neural progenitors compose a defined population of small, radial glia-like cells. In the DG, these stem like cells send out long processes that may help guide migrating neuroblasts or the outgrowth of their processes. At any given time, only a small proportion of progenitors are actively dividing. This proliferation appears tightly regulated in vivo and is likely modulated by a number of growth and neurotrophic factors, among other stimuli.

Although the SVZ and DG both support germinative zones, the plasticity of adult-born cells differs widely at each site. Adult neurogenesis occurs extensively along the rostral SVZ of the lateral ventricles. From this niche, neuroblasts migrate tangentially through the rostral migratory stream to the olfactory bulb. In the bulb, they disperse and differentiate into granular and periglomerular interneurons. This prolific process substitutes as much as 3% of the olfactory interneuron population daily. Further, SVZ progenitors are true neural stem cells (NSCs), displaying multipotentiality in vitro. Stroke and other neurological insults recruit neuroblasts from the ipsilateral and contralateral SVZ to areas of injury, where they may survive and integrate in damaged neural circuits.

By comparison, neurogenesis in the DG is restricted to the subgranular zone. Neuroblasts subsequently migrate a short distance to the granule cell layer where they mature as dentate granule cells (DGCs). Proliferation occurs at a much lower rate in the DG than in the SVZ, replacing a mere 0.05% of dentate granule cells daily. And while injury similarly stimulates neurogenesis in the DG, adult-born neurons are confined to the hippocampus and only appear capable of differentiating into DGCs and not other neuronal subtypes. Whereas SVZ neuroblasts are thought to participate in olfactory discrimination and memory, dentate neuroblasts are implicated in hippocampal learning and memory.

Aberrant Neurogenesis in mTLE

Rodent models of mTLE show a dramatic increase in DGC neurogenesis (5–10 fold) after chemoconvulsant- or electrically induced status epilepticus (SE). After a latent period of several days, the newborn neurons migrate, differentiate, and display markers of mature DGCs. Many of the DGCs generated after SE survive for months to years. At the same time, seizure-related excitotoxicity causes extensive damage and neuronal loss, and the epileptic hippocampus undergoes considerable network reorganization. As a result, the relationship between dentate neurogenesis and neural plasticity has been the focus of considerable research.

Seizure-induced DGC neurogenesis is associated with at least three forms of aberrant plasticity during epileptogenesis. The first is mossy fiber sprouting, the remodeling of DGC axons (mossy fibers) into the supragranular molecular layer of the DG. This remodeling is believed to result in recurrent excitatory circuits and subsequent hippocampal hyperexcitability. Although initially implicated in sprouting, the role of newborn cells in this process appears to be more complex than originally suspected (see below). Another form of plasticity, one that seems to involve only adult-born cells, is the persistence of hilar basal dendrites. Mature DGCs typically extend only a single apical dendrite into the molecular layer, but in experimental mTLE models, a much larger percentage show basal dendrites. Recent work suggests that these are

all adult-born DGCs, and the basal dendrites persist indefinitely. A third form (or two unrelated forms) of abnormal plasticity involves DGC layer dispersion and the finding of DGCs in ectopic locations in the dentate hilus and molecular layer after an epileptogenic insult. At least in the latter instance, the heterotopic appearance of DGCs appears limited to effects on DGC progenitors. Ongoing investigations in these areas are discussed under the 'Recent Results' section.

Methodological Approaches

The study of adult NSCs and neurogenesis first and foremost involves the ability to identify proliferating and migrating cells. The simplest way to label dividing cells is to immunostain them, using an antibody that recognizes a protein expressed only during active cell cycling, such as Ki67, proliferating cell nuclear antigen (PCNA), or mini-chromosome maintenance protein-2 (MCM-2). This approach, however, only allows a single 'snapshot' of the dividing cells and does not allow one to determine their eventual phenotypes. The time-honored approach for labeling dividing cells, then and determining their phenotypes after they differentiate, has been labeling with tritiated thymidine, a hydrogen radionuclide-labeled nucleotide that is incorporated into the DNA of dividing cells and can later be detected by autoradiography. This approach proved cumbersome and insensitive, however, and was largely replaced by the development of an antibody to the thymidine analog, bromodeoxyuridine (BrdU). BrdU also is taken up in dividing cells but is more easily identified using immunohistochemistry, with an anti-BrdU antibody. To 'see' the BrdU, DNA must first be denatured to single strands, usually by hydrochloric acid treatment so that the antibody can bind to the BrdU. BrdU can be used to 'pulse' label dividing cells, by injecting it and looking at the cells within hours. Alternatively, one can use a 'chase' period in which one waits days to weeks after the BrdU is administered to identify cells that had earlier incorporated the BrdU; this approach also allows the investigator to determine the cell types into which the newborn cells developed after becoming postmitotic.

A major disadvantage of tritiated thymidine or BrdU labeling of dividing cells is that only the nuclei of labeled cells are visualized, and thus no information on cell morphology is available. In the nervous system, therefore, the cell bodies, axons, and dendrites of recently dividing cells cannot be visualized. Several strategies have been developed more recently to overcome this limitation. The first is the use of retroviral reporter labeling. Retroviruses only infect dividing cells because they require dissolution of the nuclear envelope to incorporate into the genome. A retrovirus can be engineered to carry a reporter gene, such as green fluorescent protein (GFP) that will label the entire cell within a few days. Because the retrovirus integrates into the genome, it is faithfully passed on to each generation such that all progeny of the initially infected, dividing cell are labeled. One potential disadvantage of this approach is that some retroviral promoters are silenced, or turned off, over time so that the cells can no longer be tracked. This problem has been overcome by using strong internal promoters that are less likely to be silenced for driving expression of GFP (or other reporter genes). In conjunction with the definitive identification of neurons by immunohistochemistry, therefore, the location, differentiation, migration, and regulation of adult-born neurons are now subject to investigation.

Several other new techniques have led to advances in NSC biology and adult neurogenesis. One technique is cell culture to isolate neural progenitors from adult brain, creating neurospheres that display self-renewal and are multipotent in vitro. Another recently developed and powerful approach is to label NSCs and their progeny during embryonic or adult life using transgenic mouse technology. Mice have been generated in which neural progenitor cell- or immature neuron-specific promoters drive reporter gene expression (**Figure 1**). If this technique is combined with inducible gene recombination (e.g., using the Cre–LoxP system), one can permanently and specifically label NSCs and all their progeny in the adult. Such transgenic mice have proven very useful for labeling neural progenitors or adult-born neurons in the mature brain. This approach, which also allows for the specific deletion of 'floxed' genes in these same cells, combined with the retroviral reporter gene method, likely

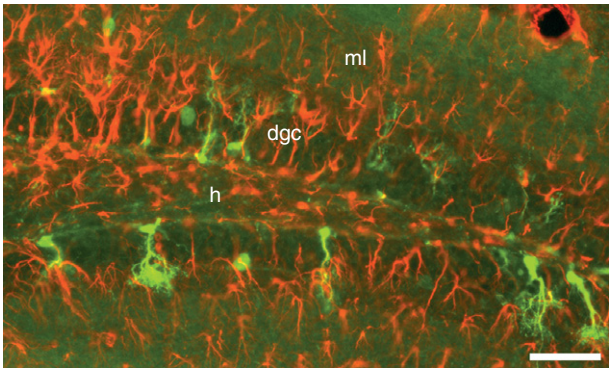


Figure 1 This photomicrograph shows immunofluorescence double labeling for GFP (green) and glial fibrillary acidic protein (GFAP; red) in a coronal brain section through the hippocampal DG of an adult nestin-GFP transgenic reporter mouse. GFP-labeled (green) neural stem cells appear in the dentate subgranular zone and inner DGC layer. GFAP labels astrocytes and neural stem cells, and many of the GFP-positive cells have slightly yellow processes because they also express low levels of GFAP. DGC, dentate granule cell layer; h, hilus; m, molecular layer. Scale bar, 50 μm.

will usher in tremendous advances in the field of adult neurogenesis over the next decade.

Recent Results

Using the adult rat pilocarpine-induced SE model of mTLE, our group first investigated the role of dentate neurogenesis in relation to aberrant mossy fiber sprouting to the molecular layer of the DG and within area CA3. As increased DGC neurogenesis precedes mossy fiber remodeling in rodent seizure models, and the differentiation of newborn neurons occurs in parallel with axonal reorganization, it is plausible that newborn DGCs lead to mossy fiber sprouting. Inhibition of neurogenesis by irradiation prior to SE, however, fails to inhibit mossy fiber remodeling at four weeks after pilocarpine treatment. These data indicate that although newborn neurons may contribute to mossy fiber sprouting, preexisting DGCs are predominantly responsible for these aberrant fibers (and associated synapses). Interestingly, recent work using retroviral reporter labeling of adult-born DGCs, before or after SE, showed that (consistent with our radiation findings) cells labeled after SE did not contribute to sprouting. Those labeled 4 weeks prior to SE, however, did show mossy fiber sprouting, suggesting that differentiating 'juvenile' DGCs (i.e., those born shortly before the SE insult) respond to injury by reorganizing their axons. The fact whether fully mature DGCs are also capable of sprouting remains to be determined. In contrast, hilar basal dendrite formation and the ectopic migration of DGC progenitors appear to be limited to DGCs generated just before or after SE. These abnormalities also may form continually during chronic epilepsy rather than just during the acute epileptogenic phase.

Recent research has explored more closely the role of aberrant neurogenesis in granule cell layer dispersion and migration of adult-born DGCs to ectopic locations in the hilus and molecular layer. This disruption of the normal hippocampal architecture may contribute to the formation of aberrant excitatory circuits. Within 2 weeks of chemoconvulsant-induced SE in adult rodents, DGCs appear in ectopic locations in the DG, accumulating for at least several weeks (**Figure 2**). Rather than migrating from the SGZ to the GCL, chains of neuroblasts extend to the hilus and, to a lesser degree, molecular layer. Of note, the degree of GCL dispersion in human mTLE correlates with decreased expression of mRNA for reelin. Reelin is a secreted neuronal migration guidance cue that is important for embryonic cortical development; it is expressed in the adult cortex and hippocampus for unknown reasons. We have found that reelin protein expression also decreases in experimental mTLE in association with ectopic migration of neuroblasts, and that reelin directly influences chain migration of DG neuroblasts grown in tissue culture. Thus, altered developmental molecules that persist in the adult may contribute to abnormalities of persistent neurogenesis in mTLE.

What is the functional effect of adult-born ectopic DGCs in terms of hippocampal network function after an epileptogenic insult? On a structural level, ectopic

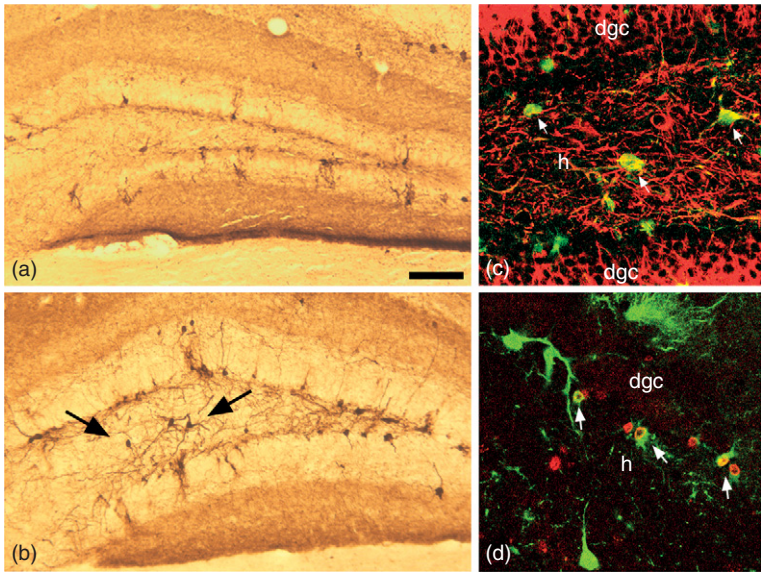


Figure 2 (a, b) Peroxidase immunohistochemistry for GFP in nestin-GFP transgenic mice treated with saline (a) or 2 weeks after pilocarpine-induced SE (b). Note the increase in GFP expression after seizures, especially in the hilus (arrows in b). (c, d) Double label immunofluorescence staining for GFP (green) and the neuronal marker MAP2 (red in b), or the cell proliferation marker BrdU (red in c) in sections from the same animal as in panel (b). Note double-labeled cells (arrows) that are hilar ectopic cells continuing to express nestin while they differentiate into MAP2+ neurons (c), and that also incorporated BrdU given 1 week earlier (7 days after SE; d). DGC, dentate granule cell layer; h, hilus. Scale bar, 100 μm for (a) and (b); 30 μm for (c) and (d).

DGCs possess more persistent basal dendrites and excitatory synaptic inputs than normal granule neurons, supporting a hyperexcitable phenotype of newborn cells in experimental mTLE. The abnormal hilar basal dendrites appear to receive remarkably increased numbers of excitatory inputs compared to typical DGC dendrites. On an electrophysiological level, study of acute hippocampal slices obtained from rats following chemoconvulsant-induced SE confirms the integration of hilar granule-like neurons in hippocampal circuits. In contrast to normal DGCs, however, these granule-like neurons demonstrate abnormal burst firing in synchrony with CA3 pyramidal cells, contributing to a possible epileptic pathway. In support of this idea, recent data indicate that inhibition of neurogenesis (with continuous infusion of an antimitotic agent) inhibits ectopic granule cell formation and reduces spontaneous seizure frequency after SE-induced injury. The antimitotic agent, however, also depletes DGC progenitors that develop normally within the layer (see below) and also has important nonspecific effects. Nonetheless, mounting evidence suggests that increased dentate neurogenesis after experimental SE leads to ectopic, dysfunctional DGCs that likely contribute to epileptogenesis.

Several considerations serve as caveats to the above conclusion. First, one group has shown that adult-born DGCs that develop normally in the DGC layer after electrically induced SE contribute to recurrent inhibition and may restore lost inhibitory tone. In addition, some investigators find that after chronic hippocampal injury in rodent epilepsy models, dentate neurogenesis declines severely, suggesting the DGC progenitor population is depleted or suppressed. Therefore, a competition of aberrant and compensatory DGC neurogenesis may occur in early epileptogenesis; however, in chronic epilepsy the suppression of neurogenesis may have additional functional significance.

Challenges for the Future

A number of questions remain to be addressed regarding the causes and consequences of seizure-induced neurogenesis. First, knowledge of the normal molecular regulation of adult hippocampal neurogenesis is incomplete, and the mechanisms underlying seizure-induced neurogenesis are even less clear. Some evidence points to the involvement of neuropeptides in modulating the increased neurogenesis seen after seizures. Many other important regulators undoubtedly will be uncovered. In addition, the causes of ectopic migration and hilar basal dendrite formation are poorly understood. As described above, existing evidence implicates reelin in ectopic migration, but other factors probably play a role. Levels of brain-derived neurotrophic factor, for example, increase after seizures and this neurotrophin is a prime candidate for stimulating both ectopic migration and abnormal dendritic development. The neural stem cell niche or local environment influences neural stem cells and neurogenesis, and almost certainly also is involved in altered neurogenesis after injury. The niche includes endothelial cells, astrocytes, and – with injury – microglia. These cell types are implicated in modulating NSCs and neurogenesis in both normal and disease states.

Another key area requiring further exploration is the functional consequences of seizure-induced neurogenesis. One approach of current research is to selectively ablate adult-born neurons and determine how this lesion impacts the development of epilepsy. Even more useful will be the development of an approach to block only *aberrant* neurogenesis and perhaps leave potentially compensatory neuronal birth unaffected. This strategy, when accomplished, will allow for a determination of whether abnormal neurogenesis contributes to epileptogenesis. A third question relates to effects of the probable exhaustion of the neural stem cell pool in chronic epilepsy as mentioned above. If DGC neurogenesis is involved in hippocampus-dependent learning and memory, the lack of neurogenesis may explain the disabling memory disturbances seen in mTLE. Careful behavioral testing in experimental mTLE models will be necessary to sort out how aberrant or depleted neurogenesis impacts cognitive function.

Finally, evidence of impaired neurogenesis in human mTLE exists but is not strong. Better techniques for identifying NSCs and neurogenesis in human hippocampus, both histologically and non-invasively, will be required to examine this problem. Once the role aberrant neurogenesis plays in epilepsy development or cognitive comorbidity is defined in the human disorder, novel stem cell-based therapies to prevent or alleviate mTLE may be forthcoming.

See also: **Development/Malformations**: Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis; **Neurogenesis**: Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; Seizures and Adult Neurogenesis; **Plasticity**: Synaptic Reorganization in the Hippocampal Dentate Gyrus.

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Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis

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Introduction

New neurons develop in the adult mammalian brain in several locations, but primarily within the subventricular zone and the dentate gyrus. In the dentate gyrus, most progenitors are thought to arise from the subgranular zone, a thin layer just below the granule cell layer. The majority of these progenitors become granule cells, and most migrate to the granule cell layer and become integrated into the normal circuitry. Earlier studies of granule cells born in the adult brain suggested that many of these new cells did not survive for long periods of time, which led to questions related to their functional importance. However, more recent experiments suggest that these newly born cells have a role in basic hippocampal functions such as learning and memory.

The process of dentate gyrus neurogenesis is not static in the mature animal. Many stimuli can influence the rate of proliferation, the cell type specificity of newly born cells, and their survival. Neuronal activity typically increases the rate of proliferation, as does exercise, environmental enrichment, and various growth factors and neuropeptides such as BDNF (brain-dervied neurotrophic factor), VEGF (vascular endothelial growth factor), and neuropeptide Y. The rate of neurogenesis also changes in various neurological disorders and psychiatric illnesses. For example, in depression, neurogenesis is decreased and antidepressant medication may alleviate symptoms by increasing neurogenesis.

Seizures appear to be a robust stimulus that increases the rate of neurogenesis. Remarkably, almost every type of seizure-like event that has been examined to date has this effect. After discharges, electroconvulsive shock and other brief seizures have been reported to increase the rate of dentate gyrus neurogenesis, and appear to do so in diverse species. Status epilepticus leads to perhaps the most marked increase in dentate gyrus neurogenesis, possibly because it is the most severe and most prolonged of the types of experimental seizures that have been studied. However, the relationship between seizures and neurogenesis is not necessarily as simple as this statement might imply. Stronger and more prolonged seizures may increase proliferation more than milder seizures, but the increase in neurogenesis may be different; seizure-induced damage is greater as the severity and duration of a seizure increases, and this effect can lead to fewer surviving progenitors, and may damage the subgranular zone pool of precursors. For example, unilateral

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New neurons develop in the adult mammalian brain in several locations, but primarily within the subventricular zone and the dentate gyrus. In the dentate gyrus, most progenitors are thought to arise from the subgranular zone, a thin layer just below the granule cell layer. The majority of these progenitors become granule cells, and most migrate to the granule cell layer and become integrated into the normal circuitry. Earlier studies of granule cells born in the adult brain suggested that many of these new cells did not survive for long periods of time, which led to questions related to their functional importance. However, more recent experiments suggest that these newly born cells have a role in basic hippocampal functions such as learning and memory.

The process of dentate gyrus neurogenesis is not static in the mature animal. Many stimuli can influence the rate of proliferation, the cell type specificity of newly born cells, and their survival. Neuronal activity typically increases the rate of proliferation, as does exercise, environmental enrichment, and various growth factors and neuropeptides such as BDNF (brain-dervied neurotrophic factor), VEGF (vascular endothelial growth factor), and neuropeptide Y. The rate of neurogenesis also changes in various neurological disorders and psychiatric illnesses. For example, in depression, neurogenesis is decreased and antidepressant medication may alleviate symptoms by increasing neurogenesis.

Seizures appear to be a robust stimulus that increases the rate of neurogenesis. Remarkably, almost every type of seizure-like event that has been examined to date has this effect. After discharges, electroconvulsive shock and other brief seizures have been reported to increase the rate of dentate gyrus neurogenesis, and appear to do so in diverse species. Status epilepticus leads to perhaps the most marked increase in dentate gyrus neurogenesis, possibly because it is the most severe and most prolonged of the types of experimental seizures that have been studied. However, the relationship between seizures and neurogenesis is not necessarily as simple as this statement might imply. Stronger and more prolonged seizures may increase proliferation more than milder seizures, but the increase in neurogenesis may be different; seizure-induced damage is greater as the severity and duration of a seizure increases, and this effect can lead to fewer surviving progenitors, and may damage the subgranular zone pool of precursors. For example, unilateral

hippocampal kainic acid infusion has been shown to increase dentate gyrus *proliferation* bilaterally, but *neurogenesis* was only increased contralateral to the infusion site, where the least damage occurred.

In light of the robust nature of seizure-induced neurogenesis, a great deal of attention has been paid to its implications. Do the new neurons play a role in epilepsy? Do they contribute to network excitability, either by increasing it or decreasing it? These questions have become the focus of current studies.

Background

There are several suggestions for the role that new neurons in the dentate gyrus might play in epilepsy. Most of these possibilities are germane to TLE, since the hippocampus is often involved in TLE.

How might new neurons contribute to TLE? The fact that there is generally more plasticity in newly generated neurons than in mature neurons makes seizure-induced neurogenesis a potential explanation for several abnormal anatomical findings that are associated with TLE. For example, newly born granule cells could be responsible for the outgrowth of the granule cell axons (mossy fibers) in abnormal locations (mossy fiber sprouting) or the emergence of basal dendrites in granule cells of the granule cell layer. It is also possible that the dispersion of the granule cell layer in epileptic tissue is due to the inability of adult-generated granule cells to terminate in the exact location of the normal granule cell. However, irradiation that reduces seizure-induced neurogenesis does not abolish mossy fiber sprouting. Regarding granule cell dispersion, new data suggest a role of seizure-induced reduction in reelin, which normally hinders granule cell migration outside the boundaries of the granule cell layer. Therefore, new neurons may not necessarily play a seminal role in mossy fiber sprouting or granule cell dispersion.

There are other ways that seizure-induced neurogenesis could play a role in TLE. For example, an initial precipitating insult (which many believe initiates epileptogenesis) might stimulate neurogenesis in the dentate gyrus, contributing to abnormal structure and/or function associated with epileptogenesis (**Figure 1**). New neurons might promote increased excitability, because they are more excitable (at least in the normal rodent brain) than are granule cells born during development. It might also be the case that seizures occur chronically in TLE because new neurons are born after each seizure – and each time this neurogenesis occurs, excitability increases further. It is important to note, however, that some new neurons do not exhibit characteristics associated with increased excitability, suggesting that dentate gyrus neurogenesis may not always lead to changes that promote seizures. Indeed, it is possible that the net effect of new neurons is not directly related to seizures, but to cognitive or mood-related changes. The new neurons may simply disrupt normal circuitry, because they change the normal network connectivity, and in so doing impair cognitive function. This disruption could be relevant to the defects in learning and memory, and depression, which have been observed in TLE.

Note that seizure-induced changes in neurogenesis may change over time. It appears that the rate of neurogenesis increases soon after an initial precipiating event (such as status epilepticus); however, several weeks later, when chronic recurrent seizures develop, data from animal models of TLE and patients with intractable TLE suggest that the rate of neurogenesis declined below normal. The initial rise in neurogenesis could stimulate epileptogenesis, and the chronic decline could be responsible for cogntive and mood-related changes.

A contrasting perspective is that seizure-induced neurogenesis is not a factor in epileptogenesis or cognitive dysfunction at all, but actually is restorative (**Figure 1**). In other words, neurogenesis may be a compensatory mechanism to replenish neurons, because the initial precipitating event leads to neuronal loss. Other effects of seizures, independent of neurogenesis, would be responsible for increased excitability and cognitive effects.

Methods

If one wanted to study seizure-induced neurogenesis, what would be the ideal approach? The answer to that question depends on the goal of the study. If the goal is to examine potential mechanisms of TLE, using an animal model of TLE is appropriate. Which model? Of those that have been developed, models that use status epilepticus as an initial precipitating insult appear to lead to the most reliable induction of spontaneous seizures. However, status epilepticus leads to severe brain damage, and the degree of damage often appears to be more than that occurs in TLE. Therefore, a modification to reduce status-induced brain damage, but maintain the spontaneous seizures, is ideal. Toward that end, many laboratories administer an anticonvulsant drug to limit status epilepticus. When diazepam is administered one hour after the onset of pilocarpine- or kainic acid-induced status epilepticus, neuronal damage is decreased greatly, but animals still develop robust spontaneous seizures after several weeks, and structural changes such as mossy fiber sprouting and granule cell dispersion are robust.

How does one study new granule cells? Many laboratories use a combination of approaches to distinguish neurons that are born after status from those that were born during development. A useful tool is the granule cell marker, Prox1, which does not label other hippocampal neurons. In addition, intracellular labeling is useful to establish granule cell characteristics, including the

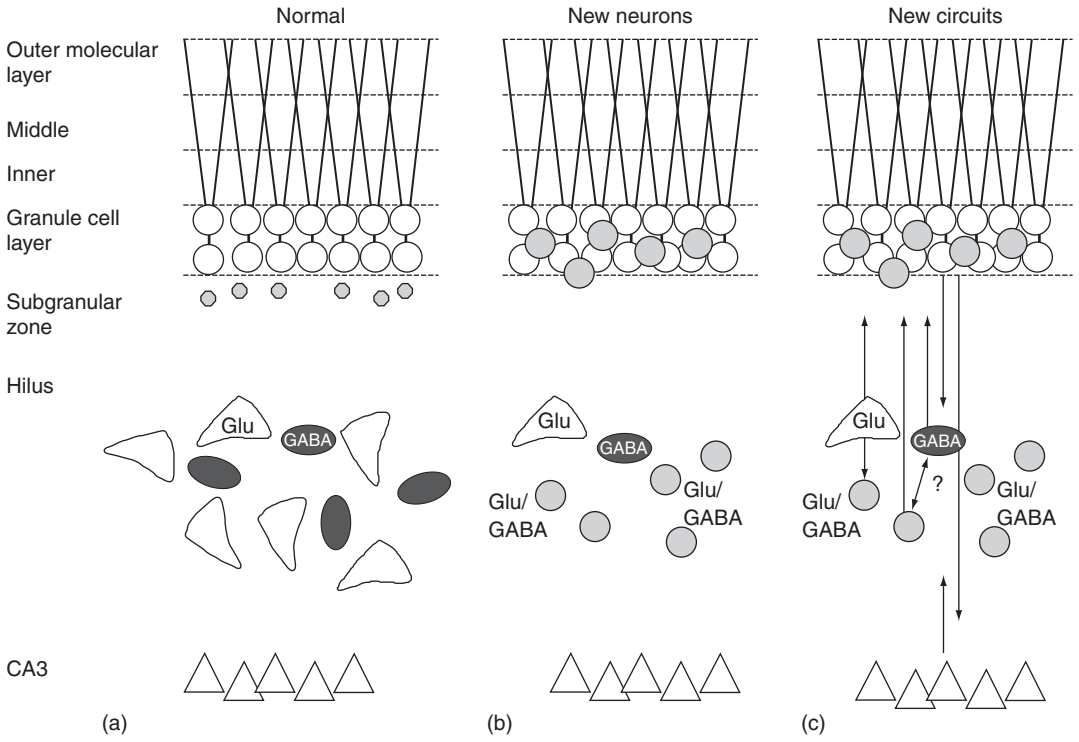


Figure 1 Seizure-induced neurogenesis after status and its potential influence on excitability in the dentate–CA3 network. (a) A schematic of the dentate gyrus illustrates the laminar organization of the dentate gyrus and several of the prominent neuronal subtypes in the normal adult rodent. White indicates glutamatergic cells, which are granule cells and mossy cells. Black indicates inhibitory neurons, which use GABA as a primary neurotransmitter. (b) After status in animal models of TLE, new neurons develop from progenitors in the subgranular zone and migrate into the cell layer as well as ectopic locations, such as the hilus. The new neurons, like granule cells in the granule cell layer, synthesize glutamate as their primary transmitter, but also synthesize GABA and potentially have inhibitory actions. Many of the normal hilar neurons are lost after status-induced damage occurs. (c) Circuitry involving newly generated neurons after status include glutamatergic and GABAergic pathways. Electrophysiological recordings in slices of animals after status have shown that hilar cells often develop epileptiform bursts, synchronized with area CA3 pyramidal cells, and the generator appears to be within CA3. These bursts appear to influence granule cells primarily by hilar projections to the granule cells.

granule cell axon or 'mossy fiber,' which has unique specializations and projections. The mitotic marker, bromodeoxyuridine (BrdU) is taken up into dividing cells for approximately 2 h after peripheral injection, leading to BrdU uptake into newly generated cells that could subsequently be identified by immunocytochemistry using an antibody to BrdU. The use of BrdU, though common, has its pitfalls in studies of seizures. A seizure that opens the blood brain barrier could increase brain BrdU to a greater extent than the rise in BrdU that would occur in a control animal. Some investigators have argued that BrdU uptake by damaged cells occurs during DNA repair, although studies in culture have indicated that this problem does not occur in practice as much as might be expected.

Alternatives to the use of BrdU involve viral vectors that specifically target newly born neurons and label them with green fluorescent protein (GFP) so that they can be visualized. One drawback is that all new cells may not be labeled. Another problem with this approach is that the injection required to introduce the viral vector into the dentate gyrus lead to injury and reactive changes which are confounding.

Recent Results

Seizure-Induced Neurogenesis of Granule Cells: Does it Contribute to Epileptogenesis or is it Restorative?

There are several lines of evidence that support a proexcitatory role of adult neurogenesis in the dentate gyrus under normal conditions, as well as in the context of epilepsy. Under normal conditions, electrophysiological studies have identified characteristics of granule cells that are consistent with increased excitability, such as depolarizing responses to GABA. In addition, it appears that newly generated granule cells contribute to perforant path-induced LTP, whereas granule cells that are born during development play a more limited role.

In animal models of TLE, there also is evidence that the neurons born in the adult brain are proexcitatory, and could be epileptogenic. Support comes from studies of the new granule cells that do not migrate correctly. These granule cells migrate into the hilar region after status, and do so in robust numbers. The 'ectopic' hilar granule cells develop independently of how status was induced – i.e.,

following pilocarpine, kainic acid, lithium followed by pilocarpine, or stimulation using implanted electrodes (**Figure 1**).

The cells that are located ectopically in the hilus have some characteristics of normal granule cells, despite their abnormal location. For example, they are labeled by calbindin and Prox1. Electrophysiological studies have shown that their intrinsic properties are similar to normal granule cells. The axon forms a mossy fiber that innervates CA3 pyramidal cells in stratum lucidum, as well as targeting diverse hilar neurons and granule cells located in the cell layer. The fact that the ectopic granule cells innervate the granule cells of the cell layer, and do so by sending axon collaterals into the inner molecular layer, suggests that ectopic hilar granule cells contribute to mossy fiber sprouting. The axonal projections of ectopic granule cells within the hilus are also potentially important, since they are likely to be a part of additional recurrent excitatory circuits that are not present normally (**Figure 1**). It is also possible that the ectopic neurons innervate inhibitory neurons, but evidence for such inhibitory circuits is not available to date.

Despite the fact that the axon arbor of ectopic hilar granule cells is so similar to granule cells of the granule cell layer, and the expression of calbindin and Prox1 is similar, ectopic hilar granule cells are distinct in some ways. The most striking distinction is the dendritic tree, which is highly polarized for the typical granule cell in the granule cell layer, but rarely polarized in ectopic hilar granule cells.

Despite the structural distinctions of dendrites between ectopic and correctly positioned granule cells, their response to inputs such as the perforant path are similarly robust. The dendrites of ectopic hilar granule cells are covered with synapses, showing that they are well integrated into the network. Studies of c-fos expression in animal models of TLE have also supported this perspective, because c-fos is expressed shortly after a seizure in ectopic hilar granule cells, as well as adjacent hilar neurons. Interestingly, the delay from a spontaneous seizure to c-fos expression is similar, whether the hilar cell is an ectopic hilar granule cell or another hilar cell type (such as parvalbumin- or neuropeptide Y-immunoreactive GABAergic neurons). Yet, the granule cell layer in the same animals typically shows c-fos weak immunoreactivity. These data suggest that hilar ectopic granule cells behave like hilar neurons when a seizure occurs, but in other ways are structurally and functionally similar to granule cells of the granule cell layer.

Interestingly, recordings from hilar ectopic granule cells during the weeks and months after pilocarpine-induced status, we have shown that many develop burst discharges that occur spontaneously, an abnormal characteristic for a granule cell. These bursts are 20–50 ms in duration, typically involve 3–5 action potentials, and are synchronous with spontaneous, rhythmic CA3 pyramidal cell bursts that occur at frequencies up to 0.25 Hz. Simultaneous recordings of dentate neurons and CA3 pyramidal neurons showed that CA3 pyramidal cells discharge first, suggesting that they are generators of the epileptiform activity. Other recordings demonstrate that subsequent to the bursts of these hilar cells, granule cells in the granule cell layer typically exhibit subthreshold synaptic potentials, usually EPSPs (but IPSPs can also occur). These recordings have important implications, because they show that the ectopic hilar neurons may contribute to a local network discharge in epileptic animals. Because ectopic granule cell bursts precede EPSPs or action potentials in granule cells of the cell layer with a single synaptic delay, it is reasonable to hypothesize that ectopic granule cells may contribute to the effects of the CA3 burst discharge on granule cells of the granule cell layer,. However, other hilar neurons are also likely to contribute to effects on granule cells; many of these hilar neurons innervate the granule cell dendrites in the molecular layer or somata/axon hillock.

The inhibitory interneurons in the hilus also have burst discharges synchronized with CA3 and other hilar neurons, and they may serve as a brake on the ability of CA3 to initiate epileptiform activity in the dentate gyrus. Interestingly, ectopic granule cells might also be inhibitory under some conditions, if they synthesize GABA. It is likely that GABA synthesis occurs in ectopic granule cells, because granule cells within the granule cell layer express GABA after seizures. A prediction based on these findings is that the dentate-hilus-CA3 network could become a seizure focus for the spontaneous seizures that occur in the animal that has had status, particularly under conditions that would 'unmask' the excitatory network by reducing inhibitory function. Furthermore, the presence of ectopic granule cells might facilitate the development of a focus, if they are primarily excitatory. However, these ectopic cells could also inhibit the formation of a focus – if they are primarily GABAergic and/or primarily activate GABAergic neurons. Support for the hypothesis that they are proconvulsant comes from studies showing that they innervate glutamatergic neurons (granule cells, mossy cells, pyramidal cells). In other experiments, the numbers of ectopic granule cells was reduced by infusion of arabinoside C after status and spontaneous seizures decreased. However, because arabinoside C is a general mitotic inhibitor, there were other effects of arabinoside C besides decreasing ectopic hilar neurons. Therefore, the more cautious interpretation of this experiment is that neurogenesis can contribute to epileptic seizures in an animal model of TLE, but a critical role of ectopic hilar granule cells in epileptogenesis or chronic epilepsy is not yet clear.

Interestingly, specimens from patients with intractable TLE exhibit ectopic hilar granule cells also. However, not all studies of patients with pharmacoresistant TLE have reported ectopic granule cells. One reason could be that ectopic granule cells are not detected (e.g., because a

granule cell specific marker, such as Prox1, has not always been used). Calbindin is a good marker of normal granule cells, and is often used in human specimens, but it can be downregulated in granule cells after seizures. Indeed, some studies have suggested a limited influence of neurogenesis in TLE because markers of proliferating neurons – examined without granule cell specific marker – were expressed at low levels. Furthermore, human tissue specimens typically are obtained at the end stage of a chronic disease process, at a time when proliferation may be reduced or ectopic granule cells may have died. Indeed, in chronic seizures in animal models of TLE, proliferation in the dentate gyrus appears to be downregulated, and there is evidence that progressive neuronal loss occurs in TLE.

There are other arguments against a role of neurogenesis in TLE. For example, febrile seizures in immature rats do not initiate a rise in neurogenesis, but animals exhibit a lower seizure threshold and many develop spontaneous electrographic seizures. Intrahippocampal kainic acid in the mouse, a different model of TLE, also shows little evidence of increased dentate gyrus proliferation, although this finding could simply reflect the severe damage at the injection site. In the kindling model, ectopic granule cells are a less robust phenomenon than in status models of TLE, but animals that have been kindled to the point of spontaneous seizures have not yet been explored. It is possible that seizure-induced neurogenesis plays an important role in some types of TLE but not in others, and the diversity of findings in animal models reflects the clinical variation.

Future Goals

The ultimate effect of new neurons in the adult brain, particularly the epileptic dentate gyrus, is a subject of debate. Studies of ectopic hilar granule cells suggest a proconvulsant or epileptogenic role, but experiments that have been conducted on new granule cells in the cell layer – those that develop after status – have led to the conclusion that seizure-induced neurogenesis may be a homeostatic mechanism. In fact, both views may be correct. Neurons born in the adult brain that migrate correctly could be important to maintain normal function, but those that migrate to ectopic locations could lead to dysfunction. Indeed, genes that regulate migration are often critical to normal function, a lesson learned from studies of transgenic animals and clinical research.

Whether neurogenesis is 'curative' or whether it leads to impaired function will require advanced tools to selectively ablate new neurons in the adult brain. For example, silencing or killing ectopic neurons, without influencing the new neurons that migrate correctly, would be a powerful approach to address the current questions about seizure-induced neurogenesis. Such approaches may ultimately be useful as novel therapeutic strategies.

See also: **Development/Malformations**: Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; Seizures and Adult Neurogenesis; **Neurotrophic Factors**: The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy; **Plasticity**: Synaptic Reorganization in the Hippocampal Dentate Gyrus.

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Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain

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Introduction

Experimental animals that experience pharmacologically or electrically induced status epilepticus (SE) start to exhibit spontaneous unprovoked epileptic seizures a few weeks later. This pattern is paralleled by cell death, neuronal circuit reorganization, and chronic inflammation in the hippocampal formation and other brain areas, as well as increased neurogenesis in the dentate gyrus. Whether and how these events are involved in the mechanisms of epileptogenesis is not well understood. In particular, it is not known what the role of increased neurogenesis in epileptogenesis is.

Background

Some data indicate that a certain subpopulation of SE-induced neuroblasts migrates into the hilus of the dentate gyrus, where they differentiate into ectopic granule cells. These cells seem to receive excitatory synaptic connections from sprouted axons (mossy fibers) of granule cells in the granule cell layer, as well as excitatory afferents from entorhinal cortex. These afferents have been proposed to create abnormal excitatory circuits, promote increased excitability of the dentate gyrus, and thus contribute to epileptogenesis. However, aberrant granule cell formation appears to be associated with a particular pharmacological model of SE, and therefore may have limited impact on epileptogenesis induced by other (e.g., electrical) models or in the human epileptic brain. Moreover, the majority of newborn cells generated after SE migrate appropriately into the granule cell layer, and differentiate into morphologically normal granule cells. Whether these cells, which have been born in a pathological environment, also receive abnormal excitatory or inhibitory afferents remains to be elucidated. Our recent data indicate that the afferent connections to the newly formed granule cells located in the granule cell layer develop in such way that they mitigate the increased excitability of the epileptic hippocampus.

Methods

Status epilepticus was induced in rats by electrical stimulation of the ventral hippocampus. As a control for increased neurogenesis after SE, a separate group of animals was exercised on a running wheel in their home cages. The latter procedure leads to increased neurogenesis without any significant pathological changes in the hippocampus. To label newly born granule cells, a retroviral vector carrying the GFP gene was injected into the dentate gyrus one week after SE or running. Four to six weeks later, acute hippocampal slices were prepared for whole-cell patch-clamp recordings from the prospectively identified GFP-labeled newborn granule cells. The slices were maintained in an artificial cerebrospinal fluid (aCSF) gassed with a mixture of 95% O_2 and 5% CO_2. Whole-cell recordings were made with patch-clamp pipettes filled with solutions specifically designed for certain experimental requirements, resulting in pipette tip resistances of 4–6 MΩ. Whole cell currents or potentials were amplified with patch-clamp amplifier (HEKA Elektronik, Germany), and recordings were saved for off-line analysis on the computer. IgorPro and FITMASTER software were used for off-line analysis, and appropriate statistical tests were applied for group comparisons. Detailed method description can be found elsewhere (see Further Reading).

Recent Results

Status epilepticus induced pronounced changes in the hippocampus of the animals, leading to neuronal degeneration in hilus (**Figure 1(a)**, upper panel) and CA1 and CA3 regions, as revealed by Fluoro-Jade staining, and increased inflammation (**Figure 1(a)** lower panel) as judged by counting activated Iba1-positive microglia. In 'runners,' no cell death was observed, and the number of activated microglia was significantly lower. Moreover, EEG recordings in SE animals displayed interictal spikes, and animals were noted to experience spontaneous seizures, none of which was seen in the runner group. These data confirmed that the new cells in SE animals were born in a pathological environment, while the new cells in runners were not. The intrinsic properties of the new cells in SE animals were not different from those of cells born after running, maintaining normal passive membrane properties and characteristics of depolarization-induced action potentials (**Figure 1(b)**). Newly born cells were identified by GFP labeling and verified by simultaneous staining for biocytin, which diffused into the cells during whole-cell recordings (**Figure 1(c)**).

The newborn cells in SE animals, however, were substantially different from their counterparts in animals

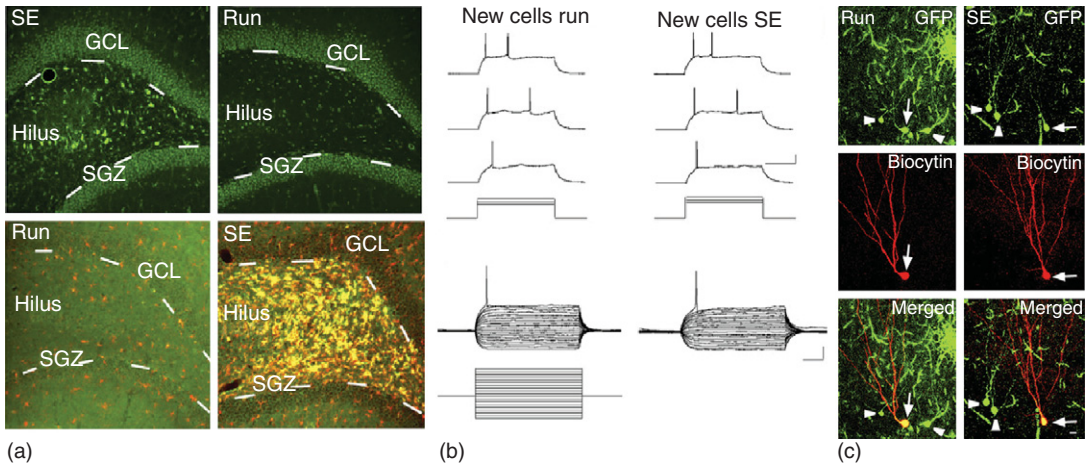


Figure 1 Intrinsic membrane properties of newborn cells in SE environment is unaltered. (a) Upper panels: Fluoro-Jade-stained, degenerating neurons in the hilus of dentate gyrus at 1 week after SE or running; Lower panels: Number and distribution of microglia (Iba1+ = red, depicts all microglia; ED1+ = green, shows activated, phagocytic microglia) in dentate gyrus at 1 week after SE or running. (b) Upper panels: Depolarization-induced action potentials in newly born cells in runners and SE animals. Scale = 200 ms, 10 mV; Lower panels: Voltage responses in new cells born after running or SE recorded in current-clamp mode at resting membrane potential (77.1 ± 6 1.4 mV, $n = 10$, and 79.0 ± 6 1.8 mV, $n = 8$). Scale bar = 100 ms, 20 mV. Recordings were performed 4.5–5 weeks after RV-GFP injection; 500 ms duration current steps with 15 pA increments. (c) Confocal images of cells in runners and SE animals. Cells expressing only GFP (not recorded) are indicated with arrowheads, and GFP-expressing, recorded cells filled with biocytin are depicted by arrows. Merged images show coexpression of GFP and biocytin in recorded cells.

that experienced only running in terms of their synaptic integration. This difference was revealed by analyzing in detail the excitatory and inhibitory drive on these cells, using spontaneous and miniature EPSCs [s(m)EPSCs] and IPSCs [s(m)IPSCs]. Such analysis demonstrated significantly lowered frequency of sEPSCs (**Figure 2(a)–2(c)**) and increased frequency of sIPSCs (**Figure 2(d)** and **2(e)**) in newborn cells in SE as compared to that in runners. In both cases, altered synaptic drive onto the newborn cells in SE animals seemed to be mostly due to presynaptic features: lower sEPSC frequency was a result of decreased overall release probability of glutamate, as estimated by the rate of NMDA receptor-generated EPSC blockade by MK-801 (an NMDA receptor activity-dependent blocker); increased sIPSC frequency was due to decreased sensitivity/expression of presynaptic $GABA_B$ receptors, as measured by changes in paired-pulse depression of monosynaptic IPSCs induced by baclofen.

These findings suggest that newborn cells in the SE environment somehow 'sense' increased excitability and/or inflammation in the dentate gyrus, and by retrograde messenger modulate the functional properties of the presynaptic terminals of the sprouted axons of the existing cells in the neuronal network (**Figure 3**). In this scenario, the overall synaptic excitability of these newborn cells would be decreased. Therefore, taken together, these data suggest that those granule cells that are born in an epileptic environment, and migrate and differentiate in the granule cell layer, integrate into the neuronal circuitry in such a way that they mitigate pathological hyperexcitability. Indeed, in case these newborn cells become active part of the granule cell-CA3 neuronal network, the overall excitatory drive of the whole dentate gyrus output (synapsing onto the CA3 cells) will be relatively diminished, and therefore these new cells would strengthen the inhibitory 'gate' function of dentate gyrus against incoming seizure activity.

Future Goals

Our findings raise a number of questions that are directly related to the possible functional outcome of increased neurogenesis in the epileptic dentate gyrus. One of the main questions is how newborn cells sense the pathological environment. It is not clear at the moment whether seizure activity per se, or inflammatory processes associated with seizures are the main factors signaling to newborn cells to adjust their synaptic integration according to the needs of neuronal networks for normalizing their excitability. Furthermore, it is not known whether the synaptic outputs of the newborn cells also possess adaptive properties, and can be modified by an epileptic environment. Most importantly, it will be central to determine if neurogenesis is capable of becoming a possible therapeutic target for brain repair, and particularly for epilepsy treatment. To address these questions, future research will require a broad approach and intense efforts in the field of normal brain function as well as in the animal models for various diseases in the brain. In this regard, experimental epilepsy could provide a fruitful platform, since studying animal models of seizures and epileptogenesis not only reveals pathological processes associated with abnormal excitability of neurons and

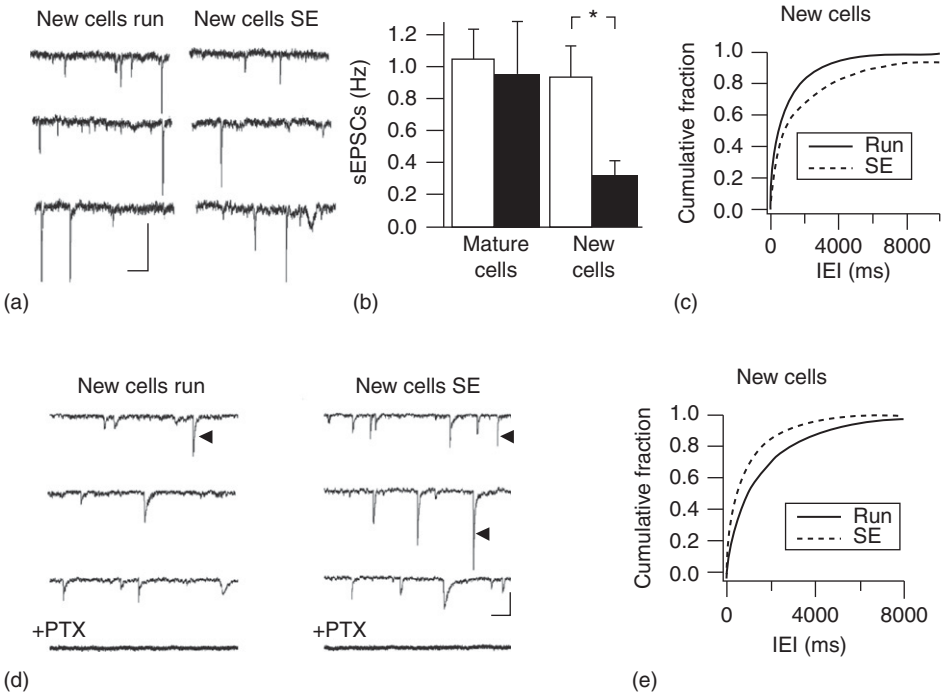


Figure 2 Decreased excitatory and increased inhibitory drive on newborn granule cells in SE animals. (a) Examples of sEPSCs in cells born after running and SE. Note fewer sEPSCs in traces from SE animals. Scale bar = 200 ms, 10 pA. (b) Average frequency of sEPSCs in new and mature cells from runners (white) and SE (black) animals. Frequency of sEPSCs was lower in new cells born after SE than in those born after running (*$p < 0.05$, Student's unpaired *t*-test, $n = 7$ and 6 cells, respectively), but similar in mature cells in runners and SE animals ($p > 0.05$, $n = 10$ and 6 cells, respectively). (c) Cumulative fraction curves of interevent intervals (IEIs) for sEPSCs in new cells showing longer IEIs in cells born after SE compared with those born after running ($p < 0.05$, K–S test). (d) Examples of sIPSCs in cells born after running and SE. The sIPSCs were blocked by PTX, confirming that they were mediated by GABAA receptor activation (+PTX). Note the larger, more frequent sIPSCs in the cells from SE animals. Scale bar = 200 ms, 50 pA. (e) Cumulative fraction curves of IEIs for sIPSCs recorded in new cells in SE animals and runners. The IEIs were shorter in new cells from SE animals compared with runners.

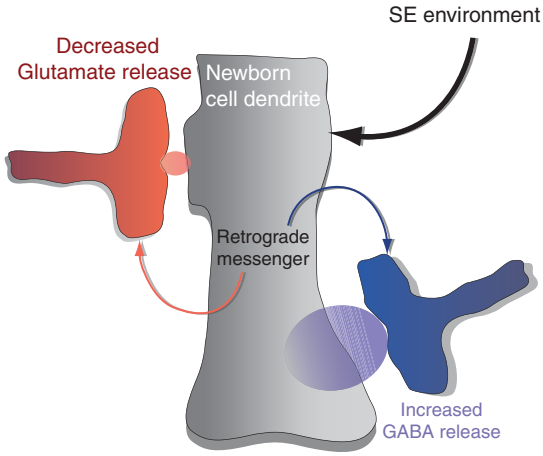


Figure 3 Cartoon depicting overall changes in synaptic drive onto the new granulce cells in SE animals. Newborn granule cells 'sense' environmental cues (black arrow) that guide formation of new functional synapses in network-normalizing manner. This is likely to be achieved by retrograde transporter (red and blue arrows selective for excitatory and inhibitory synapses, respectively) from the new granule cells that travel to the arriving or newly established synaptic endings of existing neurons to alter their functional properties during the final synaptogenesis.

neuronal networks, but also provides a basis for better understanding of normal brain function.

See also: **Development/Malformations**: Neonatal Progenitor Contributions to Epilepsy-Associated Cortical Malformations: A Hypothesis; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Seizures and Adult Neurogenesis; **Neurotrophic Factors**: GDNF: A Neurotrophic Factor Against Epilepsy; **Plasticity**: Synaptic Reorganization in the Hippocampal Dentate Gyrus; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Resculpturing Synaptic Transmission with Neuropeptides.

Further Reading

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Seizures and Adult Neurogenesis

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Introduction

Mesial temporal lobe epilepsy (mTLE) is one of the most common idiopathic epilepsies of the adult. The syndrome of mTLE frequently involves changes in cognitive function, leading to substantial learning and memory disabilities and marked reduction of life quality. Although antiepileptic drug treatment often leads to substantial seizure control, lasting cognitive disturbance remains a frequent clinical feature of mTLE. A large variety of animal models of TLE have been established over the last decades, in which investigators can study the progression from an initial insult to the development of epilepsy, a process called 'epileptogenesis.' In most rodent models, the initial insult is a prolonged seizure called 'status epilepticus' (SE), which is commonly induced by chemoconvulsants such as kainic acid (KA) or pilocarpine. Importantly, SE not only affects mature structures in the adult brain but also leads to a marked increase in the number of newborn cells generated in the subventricular zone of the lateral ventricles and the dentate gyrus (DG) of the hippocampus. Interestingly, many of the newborn neurons in the dentate extend aberrant basal dendrites and migrate ectopically into the hilus – two features of adult neurogenesis that are absent under normal conditions. The functional association between neurogenesis and mTLE remains unclear and there is an ongoing debate if seizure-induced neurogenesis represents an attempt of the injured brain for repair or if newborn neurons after SE participate in the disease process.

Background

Hippocampal neurogenesis is a dynamically regulated process and the number of newborn neurons is increased or decreased by a large number of factors. In the 1990s several laboratories showed in parallel that seizure activity strongly increases the number of new granule cells born in the DG. Early on it was noted that SE leads to marked alterations in granule cell localization and morphology, as many cells born after SE migrate into the hilus and extend abnormal dendrites. This observation has led to the hypothesis that seizure-generated neurons (i) might interfere with synaptic transmission through the hippocampal circuitry, leading to cognitive impairment associated with SE, and (ii) might contribute to epileptogenesis by forming aberrant recurrent excitatory networks with area CA3. Several studies showed that inhibition of seizure-induced neurogenesis was associated with improved hippocampus-dependent learning and less seizure activity in the chronic phase of the disease, supporting a functional link between newborn cells and the epileptic disease process. However, there is also recent evidence showing that seizure-generated granule cells receive more inhibitory input compared to new granule

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cells born under normal conditions. Thus, it is also possible that the function of newborn neurons that are born after SE might be to decrease overall excess excitation in the epileptic hippocampus. In the following sections, we will discuss the effects of SE on hippocampal neurogenesis and the potential contribution of seizure-generated granule cells to epileptogenesis.

Methods

Several approaches are used to study the effects of SE on cell proliferation, differentiation, neuronal migration, and functional integration of hippocampal granule cells born after SE. Dividing cell and their progeny can be either labeled with the thymidine-analogue, bromo-deoxyuridine (BrdU) – alone or in combination with transgenic markers of hippocampal precursor cells. In addition, several laboratories use retroviral vectors that specifically label dividing cells and their progeny in order to study the effects of SE on the morphology and synaptic integration of seizure-generated granule cells. Furthermore, video-EEG monitoring and hippocampus-dependent behavioral tasks have been used to analyze the contribution of seizure-associated neurogenesis to impairment of hippocamapal function after SE.

There are various rodent models of mTLE. The most popular models involve systemic injections of chemoconvulsants (such as kainic acid or pilocarpine) to induce SE, but electrical stimulation has also been used. Differences in the choice of model (i.e., which method is used to induce SE), of rodent species (e.g., mouse or rat), strain, age, and gender may lead to markedly different results. This range of options between different laboratories undoubtedly accounts for many of the apparent discrepancies in the field. Even though it is reasonable to choose a rodent model in which one can reproduce the pathological changes seen in human mTLE brains (such as hippocampal sclerosis), we are then faced with the problem that the human phenomenology – largely determined from brain samples obtained following surgery or post-mortem – might represent only the structural end point of the disease. The human samples from which the neuropathological hallmarks of mTLE have been derived, in most cases, had experienced a very long disease history, often several years or longer. Thus, rodent models that reproduce this morphological stage of disease within days – as seen, for example, with intrahippocampal KA injections – might not be ideal for studying epileptogenesis as it occurs in humans.

On the basis of these considerations we have used two different seizure models in our studies. To characterize the progenitor cell type that is activated in response to seizures, we used transgenic mice expressing green fluorescent protein under the nestin promoter; such animals provide an experimental means of dissociating subtypes of progenitors in the adult hippocampus. In these experiments, intraperitoneal injections of KA were used to induce SE. However, we chose a mouse strain – C57Bl/6 – that does not develop spontaneous recurrent seizures and is resistant to KA-induced excitotoxicity. Thus, while KA injections into C57Bl/6 mice are not well suited to study epileptogenesis and mechanisms of the chronic disease phase of epilepsy, they are ideal for studying the impact and long-term consequences of single bouts of seizures. In our later studies that were designed to investigate the effects of SE on cognition and lasting morphological alterations of newborn granule cells, we switched to an animal model that better reflects a chronic epileptic condition; these studies were carried out using adult rats that were injected with KA, leading to the development of spontaneous recurrent seizures and seizure-associated cognitive impairment.

Recent Results

The work of several laboratories has provided an astoundingly sound picture describing the phenotype of dividing cells in the DG, and the morphological maturation of newborn granule cells. Our initial experiments tried to answer the question of which precursor mediates the massive increase in cell proliferation following SE. Toward this end, we used a transgenic animal expressing green fluorescent protein under the control of the nestin promoter (which is active in hippocampal precursors). This approach enabled us to identify doublecortin (DCX)-expressing neuroblasts (type-3 cells) as a major contributor to increased cell proliferation 1 week after SE. Furthermore, SE led to the dispersion of cells expressing DCX (which is also expressed during later stages of neuronal maturation) compared to newborn DCX-expressing cells under control conditions (**Figure 1(a)**). Together with earlier results from other laboratories, these experiments pointed towards a marked alteration in the behavior of granule cells born after SE. We next used a retroviral strategy, which allowed us to visualize whole cells, so that we could analyze potential morphological alterations of newborn granule cells. Using this approach, we confirmed the previously described ectopic migration and synaptic integration of aberrant granule cells in the hilus. We showed that some of the newborn granule cells extended aberrant basal dendrites towards the hilus, became stably integrated into the DG circuitry, and made ectopic synaptic connections (**Figure 1(b)**). These new connections altered DG connectivity. Furthermore, we determined that granule cells born 4 weeks prior to SE are the cells that participate in SE-induced axonal sprouting (mossy fiber sprouting into the molecular layer); these findings supported previous reports that granule cells born after SE do not contribute to mossy fiber sprouting. We also found that only granule cells born after SE were those that extended hilar basal dendrites.

Even though the cellular events and morphological effects of SE on dentate granule cell neurogenesis are

quite well described, many of the molecular mechanisms responsible for increased neurogenesis and seizure-induced structural alterations, and many of the consequences of SE-induced neurogenesis remain largely unknown. We were interested in studying the possibility that neurogenesis contributes to the cognitive changes seen in mTLE. To carry out these studies, we treated animals with valproic acid (VPA). This histone deacetylase inhibitor – one of the most commonly used antiepileptic drugs – has a marked effect on the proliferation and differentiation of hippocampal precursor cells. We used VPA to analyze the contribution of seizure-generated granule cells to SE-associated cognitive impairment. In the VPA-treated groups we started to inject VPA (150 mg kg^{-1}) 5 h after the initial SE, and rats of the VPA groups received two injections per day throughout the entire length of the experiment. VPA proved to be a potent inhibitor of seizure-induced cell proliferation (judged by the numbers of BrdU positive cells in animals that were killed immediately after 5 days of daily BrdU injections) and net neurogenesis (judged by the numbers of BrdU positive cells in animals that were killed 4 weeks after 5 days of daily BrdU injections). VPA treatment also reduced the formation of basal dendrites extending from newborn granule cells born after SE compared with seizure animals that had not received VPA injections. Next we asked if the reduction of seizure-generated neurons in VPA-treated rats (compared to seizure animals without VPA treatment) affected behavior depending on proper hippocampal function. To this end, we used a well-established hippocampus-dependent object recognition memory task, with a delay of 3 h between the familiarization and testing phase. Similar to previous reports, we found an impairment in the object recognition task in seizure rats that had not received VPA injections. Strikingly, VPA-mediated inhibition of aberrant neurogenesis after SE attenuated the hippocampus-dependent behavioral deficits (seen in non-VPA treated, seizure control animals), supporting the notion that seizure-induced neurogenesis might contribute to the epileptic disease process.

Future Goals

Even though the initial reports on seizure-induced adult neurogenesis were published more than ten years ago, many basic questions remain unanswered: (1) how is seizure-activity 'translated' into increased cell proliferation? (2) what are the molecular mechanisms that lead to the ectopic migration of newborn granule cells and aberrant

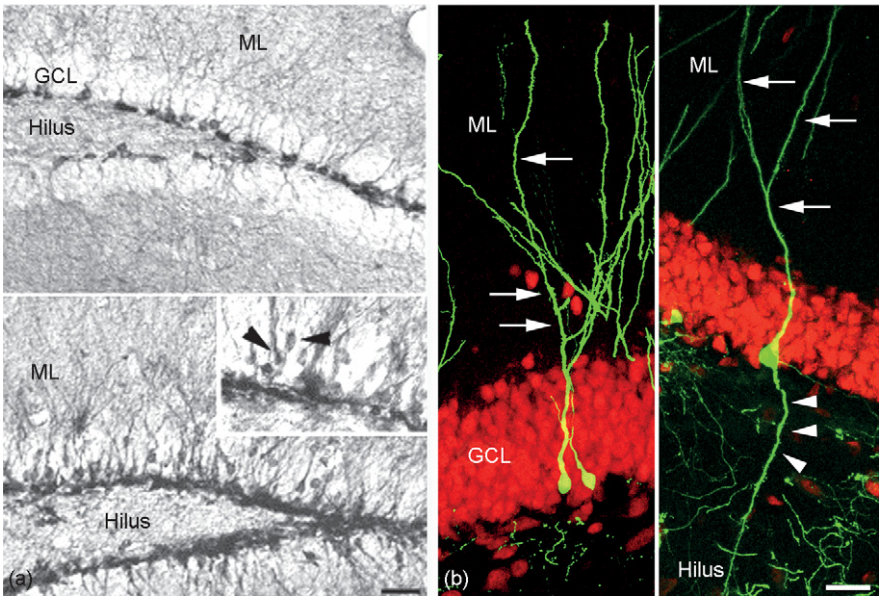


Figure 1 Aberrant neurogenesis after SE. Newborn cells expressing the microtubuli-associated protein doublecortin (DCX) line up along the inner parts of the granule cell layer (GCL) under control conditions (a, upper panel). One week after KA-induced seizures the number of DCX-expressing cells is greatly increased (a, lower panel). New neuronal cells born after seizures also migrate further into the GCL compared to control conditions (see high power view in the inset in panel a). Retrovirus-labeled, 4-weeks old granule cells born under control conditions have a highly polarized architecture with an apical dendrite (arrows in panel b) extending towards the molecular layer (ML; b, left panel). In contrast, 4-weeks old virus-labeled granule cells born two months after SE commonly extend an additional basal dendrite towards the hilus (arrowheads in b; b, right panel) and that synaptically integrate into the preexisting dentate circuitry. ML, molecular layer; GCL, granule cell layer. Scale bars in panel a: 50 μm, in panel b: 20 μm. **Figure 1a** is adapted from Jessberger S, Romer B, Babu H, and Kempermann G (2005) Seizures induce proliferation and dispersion of doublecortin-positive hippocampal progenitor cells. *Experimental Neurology* 196: 342–351, with permission from Elsevier. **Figure 1b** is adapted from Jessberger S, Zhao C, Toni N, Clemenson GD, Jr., Li Y, and Gage FH (2007) Seizure-associated, aberrant neurogenesis in adult rats characterized with retrovirus-mediated cell labeling. *Journal of Neuroscience* 27: 9400–9407, with permission from society for Neuroscience.

extension of basal dendrites? (3) what are the functional consequences of seizure-induced neurogenesis?

The fact that SE leads to dramatic increase in cell proliferation in the DG is unquestioned. Furthermore, it seems clear that excitation alone is sufficient to increase cell proliferation and that SE-associated cell death is not required for the proliferative response. Previous reports suggested that neural precursors appear to 'sense' excitation, influencing proliferation and differentiation behavior. Furthermore, proliferative activity of dividing precursor cells in the adult DG is regulated by synaptic GABA transmission. Clearly, excitatory activity and GABA-mediated currents are altered in the course of epileptogenesis, suggesting a role for these activity-dependent processes in seizure-induced neurogenesis. However, future experiments manipulating those activity-sensing mechanisms – specifically in dividing and newborn immature cells – are needed to understand the molecular mechanisms of seizure-induced cell proliferation. In addition to changes in neurotransmitter concentrations in the DG after SE, there is an emerging understanding for the role for neuropeptides such as neuropeptide Y in the pro-proliferative effects of SE.

Another exciting question is how newborn cells in the DG find their correct position. What are the signals that regulate migration and that direct dendritic and axonal growth? Knowledge regarding mechanisms of neurite extension and pathfinding remains scant, even for normal conditions. The first contributions towards understanding the molecular mechanisms underlying ectopic migration of granule cells after SE were only recently published. The mechanistic hypothesis derived from these data is that the expression of the secreted glycoprotein reelin, which is a critical neuronal guidance molecule during development, is dramatically decreased after SE, and that this decrease might help explain the ectopic migration of granule cells following SE. Without a doubt, however, additional factors will be involved in ectopic migration and aberrant dendrite extension. Potential candidates include, for example, cdk5/p35 signaling and growth factor (e.g., VEGF and BDNF) signaling.

As adult neurogenesis obviously occurs in the midst of a mature neural structure, it seems reasonable to speculate that the environmental niche is a critical factor in governing distinct aspects of progenitor proliferation and neuronal maturation. This consideration is probably particularly important within the context of the extensive structural alterations that occur in the DG after SE. To date, the distinction between cell-autonomous effects within the domains of dividing precursors and immature neurons, in contrast to changes within the neurogenic niche of the hippocampus, is not clear.

Probably the currently most debated question in this field is whether seizure-generated granule cells are an attempt of self-repair or exert additional pathologic influences on the epileptic hippocampus. Current evidence actually points toward both possibilities. Four weeks after they were born, seizure-generated granule cells were found to have intrinsic electrophysiological properties similar to cells that were born in running animals, but appeared to receive less excitatory input. These results suggested that the SE brain was attempting to 'balance' surplus excitation. Along the same lines, another study showed that brain irradiation – which suppresses adult neurogenesis – transiently increased excitation after SE. In contrast, extensive analyses strongly suggest that ectopic granule cells are part of a recurrent excitatory circuitry with area CA3, which might contribute to epileptogenesis. Similarly, the basal dendrites extending from seizure-generated granule cells might be at least partially responsible for DG hyperexcitability observed after SE. We and others have found that inhibition of aberrant neurogenesis attenuates seizure-associated deficits in hippocampus-dependent learning and memory. Thus, it seems probable that seizure-induced neurogenesis might have both positive and negative effects on the integrity of the DG circuitry. The challenge will be to design experiments that specifically target seizure-generated granule cells in isolation from the rest of the DG circuitry.

The involvement of neurogenesis in the epileptic disease process might also be dependent on the disease stage. Although early stages of the disease apparently lead to aberrant newborn granule cells that might interfere with proper hippocampal function caused by ectopic synaptic integration, there is emerging evidence that the levels of adult neurogenesis might be strongly decreased (compared to control animals) in the chronic phase of the disease process. Such decline of neurogenesis might account for cognitive or affective clinical syndromes at later stages of the disease. With this possibility in mind, it is interesting to consider the possibility that stimulation of endogenous neurogenesis (or the addition of transplanted precursors) might be a future strategy to treat epileptic syndromes.

Even though the molecular basics remain largely unclear and the functional significance of seizure-induced neurogenesis is to some extent controversial, there is no doubt that newborn cells after SE do change the dentate circuitry. Seizure-generated granule cells integrate synaptically and make contacts with neural structures that are not targeted by cells born under normal conditions. Understanding the cause for these changes, and identifying the stages of the disease process in which new neurons may be good or bad for the epileptic brain, will require the concerted effort of molecular biologists, electrophysiologists, behavioral biologists, and clinical neurologists.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Effects of Seizures in Animal Models; **Models**: Temporal Lobel Epilepsy in Humans: Searching for Answers through the Pilocarpine Model; **Neurogenesis**:

Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain..

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Synaptic Activities and Transmitter Function: Receptor Trafficking

Changes in Cholinergic Synaptic Activity and Receptor Function Associated with Seizures and Epileptogenesis

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Introduction

Endogenous ACh acts on several subtypes of nicotinic and muscarinic receptors. The brain is rich in cholinergic receptors, including those areas, like hippocampus and cortex, that have a low threshold for the generation of seizures. The importance of this neurotransmitter for cognitive function has been established by findings in neurodegenerative diseases, where cognitive and behavioral dysfunction is accompanied by abnormalities in the cholinergic system.

There is substantial evidence that early life seizures influence brain development. Moreover, the probability of

Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain..

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NEUROTRANSMITTER FUNCTION

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Changes in Cholinergic Synaptic Activity and Receptor Function Associated with Seizures and Epileptogenesis

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Introduction

Endogenous ACh acts on several subtypes of nicotinic and muscarinic receptors. The brain is rich in cholinergic receptors, including those areas, like hippocampus and cortex, that have a low threshold for the generation of seizures. The importance of this neurotransmitter for cognitive function has been established by findings in neurodegenerative diseases, where cognitive and behavioral dysfunction is accompanied by abnormalities in the cholinergic system.

There is substantial evidence that early life seizures influence brain development. Moreover, the probability of

long-lasting effects is associated with the characteristics of the seizure. In addition, epidemiologic evidence shows that the incidence of seizures, in a variety of syndromes associated with cognitive dysfunction or deterioration, is significantly higher than in the general population – indicating either a common cause or a causative link between the two. These findings, taken together with those implicating ACh in cognitive processes, suggest that this neurotransmitter is a very good candidate if one wants to investigate the cellular mechanisms linking seizures to (long-term) cognitive function, and more particularly, early life seizures to brain development.

We have had two major targets for our research program. First, we have designed studies to examine whether there is evidence to implicate cholinergic mechanisms in the generation of epileptiform discharges in immature brain, and to examine whether the occurrence of early seizures had any long-lasting effects on adult cholinergic responses. We chose a model within which we could provoke generalized and relatively long-lasting convulsions, since the severity and duration of seizures are both factors positively correlated with the appearance of long-lasting effects. Our second target was to determine whether adult (brain) cholinergic responses differed in animals that had experienced early convulsions (vs. age-matched controls), and if so, to investigate the cellular/synaptic basis for this difference. This latter aim has been investigated with a variety of approaches, including electrophysiology, cell and molecular biology, and behavioral testing.

Background

Cholinergic agents have been extensively used in electrophysiological recordings in vitro to manipulate synaptic potentials and/or neuronal membrane properties. Thus, we have gained to date substantial insight into their mechanism(s) of action and their probable effects on the integrated neuronal activity, that is, brain function. Muscarinic agents mostly inhibit synaptic activity by acting on presynaptic receptors that decrease transmitter release, but on the other hand, also exert excitatory effects by blocking K^+ channels and also by facilitating intracellular Ca^+ accumulation (see Further Reading section). Several reports have suggested their potential in modulating epileptiform discharges in a way that would add up to the excitatory process. Nicotinic receptors exert mostly excitatory effects although there is less evidence linking them to epileptic paradigms (see Further Reading section).

Cholinergic agonists have been effectively used in vivo for the provocation of convulsions, in immature as well as in adult animals. In this respect, they are not different from many neuroactive agents with varying mechanisms of action, where administration at a sufficiently high dose may provoke seizures. Pilocarpine, in particular, has been extensively used, often in combination with lithium (which reduces animal mortality – although with a still unresolved mechanism). Because of the uncertainties associated with pilocarpine's actions, we have used the $GABA_A$ receptor antagonist, pentylenetetrazol (PTZ) for our studies; this drug is eliminated within 24 h of injection from the animal's system, and has no known toxic or long-lasting effects of its own.

A number of reports have examined the effects of experimental seizures on brain cholinergic neurotransmission, with attention to post seizure ACh release, changes in the number of cholinergic terminals, and changes in receptor subtypes. Fewer studies have examined post seizure electrophysiological cholinergic effects. Findings from these reports are not easily comparable for three reasons: (1) Differences in the age and species in which convulsions were provoked; (2) Differences in the experimental protocol, including the choice of pharmacological agent used, method of administration, dose, and the resulting type of convulsion (severity, duration, single vs. repetitive); (3) Differences in timing – that is, interval between 'conditioning' (seizure provocation) and assessment. This third factor is critical, since many studies have shown that long-term effects of brain insults of varying etiology, including seizures and stroke, differ from those observed in the short term. Indeed, changes assessed immediately or at a short interval after the seizure are often not evident in the long term, and changes assessed after a long interval are not evident in the short term. Short-term effects in cell physiology often dissipate with time, while some long-term effects are not detectable shortly after the insult, indicating that the initial insult starts a cascade of 'mostly unknown,' events leading to the long-term alteration. The time interval between the initial insult and the appearance of long-term (permanent) effects is called the 'silent period' precisely because no changes are seen within this time window; this period may vary in length, depending on the type of insult and the specific effect (i.e., change in physiology) to be investigated.

Methods

Sprague–Dawley rat pups of postnatal day (P) 10 or 20 were subjected to generalized and sustained convulsions (>20 min duration, a model for status epilepticus, SE), provoked by i.p. injection of pentylenetetrazol (PTZ, 70–80 mg kg^{-1}); some pups of each litter were used as controls. After a period of at least 40 days, the (adult) conditioned animals were decapitated under volatile agent anesthesia, and the brains were either frozen for the assays described in the later sections, or used for the

preparation of hippocampal slices for electrophysiological recordings. We measured the frequency of occurrence of spontaneous synchronous bursts and the features (amplitude, duration, etc.) of evoked potentials in vitro, using either extracellular or intracellular recording techniques.

Whole brain or hippocampal tissue obtained 40 days post seizure was used to determine ACh innervation density (using choline acetyltransferase immunocytochemistry) and endogenous ACh and GABA content by high-performance liquid chromatography (HPLC) electrospray ionization mass spectrometry, a technique that allows the measurement of small concentrations of endogenous substances (nM) from small volume samples. Whole brain sections were used for (i) quantitative radioligand binding autoradiography, (ii) in situ hybridization, and (iii) [^{35}S]GTPγS autoradiography, to assess the properties of hippocampal and neocortical muscarinic receptors.

Results

In the first set of experiments, we sought to determine whether cholinergic receptors played any role in the generation of epileptiform activity in immature brain, by recording extra- and intracellularly from P10–P20 hippocampal slices. We found that cholinergic agonists greatly increased the frequency of spontaneous epileptiform discharges when $GABA_A$-mediated inhibition was blocked, but did not have such an effect when $GABA_A$-mediated inhibition was intact (**Figure 1**). This result suggested that cholinergic receptors played a facilitatory but not a generating role, since another excitatory condition (i.e., the $GABA_A$ receptor block) was a prerequisite. This cholinergic effect was completely reversed by the mucarinic antagonist atropine, and was also mimicked by several muscarinic agonists. Interestingly, the anticholinesterase eserine (which inhibits the catabolism of

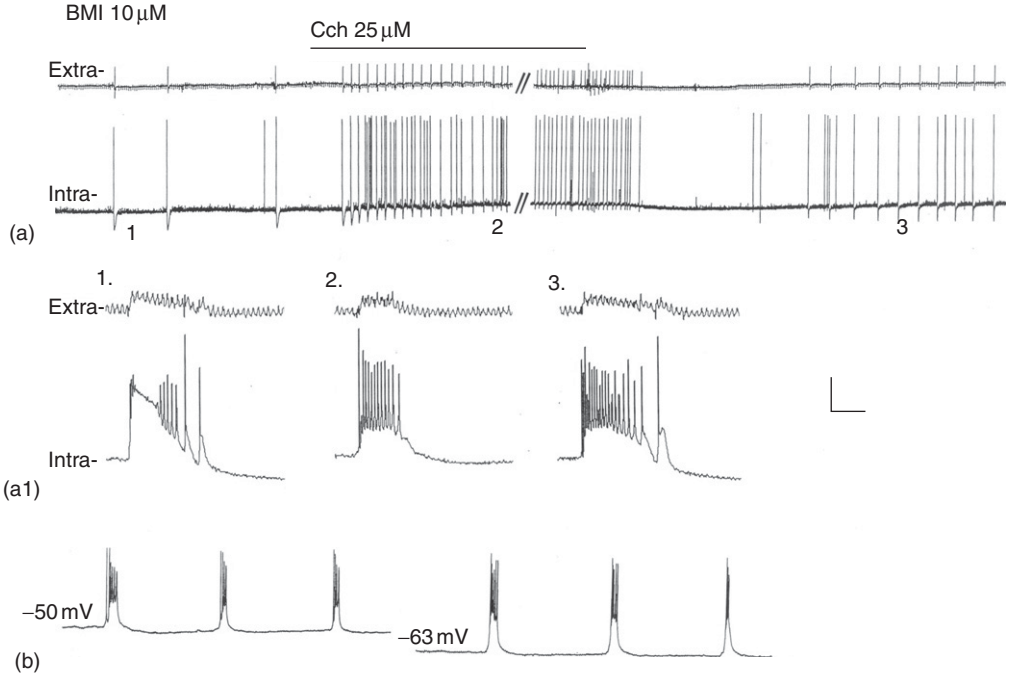


Figure 1 Effects of CCh on spontaneous synchronous activity in the presence of BMI. (a) A P14 CA3 neuron (RMP −57 mV) responded to the application of 10 μM BMI with rare synchronous discharges corresponding to intracellular bursts (freq. 0.012 Hz). Perfusion with BMI had started 14 min before the beginning of – and lasted throughout – the trace shown here, so the rate of occurrence of spontaneous discharges was maximal, under these conditions. Addition of CCh (25 μM) to the perfusion medium depolarized the neuron by 4 mV and increased the frequency of synchronous discharges to 0.202 Hz (16.83 times the original frequency). These effects of CCh were reversible upon wash. Three of the discharges recorded extra- and intracellularly (marked with numbers 1, 2, 3) are depicted at a higher speed in (a1). (a1) The amplitude and duration of the synchronous spontaneous discharges recorded in BMI (1) was reduced by the addition Cch (2), an effect that was reversible upon CCh wash (3). CCh also reduced the number of APs associated with the burst, an effect evident when comparing traces 3 and 2, but not 2 and 1. The reason for that is the high amplitude of the burst in 1, resulting in AP block at its peak. (b) Intracellular recording from another P14 neuron in the presence of 10 μM BMI and 25 μM CCh. The amplitude but not the frequency of the spontaneous bursts recorded at RMP (−50 mV) changed following artificial hyperpolarization by dc current injection to −63 mV. Calibration bars: 4 mV (extra-), 20 mV (intra-in a, a1, b), 40 s (a), 100 ms (a1), 285 ms (b) Reproduced from Psarropoulou C and Dallaire F (1998) Activation of muscarinic receptors during blockade of $GABA_A$-mediated inhibition induces synchronous epileptiform activity in immature rat hippocampus. *Neuroscience* 82: 1067–1077, with permission from Elsevier.

endogenous ACh) had effects similar to those of exogenous agonists under the same conditions of $GABA_A$ receptor blockade, suggesting that elevations in endogenous ACh would presumably suffice to play this role in vivo. This effect of eserine was manifested after P10 but not earlier (**Figure 2**), suggesting a developmental onset for this synergistic excitatory effect. P10 in a rat pup is comparable to a human neonate, according to some earlier studies comparing some biochemical indices and anatomical data. Taken together, these data suggest that endogenous ACh could play a significant role (as a co-factor) in precipitating seizures in neonates or infants.

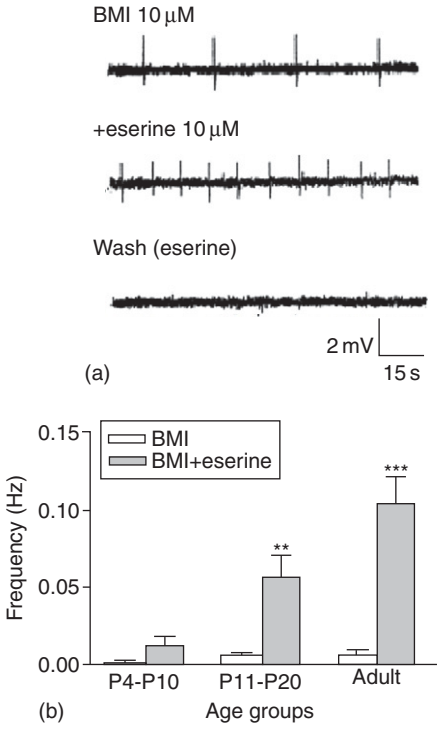


Figure 2 (a) Effects of eserine 10 mM on the frequency of the synchronous spontaneous discharges recorded extracellularly during blockade of the inhibitory GABA-mediated potentials by BMI 10 mM. *Upper trace*: BMI induced infrequent synchronous spontaneous discharges in the CA3 area of a hippocampal slice from a P10 rat pup. In this and all slices tested, no synchronous spontaneous discharges were recorded in control ACSF. *Middle trace*: Addition of eserine more than doubled the frequency of synchronous spontaneous discharges. *Lower trace*: The effects of eserine were reversible. (b) Bar chart showing the mean frequencies in Hz of the spontaneous synchronous discharges recorded in 10 mM BMI as well as following the addition of 10 mM eserine. All slices tested were included in the graph and 0 Hz was entered if no synchronous discharges were recorded. Standard errors of the mean values are shown on top of each bar. BMI versus BMI-eserine data were compared by the paired Student's *t*-test; significant differences in the mean values are marked by asterisks p 0.01, p 0.001. Reproduced from Gruslin E, Descombes S, and Psarropoulou C (1999) Epileptiform activity generated by endogenous acetylcholine during blockade of GABAergic inhibition in immature and adult rat hippocampus. *Brain Research* 835: 290–297, with permission from Elsevier.

Although the cholinergic excitatory effects appeared to be muscarinic, we conducted a separate in vitro study to determine whether nicotinic agonists displayed similar activity in the same paradigm. We found that nicotinic agonists did not increase significantly the frequency of spontaneous field discharges recorded during $GABA_A$ receptor block.

We then investigated whether the provocation of seizures in vivo, by a similar mechanism (blockade of $GABA_A$ synaptic inhibition by PTZ) would provoke any long-term changes in central cholinergic responses. We found that a PTZ-induced generalized convulsion in P10 or P20 rat pups had led to an apparent hypersensitivity to cholinergic stimulation in hippocampal slices from adult rats (40 days or more after the convulsion). That is, following perfusion of the slice with eserine, we recorded synchronous, interical-like epileptiform discharges that were atropine-sensitive; no such activity was seen in slices from naïve (i.e., not exposed to PTZ) littermates. And no such spontaneous field activity was recorded from these same slices when they were perfused with standard ACSF (i.e., without eserine), that would suggest a generalized increase in excitability. Inhibition appeared normal; IPSPs ($GABA_A$- and $GABA_B$-dependent, mono- or polysynaptic) measured intracellularly were similar in slices from PTZ and naive rats. The spontaneous discharges provoked by eserine were not observed if the PTZ convulsion was blocked by prior injection of pentobarbital, suggesting that this effect was linked to the convulsion and not the PTZ injection.

In the same set of experiments, we compared young (P10, P20) and adult animals (>P60) with regard to this long-lasting post-convulsion effect; comparisons were made between animals with convulsions of similar duration. Interestingly, when animals were young (P10 or P20) at the time of seizure induction, the cholinergic supersensitivity was found both in the short term (10 days) and in the long term (>40 days) after the convulsion. In contrast, when animals were adult (P60) at the time of seizure induction, the cholinergic supersensitivity was found only in the short term (10 days) but not in the long term (>40 days), indicating a temporary and reversible change. These findings differentiate immature and adult animals with regard to seizure-induced effects on cholinergic neurotransmission, and moreover suggest a mechanism by which, early life seizures may influence later life brain functions that depend on cholinergic neurotransmission (cognitive, behavioral). Within a broader context, this increased cholinergic sensitivity represents an activity-dependent developmental change – that is, a paradigm of activity dependent immature brain plasticity.

This cholinergic sensitivity was not the result of (i) decreased synaptic inhibition or GABA content,

(ii) increased ACh content, (iii) proliferation of cholinergic terminals, or (iv) changes in the number and/or subtype of muscarinic receptors. Instead, it was associated with a marked increase in the cholinergic-stimulated [^{35}S]GTPγS incorporation, leading to an increase in the efficacy of G-protein coupling to muscarinic receptors (Figure 3). We do not know the pathway leading to this functional change; however, it may be a seizure-induced modification in the regulation of one or more genes in a muscarinic receptor (M1?) signaling cascade. In terms of function, this increase in the efficacy of G-protein coupling may act as an equivalent of, for example, reduced receptor desensitization.

Associated with this identification of seizure-induced increased G-protein coupling, we discovered a trend toward a higher level of total [^{35}S]GTPγS binding in female compared to male PTZ-exposed rats. Although this finding may be explained by the higher basal level of [^{35}S]GTPγS incorporation in females compared to males, it illustrates how seizures may amplify naturally occurring gender differences. Indeed, some G-protein-coupled receptors have been reported to be differently regulated in females and males, and thus may result in some of the gender-specific characteristics seen in some forms of epilepsy. To date, we have also observed this male–female difference in a number of experimental protocols involving cholinergic function.

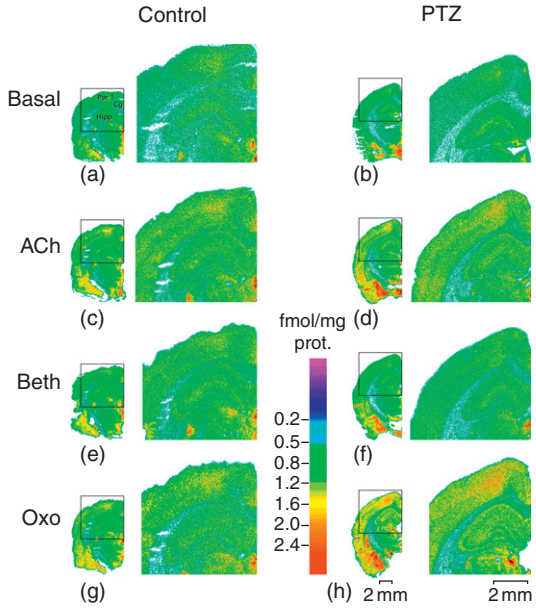


Figure 3 Color-coded autoradiographs illustrating the basal and agonist-stimulated [^{35}S]GTPγS incorporation in the cerebral cortex and hippocampus of control (a, c, e, and g) and pentylenetetrazole (PTZ)-treated rats (b, d, f, and h), at a transverse level near stereotaxic plane A 4.7 mm according to Paxinos and Watson (1986). The rat brain in stereotaxic coordinates, 2nd edition, Academic Press, Sydney. The enlarged area includes the Par1 (primary somatosensory) region of cerebral cortex and dorsal hippocampus. ACh, acetylcholine; Beth, bethanechol; Oxo, oxotremorine. There are noticeable increases in [^{35}S]GTPγS incorporation in both the cerebral cortex and hippocampus of PTZ-treated compared with control rats after stimulation by ACh and Oxo. Reproduced from Potier SSJ, Chabot J-G, Psarropoulou C, and Descarries L (2005) A pentylenetetrazole-induced generalized seizure in early life enhances the efficacy of muscarinic receptor coupling to G-protein in hippocampus and neocortex of adult rat. *European Journal of Neuroscience* 21: 1828–1836, with permission from Wiley-Blackwell.

Directions for Future Research

The accumulated evidence shows that the CNS cholinergic-muscarinic system undergoes a functional change following an early life prolonged and generalized convulsion. Seizures of this type are considered to model status epilepticus (SE), a condition that is associated with long term effects in brain anatomy and function. It is possible, but not yet proved, that this change in the cholinergic system may contribute to changes in these animals' response to environmental stimuli. It is also conceivable that this change may play a role only in specific conditions – for example, in behavioral or pathophysiologic conditions that increase endogenous ACh release. Several such conditions have been reported in the literature. These findings provoke a number of further questions that have guided our current efforts. For example:

What may be the consequences of this cholinergic system change on the animal's general behavioral phenotype and/or the animal's susceptibility to future insults? Is this alteration in the ACh system simply an adaptive and/or compensatory change, or does it facilitate the precipitation of future seizures ('seizures beget seizures')? In an effort to find out, whether the early life SE episode and the ensuing cholinergic change were associated with any phenotypical changes, we carried out a series of behavioral tests for assessing the animal's reaction to novelty or to different aspects of memory and learning. We found statistically significant differences in behavioral responses, not only between control and PTZ-treated rats of the same sex, but also between male and female rats of the same group (control or PTZ-treated). In the open field test, spontaneous motor activity was estimated as an index of the animal's reaction to a novel environment. Female PTZ-treated rats had significantly reduced spontaneous motor activity as compared to controls, a finding that was not replicated in the male PTZ-treated rats. In the object recognition test, which models nonspatial memory and learning, both male and female PTZ-treated rats performed significantly worse compared to controls. In the water maze test modeling spatial memory and learning, only male PTZ-treated rats

performed worse than controls. Overall, male PTZ-treated rats displayed impaired performance related to memory and learning abilities as compared to female PTZ-treated rats. These preliminary results suggest that even a single, sustained, generalized early life seizure may induce long-term consequences related to novelty behavior, spatial and nonspatial learning, and memory, which appear to be sex-specific (E. Papalexi, C. Psarropoulou, and K. Antoniou, unpublished data). Whether these findings are directly associated with the changes observed in cholinergic neurotransmission or not remains to be seen.

Is there a specific muscarinic receptor subtype that is primarily affected by the seizure insult? Our findings so far point to the M1 muscarinic receptor, but do not exclude other types. It is noteworthy, however, that disruption of the m1 gene (the pharmacologically defined M1 receptor coincides with the genetically defined m1 gene) can disrupt the muscarinic receptor-dependent M current regulation, and leads to seizure activity in mice.

How does this functional change in muscarinic receptors affect neuronal output in terms of responses to synaptic and intrinsic stimuli? By answering this question, we can elucidate the pathway leading from the seizure-dependent modification of cholinergic neurotransmission to animal behavior. It will also permit us to predict the types of effects, other than those already observed, that may stem from such a modification. This question can be answered by electrophysiological in vitro recordings, where the extracellular or intracellular neuronal environment can be manipulated to mimic different in vivo conditions.

Does this functional change in muscarinic receptors also affect the output of other neurotransmitter systems that act in synergy? Neurotransmitter systems often share metabolic pathways or interact via receptors that can be regulated by common mechanisms. Therefore, changes in any given neurotransmitter system may indirectly affect the output of another. For example, it has been shown that activation of m1 muscarinic receptors potentiates NMDA-receptor currents in hippocampal pyramidal cells. Taking our cue from this work, we examined whether the NMDA-receptor mediated excitability would differ in brain tissue of adult conditioned versus naive animals. We provoked spontaneous NMDA-receptor mediated epileptiform discharges by bathing the tissue from PTZ-treated and naïve rats in ACSF without added Mg^{+2}, and recorded from the CA3 area. We found that the rates of epileptiform discharges were significantly higher in slices from PTZ-treated animals (PTZ slices) in both the male group ($n = 55$ control slices, $n = 61$ PTZ slices, Student's t-test $p = 0.0008$) and in the female group ($n = 43$, $n = 112$, $p = 0.01$) compared to sex-matched controls. Interestingly, female slices showed significantly higher frequencies compared to male slices, in all recording locations (CA1, CA3, dentate gyrus), within both the control and the PTZ group (B. Foadjo Awoumé and C. Psarropoulou, unpublished data). The latter finding may be explained by the reported interaction between ACh, NMDA, and estrogen receptors.

Is the cholinergic change functionally important for all, or only some brain regions? The output of each region or brain formation depends on its structural (neuronal types, connections) and functional (receptor types, neurotransmitter systems) features. For example, it is known that hippocampal excitability differs along the septotemporal axis, with the temporal pole reported as 'more excitable.' It is, therefore, possible that this muscarinic receptor functional change would differentially influence the output of septal versus temporal hippocampal regions. Using septal and temporal hippocampal slices from PTZ-treated or control animals, and slice perfusion with ACSF with no added Mg^{+2}, we have tested the cholinergic modification of NMDA-mediated epileptiform discharges. Our preliminary findings indeed suggest that the degree of the ACh-mediated frequency increase of the NMDA-dependent epileptiform discharges differs along the septo-temporal hippocampal axis (A. Mikroulis and C. Psarropoulou, unpublished data).

What mechanisms lead from the seizure to the cholinergic neurotransmission changes? What is the sequence of events leading up to the establishment of long-term effects? Are there any interventions that could reverse these changes or lessen their impact? The increased cholinergic sensitivity was recorded in both immature and adult animals 10 days after the PTZ seizure, indicating that it was developed within this 10 day time window – that is, a relatively short latency response. Longer interval recordings showed, however, that this change is reversed (no longer recordable) in animals that had the seizure in adult life, but persists in animals that had the seizure during CNS maturation. Thus, it appears that the better question is not "how/when does this cholinergic change appear?", but rather "what are the factors that are responsible for its dissipation in adult the brain or its maintenance in the immature brain?"

And finally, one can add the gender factor in all these questions, especially since gender differences in G-protein receptors occur naturally, and they may be stressed or increased following seizures, as shown by our preliminary data. Because of the naturally occurring gender differences in G-protein receptors, muscarinic and other such receptors may be good candidates in which to investigate gender-related seizure phenomena

(e.g., catamenial epilepsy), and also any gender associated short- or long-term seizure effects.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain

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Introduction

Synapses regulate their own neurotransmitter supply. Once released, neurotransmitter is recycled, both through direct reuptake and by processing through the astrocytic glutamate–glutamine cycle. Inhibitory synapses exhibit tight, activity-dependent coupling of the GABA supply to the activity of the glutamate–glutamine cycle. Interrupting the glutamine cycle rapidly depletes GABA supply at active inhibitory synapses – which in turn may mediate synaptic scaling, allowing the inhibitory efficacy to ramp up during periods of high excitatory synaptic activity, thus maintaining circuit regulation. In epileptogenic foci in hippocampus resected from patients with temporal lobe epilepsy, the keystone enzyme in the glutamine cycle, glutamine synthetase, is significantly downregulated in reactive astrocytes. GABA supply will also be deficient in this tissue, causing disinhibition and a lack of circuit regulation. This condition could contribute to seizure initiation and/or propagation. Therefore,

(e.g., catamenial epilepsy), and also any gender associated short- or long-term seizure effects.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures.

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Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain

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Introduction

Synapses regulate their own neurotransmitter supply. Once released, neurotransmitter is recycled, both through direct reuptake and by processing through the astrocytic glutamate–glutamine cycle. Inhibitory synapses exhibit tight, activity-dependent coupling of the GABA supply to the activity of the glutamate–glutamine cycle. Interrupting the glutamine cycle rapidly depletes GABA supply at active inhibitory synapses – which in turn may mediate synaptic scaling, allowing the inhibitory efficacy to ramp up during periods of high excitatory synaptic activity, thus maintaining circuit regulation. In epileptogenic foci in hippocampus resected from patients with temporal lobe epilepsy, the keystone enzyme in the glutamine cycle, glutamine synthetase, is significantly downregulated in reactive astrocytes. GABA supply will also be deficient in this tissue, causing disinhibition and a lack of circuit regulation. This condition could contribute to seizure initiation and/or propagation. Therefore,

therapeutic intervention strategies targeted towards restoring the glutamine cycle and GABA levels in epileptic brain appear promising.

Background

Synapses as Autonomous Structures

Synapses are compartmentalized structures, geographically isolated from regulation by the neuronal soma. For synapses releasing small molecule neurotransmitters, complex metabolic machinery exists to recycle and package neurotransmitter from amino acid precursors. This local regulation of neurotransmitter supply is an absolute necessity for the efficient regulation of synaptic function. If it were necessary for neurotransmitter-containing synaptic vesicles to be synthesized in the Golgi apparatus (in the cell soma) and transported down axons for release at synapses, the resulting delay in supply and demand signaling would preclude economical up- or downregulation of neurotransmitter supply based on the recent history of synaptic activity.

The specialized machinery required for local neurotransmitter recycling and synthesis resides both within synapses themselves, and within the astrocytic processes investing synapses. Specialized neurotransmitter transporters are localized within presynaptic terminals, allowing for the immediate recycling of released neurotransmitters, the most efficient form of recycling. This type of recycling occurs at inhibitory synapses, where GABA transporters (GAT-1) and glutamate transporters (EAAC-1) (glutamate serving as a precursor for GABA synthesis) are localized to the presynaptic terminal membrane. However, this direct recycling capability is not predominant at excitatory synapses, where little or no glutamate is transported directly back into the presynaptic terminal following release. At these synapses, astrocytic uptake (via glutamate transporters such as GLT-1) accounts for 80–90% of glutamate sequestration.

The Astrocytic Glutamate–Glutamine Cycle

Once transported into astrocytes, the potent neurotoxin glutamate is efficiently inactivated by conversion into the nontoxic amino acid, glutamine. This conversion is accomplished by glutamine synthetase, an enzyme whose expression is astrocyte-specific in brain. However, if all amino acid transmitters were rapidly, unidirectionally, and irreversibly absorbed into astrocytes following release, synaptic transmission would rapidly fail in the absence of a supply of neurotransmitter precursors. Alternatively, neurons would be required to synthesize large amounts of neurotransmitter de novo and in an ongoing manner, a hugely expensive metabolic endeavor. To avoid this failure or metabolic cost, an additional set of transporters is localized both in astrocyte and neuron membranes, to efficiently recycle glutamine back into neurons.

Glutamine exits astrocytes through the activity of a specialized set of transporters, termed system N transporters. It is then taken up by neurons by the activity of a second set of transporters, termed system A transporters. Once inside neurons, glutamine is then converted to glutamate by the mitochondrial enzyme, glutaminase. In excitatory neurons, glutamate can then be re-packaged into synaptic vesicles by vesicular glutamate transporters. In inhibitory neurons, the glutamate must be transformed into GABA by glutamic acid decarboxylase (GAD) before packaging into synaptic vesicles by vesicular GABA transport (via the vesicular inhibitory amino acid transporter, VIAAT). This neurotransmitter recycling pathway is summarized in **Figure 1**.

The primary methodology utilized in studies characterizing the dynamics of localized neurotransmitter flux through the recycling pathway described earlier has been radiotracer flux assays. Years of research in this arena has shown that the preferred substrate for GABA synthesis is glutamine. In brains of animals infused with radiolabeled glutamine or glutamate, a significant fraction of label rapidly appears in GABA, suggesting that inhibitory neurons rely on the glutamine cycle to a significant extent for neurotransmitter synthesis. We were interested in determining how glutamine cycle dynamics regulate inhibitory synaptic function – the final, biologically significant readout regulated by neurotransmitter recycling.

Methods and Results

Regulation of Hippocampal Inhibitory Synaptic Transmission by the Astrocytic Glutamate–Glutamine Cycle

By definition, neurotransmitter recycling requires that there be ongoing activity to necessitate vesicle refilling for subsequent release. Static, inactive inhibitory synapses should not exhibit much in the way of recycling requirements, while active inhibitory synapses should experience significant demand to regenerate GABA for subsequent vesicular release. In order to assess this prediction experimentally, we utilized patch clamp recording techniques in hippocampal slices to examine the potential role of the glutamine cycle in regulating inhibitory synaptic transmission, under both static and active conditions. The glutamine cycle was perturbed at several distinct steps by perfusion of the slices with various pharmacological antagonists, including the astrocytic glutamate uptake blocker dihydrokainate (DHK), the glutamine synthetase inhibitor, methionine sulfoxamine (MSO), and the System A neuronal glutamine transport inhibitor, α-(methylamino) isobutyrate (MeAIB) (see **Figure 1**). Activity was generated by synaptic stimulation at varying frequencies, and activity

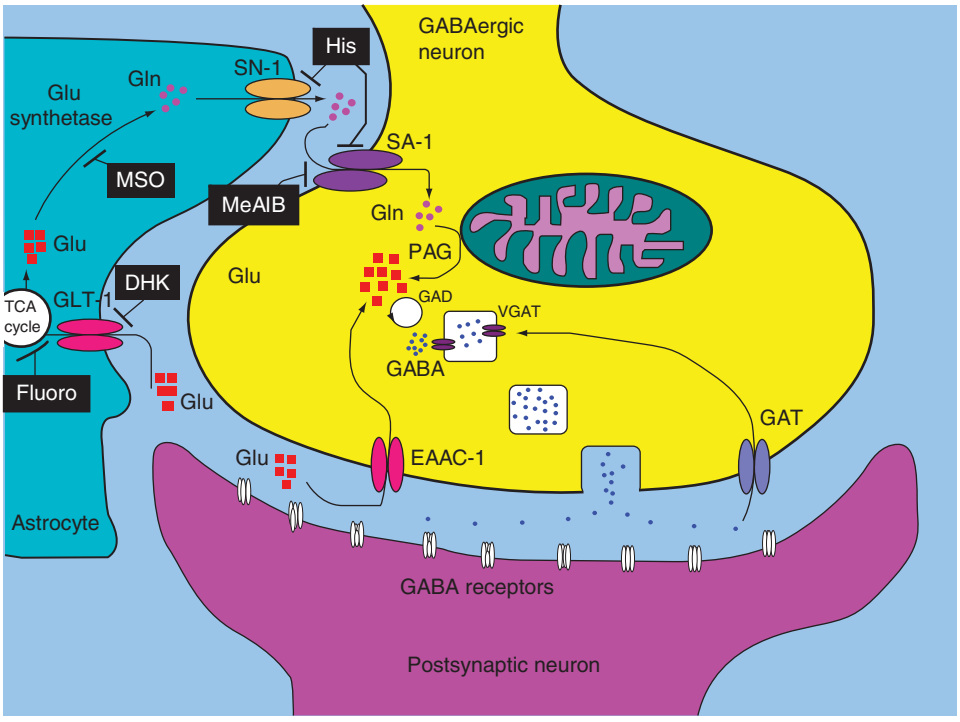


Figure 1 Schematic of the major functional players determining glutamate-glutamine cycle contributions to vesicular GABA content, and pharmacological antagonists targeting these players. Extracellular space is in blue, the presynaptic GABA terminal is in yellow, and an astrocyte is in green/turquoise. GAD, glutamic acid decarboxylase; GAT, GABA transporter; EAAC-1 and GLT-1; neuronal and glial glutamate transporters, respectively; VGAT, vesicular GABA transporter (also known as vesicular inhibitory amino acid transporter, VIAAT); SN-1 and SA-1, glial and neuronal (system N and A) glutamine transporters, respectively; PAG, phosphate-activated glutaminase; DHK, dihydrokainate; MeAIB, α-(methylamino)isobutyrate; MSO, methionine sulfoximine; his, histidine; fluoro, fluoroacetate (TCA cycle inhibitor).

was retarded in some experiments using sodium channel blockers and excitatory amino acid receptor antagonists.

In keeping with the obvious potential for an activity-dependent role of the glutamate–glutamine cycle in regulating inhibition, we found that blocking the glutamine cycle had no effect on inhibitory synaptic transmission in hippocampal neurons in the absence of activity (in medium containing sodium channel and excitatory amino acid receptor blockers). Under these conditions, little or no neurotransmitter recycling occurs. Antagonists blocking glutamine uptake into neurons (MeAIB), or enzyme antagonists targeting glutamine synthetase (MSO), had no effect on spontaneous inhibitory synaptic responses recorded in the absence of activity (miniature IPSCs, or mIPSCs, reflecting spontaneous, activity-independent vesicle fusion). Under these static conditions, in the presence of the sodium channel blocker, tetrodotoxin, neuronal glutamate uptake via EAAC-1 (localized in inhibitory terminals) and GABA reuptake via GAT-1 were responsible for maintaining GABA vesicle levels. Targeting either of these processes (by antisense oligonucleotides targeting EAAC-1, or blockade of GAT-1 by NO-711) resulted in reduced mIPSC amplitudes, slower mIPSC kinetics, and the appearance of peak mIPSC augmentation effects by benzodiazepines. The latter phenomenon is thought to reflect the lack of postsynaptic receptor saturation, due to decreased release of GABA. These data are consistent with the idea that vesicle GABA content in inactive synapses is predominantly mediated by direct recycling of glutamate and GABA, with no significant role of glutamine provided by the astrocytic glutamate–glutamine cycle.

In marked contrast to the earlier data, when we began to examine synapses under higher activity conditions (generated by sustained stimulation protocols), we found that 60–80% of synaptically released GABA was derived from the astrocytic glutamate glutamine cycle. To examine this process, we activated inhibitory synapses with a 15 min period of stimulation, consisting of a 4-pulse burst every 20 s, in normal media, and in media containing various astrocytic glutamate–glutamine cycle blockers. In control media, this stimulus protocol induced transient synaptic potentiation consisting of a 20–30% increase in inhibitory postsynaptic current amplitude, which persisted for 10–15 min. This potentiation was due to a presynaptic increase in the probability of release in the stimulated inhibitory terminals. In contrast, in media containing a glutamine cycle antagonist (either an astrocytic glutamate uptake inhibitor, dihydrokainate, a glutamine synthetase inhibitor, MSO, or a System A transport blocker, MeAIB), synapses showed a marked depression (60–80% reduction compared with the

potentiated response) in inhibitory synaptic efficacy, which gradually recovered over the next 20–40 min. This depression was due to decreased vesicular release of GABA, and occurred despite the fact that inhibitory synapses still showed an increased probability of release when stimulated in the presence of glutamine cycle blockers (assessed with paired pulse stimulation protocols, or by decreased failure rates in quantal analysis). The addition of exogenous glutamine could restore inhibitory synaptic efficacy during perfusion with MSO, but not MeAIB, supporting the concept that inhibition decreased because of glutamine depletion, and neuronal uptake of glutamine occurred through MeAIB-sensitive transporters.

Similar studies to those described earlier have been conducted by the Nicoll laboratory in hippocampal excitatory synapses, both in slices and in culture. In contrast to effects on inhibitory neurotransmitter supply, the glutamine cycle was found to play a much more indirect role in regulating glutamate neurotransmitter supply. Supply of exogenous glutamine, or blockade of the glutamine cycle with MSO, had little effect on excitatory synaptic responses in the short term (minutes to hours). Only after prolonged treatment were effects evident, consistent with the known dichotomy in cytoplasmic glutamate levels in excitatory and inhibitory neurons. Excitatory neurons have high cytoplasmic glutamate levels (mM), which would serve to provide a reserve neurotransmitter supply. Inhibitory neurons exhibit several orders of magnitude lower glutamate levels in cytoplasm.

Taken together, these data strongly support the concept that inhibitory (but not excitatory) synaptic terminals rely heavily on the astrocytic glutamate–glutamine cycle to replenish neurotransmitter stores depleted by synaptic activity. Although this concept was postulated on the basis of amino acid flux studies, these experiments provide the first demonstration that the glutamine cycle can directly regulate the inhibitory synaptic efficacy in a local, dynamic, and immediate manner. These results lead immediately to the hypothesis that any perturbation of the glutamine cycle, triggered as a consequence of either normal or pathological mechanisms, should have a direct consequence on the inhibitory efficacy, which in turn would alter circuit excitability. Thus, the regulation of the glutamate–glutamine cycle may constitute a novel mechanism for circuit plasticity, and also may be significant in pathophysiological states involving abnormal brain function, such as epilepsy.

Implications of Glutamine Cycle Coupling to Inhibitory Synaptic Efficacy: Scaling Inhibition to Regulate Enhanced Excitatory Synaptic Activity Levels

There is an intriguing implication derived from the prominent role played by the glutamate–glutamine cycle in providing neurotransmitter precursors for local GABA synthesis in inhibitory synapses: This process may directly couple the ability of these synapses to sustain activity to the immediately preceding levels of excitatory activity. That is, inhibitory synaptic power should be directly coupled to excitatory activity via the glutamine cycle. Such a synaptic scaling mechanism would be energetically efficient, and esthetically appealing. According to this scenario, inhibitory synapses will be able to release neurotransmitter in a more sustained manner during periods of elevated glutamatergic activity, and potentially release more neurotransmitter per vesicle (if vesicular content is not saturated). Similarly, during low activity states, GABAergic synapses would synthesize less transmitter, and potentially conserve energy. These combined processes of up- and downregulation of inhibitory efficacy during high and low network activity states, respectively, should stabilize network excitability through regulation of neurotransmitter supply. What might happen to network stability if the glutamate–glutamine cycle were compromised? One such state where ubiquitous disruptions in astrocytic function are evident is temporal lobe epilepsy, a disease characterized by network instability, manifested as seizures.

Loss of Glutamine Synthetase and GLT-1 in Reactive Astrocytes of the Epileptic Brain: Implications for Regulation of Network Excitability

Reactive astrocytosis is a hallmark of mesial temporal lobe epilepsy. Astrocytes in an epileptogenic focus within the human hippocampus, surgically resected from patients with temporal lobe epilepsy, exhibit dramatic changes in their anatomy and biology. Among the vast array of changes that have been described in these cells, downregulation of key proteins involved in the glutamate–glutamine cycle is notable. There is a virtually complete downregulation in the expression of the keystone astrocytic enzyme in the glutamine cycle, glutamine synthetase. This loss of glutamine synthetase is restricted to astrocytes within epileptogenic hippocampal areas – even immediately adjacent, nonepileptogenic areas (such as area CA2 and subiculum) retain the expression of this enzyme, despites its complete absence from sclerotic hippocampal cell areas, such as CA1, CA3, and the dentate gyrus. There is also a reduced expression of glutamate transporters in astrocytes from epileptic human hippocampus, accompanied by a loss of interglial gap junction coupling.

A loss of these keystone glutamine cycle enzymes and transporters in astrocytes from epileptic hippocampus has obvious implications for the regulation of circuit excitability. Neurotransmitter supply at inhibitory synapses in epileptic hippocampus would be expected to be significantly compromised. In addition, the capability of glutamine cycle-dependent synaptic scaling to maintain circuit

equilibrium in the face of changing activity levels would be lost. This condition has the potential to generate basal, steady state disinhibition in the epileptic hippocampus, which would be exacerbated during periods of elevated activity, including seizure initiation and/or propagation. Thus, a loss of glutamine cycle enzymes and transporters could contribute significantly to circuit disequilibrium and seizure susceptibility.

Directions and Challenges for the Future

Potential Novel Therapeutic Strategies Targeting the Glutamine Cycle in Epilepsy

Compromised neurotransmitter supply at inhibitory synapses in epileptogenic brain areas is a potential novel therapeutic target for seizure control. Since reactive (glutamine synthetase-lacking) astrocytes are restricted to focal epileptogenic regions, strategies to augment the glutamine supply should exhibit activity only in these pathologically modified regions, and have little effect on normal areas of brain not exhibiting gliosis. This specificity would make such strategies less prone to side effects derived from undesired actions on nonpathologic structures. Glutamine itself is innocuous, and is tolerated at high millimolar concentrations by brain and peripheral tissues without overt adverse activity. Gavage studies in rodents have shown effects on brain glutamine dialysate levels following an oral administration of high concentrations of this amino acid. Therefore, dietary intervention to augment brain glutamine levels could be a useful strategy to augment inhibition specifically in focal epileptogenic regions of brain. Since many anticonvulsant drugs target inhibitory synapses (often postsynaptic GABA receptors) as a mechanism of action, dietary supplements could increase the effectiveness of conventional antiepileptic drugs by augmenting synaptic GABA release specifically in the seizure focus, enhancing the activity of the receptors targeted by the antiepileptic medication, without effects in other brain areas. This strategy is a logical therapeutic approach deserving further attention and corroboration through a combination of basic and clinical studies.

Potential Interactions of Glutamine Cycle Deficits with Existing Therapeutic Strategies

Ameliorating glutamine cycle-mediated deficits in inhibitory synaptic transmission in the epileptic brain may be an as-yet-uncharacterized mechanism of the action of metabolic therapies in epilepsy, including the ketogenic diet. This diet is known to enhance GABA levels (although there are other, significant, potentially antiepileptic effects of this diet as well). This GABA effect is produced by the presence of high ketone body levels, which retards glutamate-to-aspartate flux and thus increases precursors for GABA synthesis. Thus, ketosis induced by the ketogenic diet may specifically augment GABA, particularly in the seizure focus where there is insufficient GABA due to glutamine cycle deficits. The effects of elevated ketones would presumably be less significant in nonfocal brain areas, where GABA levels are normal. Understanding the precise intermediaries of metabolic therapies that lead to this GABA effect (and to seizure control) could more specifically target dietary efforts to control seizures, without the difficulties involved in the ketogenic diet (which restricts patient compliance). This area deserves further research attention.

Gliosis

Clearly, understanding the role gliosis may play in epilepsy is an area of growing interest. Defining the precise consequences of neurotransmitter recycling deficits on synaptic and circuit function in focal epileptogenic brain regions is a formidable challenge worthy of considerable additional research activity. These efforts have the added benefit that they are likely to translate into significant clinical advances toward improving patient care. In a broader context, it is important to define the mechanisms mediating gliosis, so as to gain a better understanding of the origin of reactive astrocytes as well as the anatomical, molecular, and physiological plasticity occurring in these cells; the effects of these astrocyte changes on neuronal circuit function constitute a critical area about which little is known. Gliosis is a ubiquitous finding in myriad neurological and psychiatric conditions, and better understanding of how this phenomenon regulates the brain function should be a significant, relevant focus of future research.

See also: **GABA**: Pathophysiology of Status Epilepticus; **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; **Metabolic Changes**: Glucose Metabolism and $GABA_A$ Receptor Dysfunction in Human Partial Epilepsy.

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Dopamine Modulation of Neurotransmission: Relationship to Epilepsy

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Introduction

The prefrontal cortex is composed of cortical areas in the most anterior regions of the frontal lobe. This area plays an important role in governing a number of behaviors, including motivation, emotion, and learning and memory. Patients with epilepsy often have frontal lobe dysfunction. Neuropsychological studies have shown that epileptic patients have impairments of attention, episodic memory, and executive function, suggestive of frontal cortex involvement. These deficits are particularly apparent in frontal lobe epilepsy, but differences in memory processing are also seen in temporal lobe epilepsy. The deficits underlying epilepsy-associated frontal lobe dysfunction have been suggested to be intrinsic to the frontal lobe and/or due to spread of epileptiform activity from the temporal region. The neurochemical changes subserving altered cognitive functioning are unclear, but may involve changes in neuromodulatory systems involving dopamine.

Prefrontal cortex receives inputs from widespread cortical and subcortical regions, including sensorimotor and limbic areas. Integration of information from these inputs has been specifically implicated in cognitive and neuropsychiatric processes. Prefrontal cortex has a pivotal role in working memory, the ability to process information 'online.' Working memory in primates, often studied using delayed response tasks, is associated with sustained increases in firing of neurons in prefrontal cortex during the delay periods. Similarly, unit activity recorded in rats during delayed performance indicates that rodent prefrontal cortex is also involved in short-term or working memory. The local networks present in prefrontal cortex consist of interconnected inhibitory and excitatory neurons. The balance between recurrent excitation and inhibition in these local networks is important for the stabilization of ongoing activity and proper circuit functioning.

Selective lesions of the cortical dopamine input impair working memory in both primates and rats. Clinical and experimental studies have implicated dopamine in the pathogenesis of a number of neurological and psychiatric disorders, including epilepsy. Dopaminergic mechanisms contribute to excitability increases induced by transcranial direct current stimulation in human cortex, and dopamine receptor binding is decreased in temporal lobe epilepsy patients. How dopamine modulates working memory and cortical excitability is still unclear. Understanding the basic mechanisms underlying dopamine's modulation of synaptic activity in local cortical networks is critical for insights into how modulatory systems contribute to changes in frontal lobe functioning in epilepsy.

Background

Dopamine Innervation of Prefrontal Cortex

In rats, prefrontal cortex has been defined as the part of cortex that receives inputs from the mediodorsal nucleus of the thalamus and from the dopaminergic cell groups localized in the ventral tegmental area. Immunohistochemical studies in rat and primate cortex have shown that dopamine terminals, together with glutamatergic axon terminals, form so-called synaptic triads on dendritic spines of pyramidal neurons. Dopamine receptor activation can gate excitatory synaptic inputs to pyramidal neurons. Moreover, dendritic spines and shafts of pyramidal cells, along with dendrites of GABAergic interneurons, receive symmetric contacts from dopamine axon terminals. Pyramidal neurons and interneurons express

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multiple different subtypes of dopamine receptors, providing a means for differential dopamine modulation of cortical neurons. Dopaminergic activation may shift, in a complex way, the balance between excitation and inhibition in neuronal circuits, providing a wide range of possibilities for neuronal modulation in prefrontal cortex.

Dopamine Modulation of Synaptic Activity

Dopamine's action in the brain at the cellular and network level is still under investigation. Dopamine can both increase and decrease spontaneous firing of neocortical neurons in vivo. Long-lasting transitions of prefrontal cortex neurons to a more excitable *up* (depolarized) state are enhanced by dopamine. During short-term working memory processing, firing of groups of prefrontal cortex neurons increases in a dopamine-dependent manner. In vitro electrophysiological experiments suggest that dopamine has multiple effects on prefrontal cortex neurons. Both increases and decreases in postsynaptic excitability of pyramidal neurons have been reported following D1 receptor activation. In addition, changes in excitability mediated by D2 receptors have been reported. Complex effects of dopamine on synaptic responses are also present and may be layer- and species-specific. AMPA receptor-mediated excitatory postsynaptic currents (EPSCs) in layer V pyramidal cells are depressed by a D1 receptor mediated effect of dopamine, whereas NMDA responses have been reported to be both enhanced and depressed. EPSCs in layers II/III are enhanced by dopamine in rats but decreased in primates.

Anatomical and electrophysiological studies have shown that axons of layer II/III pyramidal cells make excitatory connections with each other via local axon collaterals. This circuit could provide a substrate for the sustained increase in firing seen during delayed response tasks. We have described dopamine-mediated increases in EPSCs in these cortical circuits. An important question, which has received little attention, is whether excitatory synapses onto GABAergic interneurons in various cortical layers are also potentiated. Inhibition is important for controlling the timing of neuronal activity and shaping the temporal flow of information in prefrontal cortex. The mechanisms underlying spatial working memory have been shown to depend upon $GABA_A$-receptor dependent inhibition. Dopamine-induced increases or decreases in excitatory input to inhibitory interneurons could thus affect working memory.

Given the complexity and microheterogeneity of dopamine's effects, the net action of dopamine modulation of local circuits in prefrontal cortex is not easily predicted. The presence of massive recurrent excitatory connections, which are powerfully regulated by inhibitory mechanisms, has been implicated in the susceptibility of both neocortex and hippocampus to the development of epileptiform activity and seizures. Modulatory influences strongly influence activity in thalamocortical and neocortical circuits. A shift of the balance towards excitation by neuromodulators may lead to the generation of epileptiform activity, whereas an enhanced inhibitory tone could dampen excitability.

Dopamine Regulation of Activity in Neocortical Circuits

Local neocortical circuits are formed by synaptic connections between pyramidal cells and interneurons. Excitatory connections are predominant, with 85% of synaptic connections within the gray matter being excitatory; most of these connections originate from cortical neurons. Pyramidal cells and interneurons in prefrontal cortex display correlated discharges over time-scales that range from millisecond action potential synchronization to hundreds of millisecond covariation in firing rates. Experimental and modeling studies have shown that circuits containing recurrent excitatory connections, like those among layer II/III pyramidal neurons, are inherently unstable. Feedback inhibition provided by GABAergic interneurons provides a stabilizing influence and prevents runaway excitation. In our laboratory, we have been using in vitro slices of rat neocortex to explore the role of dopamine receptors in maintaining the balance between inhibition and excitation necessary for normal functioning of neocortical circuits.

Methods

Our laboratory has been using in vitro studies of rat prefrontal cortex to study modification of synaptic activity by a variety of neuromodulators. We study both normal synaptic activity and epileptiform activity in chronic models of epilepsy. The focus in this article is on dopamine receptors, but the approach is applicable to a variety of neurotransmitters. Slices containing the anterior cingulate cortex and the shoulder (or Fr2 region) of the frontal cortex are used for recording, as these regions receive a dense dopaminergic innervation. We use a two-pronged approach in our studies. The first component exploits the power of the visualized neuron brain slice preparation. Using infrared illumination and Nomarski differential interference contrast microscopy, it is possible to record from identified cell types within prefrontal cortex. We use this method to study the effects of neuromodulators on intrinsic excitability of neurons and the changes induced by these modulators in synaptic activity. The second method used is the voltage-sensitive dye recording technique. Although single cell recordings provide useful information on biophysical properties of individual cells and synapses, they cannot simultaneously monitor vertical and horizontal spread of activity in local neocortical

circuits, an important issue in epilepsy research. Voltage sensitive dyes can be used to reliably monitor the initiation, distribution, and spread of activity in the neocortex in vitro. Modern optical methods allow reproducible detection of dye signals in single trials and without serious photo-bleaching or toxicity. We have shown that the changes in dye signals (i.e., fluorescence changes) – in response to alterations in extracellular calcium, addition of tetrodotoxin, or application of excitatory amino acid receptor antagonists – correlate well with established electrophysiological measures of electrical activity. We use this method to correlate effects seen at the local circuit level with observations made using single cell voltage-clamp recordings. Our studies have examined changes in intrinsic cell excitability, synaptic activity, and spread of activity after bath application of known concentrations of D1- and D2 receptor specific agonists and antagonists.

Results

Photomicrographs of neurons visualized during recording are shown in **Figure 1(a)**. The left-hand panel shows the oval cell body of a GABAergic interneuron in layer I, whereas the right-hand panel shows a group of pyramidal cells in layer II/III. An approaching patch electrode can be seen. Including biocytin in the recording pipette allows unequivocal identification of these cells. Camera lucida reconstructions of a layer I interneuron and a pyramidal cell are shown in **Figure 1(b)**. An important early observation was that dopamine had different effects on interneurons and pyramidal cells. Due to our ability to make recordings from GABAergic interneurons, we were able to determine that dopamine directly depolarizes the majority of cortical interneurons in frontal and cingulate areas by enhancing the hyperpolarization-activated, non-specific, cationic current I_h. This action leads to increased interneuron excitability and increased spike firing, as shown on the left part of **Figure 1(c)**. Excitability of the pyramidal cell (shown on the right part of **Figure 1(c)**) was moderately decreased, but overall there was no significant effect on resting potential or intrinsic excitability of pyramidal cells. In additional studies, we have shown that spontaneous and evoked IPSCs appear to be differentially regulated by dopamine. Spontaneous IPSCs are increased in frequency, presumably due to the enhanced interneuron excitability; however, dopamine can inhibit evoked IPSCs in layer II/III pyramidal cells. This effect appears to be mediated via a presynaptic mechanism in GABAergic nerve terminals, apparently involving D1-like receptor stimulation of an adenylate cyclase-protein kinase A-mediated process. These diverse effects raise the possibility that dopamine could decrease overall cortical excitability by enhancing GABAergic tone via an increase in spontaneous IPSCs, while at the same time facilitating specific inputs by reducing evoked IPSCs. Such differential effects could be important for control of circuit excitability in epilepsy.

Our results using single cell recordings are summarized in **Figure 2**. This illustration makes the point that dopamine can act at multiple sites within neocortex. Complex effects on inhibition and excitation are present, as are cell type specific changes in intrinsic excitability. D1 receptors seem to underlie many of the observed effects, although other investigators have shown that D2 receptors have important effects in deeper cortical layers. These studies have, in large part, used a reductionist approach where a particular cell, synapse or receptor subtype was isolated for investigation. An important issue in epilepsy is the *net effect* of a neuromodulator on cortical excitability. We turned to voltage-sensitive dye studies to examine the net effect of dopamine on normal cortical excitability.

In our recent work, we have used optical imaging with the voltage-sensitive dye RH 414 to study how spatiotemporal patterns of activity were modulated by dopamine under conditions of normal synaptic transmission. **Figure 3** (upper) shows a pseudocolor map of dye signals recorded from a prefrontal cortical slice after a single intracortical stimulation under control conditions. It can be seen that stimulation results in activation of a small area of the slice. Activity was most prominent in upper cortical layers and extended laterally for up to 900 μm. The interval between each map in **Figure 3** was 15 ms, showing that activity spreads laterally to adjacent areas and persists for ~60 ms. After application of 30 μM dopamine for 10 min, activity in response to the same stimulation was decreased (**Figure 3(a)**, bottom). To quantify these changes, peak signal amplitudes – before and after dopamine application – were calculated for diodes lying on a line corresponding to the region of the slice showing the highest levels of activity. These amplitudes were normalized to the highest peak amplitude and plotted as a function of distance from the diode showing the highest peak dye signal (**Figure 3(b)**). Our results show that peak dye signals and lateral spread of activity were decreased after dopamine application. The level of background activity is indicated by the dotted line. Taken together with results from whole cell recording, the voltage-sensitive dye imaging suggests that dopamine's net effect is to increase inhibition under these control conditions. Acting via D1-like receptors, dopamine restricts spatiotemporal spread of synaptic activity in local neocortical networks.

Future Directions

For a more complete understanding of the nature of dopamine's modulatory role in epilepsy, it is necessary

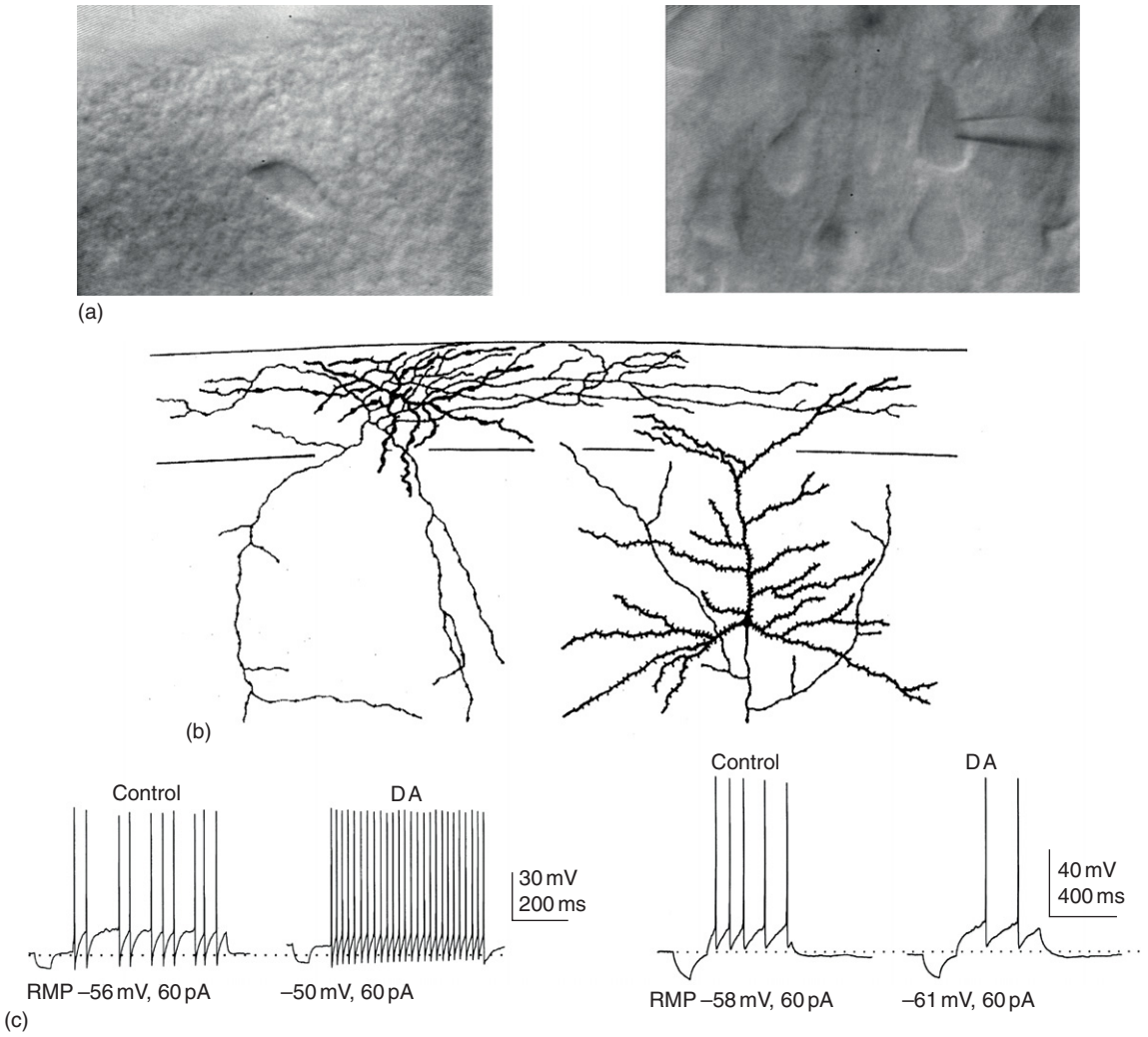


Figure 1 Effects of dopamine on intrinsic excitability of identified cells in rat prefrontal cortex. (a) Infrared Nomarski differential interference contrast images of layer I (left) and pyramidal (right) neurons, as seen during recording. The patch pipette can be seen approaching a pyramidal cell from the right. (b) Camera lucida reconstructions of the biocytin labeled neurons. Pial surface is up in all cases. (c) Dopamine increases interneuron excitability but decreases pyramidal neuron excitability. Recordings were made in the presence of 10 μM bicuculline methiodide (Bic), 20 μM D(−)2-amino-5-phosphonovaleric acid (D-APV), and 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) to block $GABA_A$, NMDA, and AMPA receptors, respectively. Left: in a layer I neuron, bath application of 40 μM DA induced a depolarization of ~6 mV and increased the number of spikes evoked by 60-pA depolarizing current pulses. Input resistance, monitored by a current pulse of −40 pA, was also increased from 230 MΩ in control to 280 MΩ in DA. Right: in a pyramidal neuron, bath application of 40 μM DA induced a hyperpolarization of ~3 mV and decreased the spikes evoked by injecting 60-pA depolarizing current pulses. Input resistance, monitored by a current pulse of −60 pA, was also decreased from 270 MΩ in control to 225 MΩ in DA. In both cells, DA effects were partly reversed on wash. Reproduced from Zhou FM and Hablitz JJ (1999) Dopamine modulation of membrane and synaptic properties of interneurons in rat cerebral cortex. *Journal of Neurophysiology* 81: 967–976, with permission from American Physiological Society.

to understand how this neurotransmitter alters activity in local neuronal networks in intact animals. The in vitro studies described here document the ability of dopamine to sharpen activation patterns by enhancing inhibition. More information is needed about the effects of endogenously released dopamine on activity in local circuits. This information could be obtained in experiments in intact animals, involving stimulation of cortically projecting dopaminergic fibers in the ventral tegmental area. Recent studies have examined the spatiotemporal dynamics of cortical sensory responses in vivo using voltage-sensitive dye imaging combined with whole-cell voltage recordings and neuron reconstructions. Similar approaches would be a powerful means of understanding dopamine modulation in intact animals.

Advances in this area will also come about by establishing the effects of dopamine agonists and antagonists in chronic animal models of epilepsy. By chronically altering dopamine levels via systemic or local administration of agents selective for activating or inhibiting specific

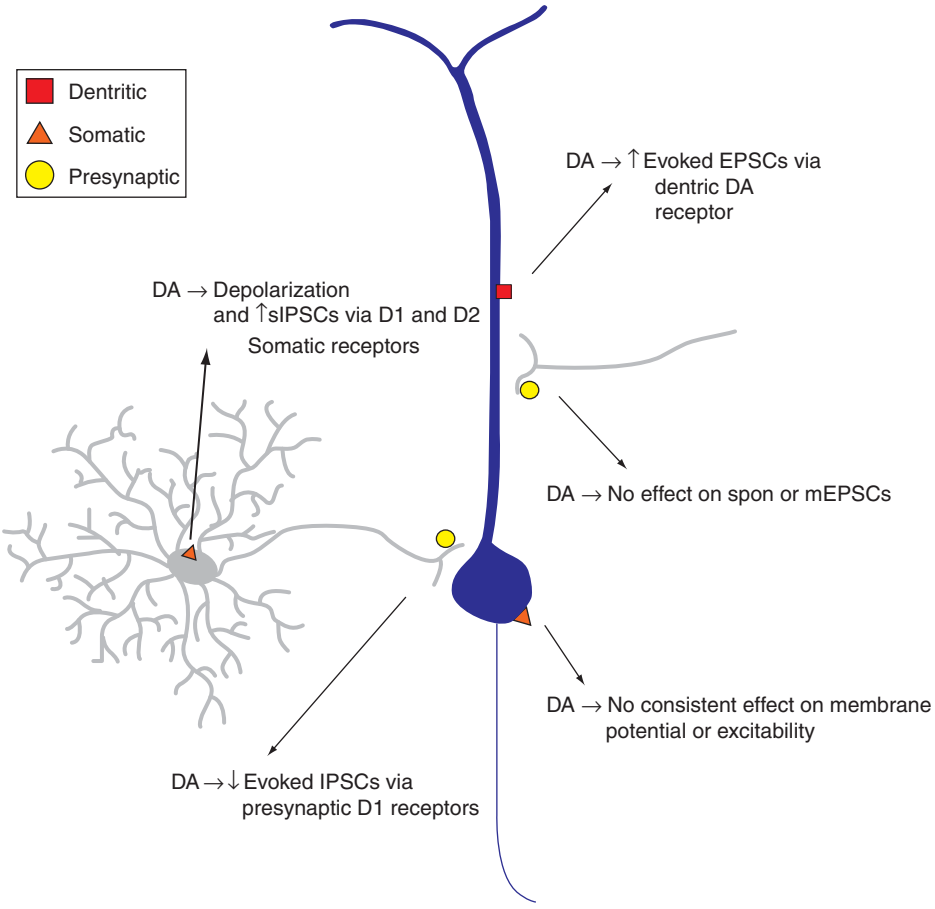


Figure 2 Schematic diagram showing the multiple sites of action and effects of dopamine in rat frontal cortex. Dopamine receptors are located both on GABAergic interneurons (gray) and pyramidal cells (blue). These receptors can be either somatic (orange triangle) or dendritic (yellow circle) or on presynaptic terminals (red box). Not indicated is the additional complexity of variability in dopamine receptor subtype (D1- or D2-like) at each location.

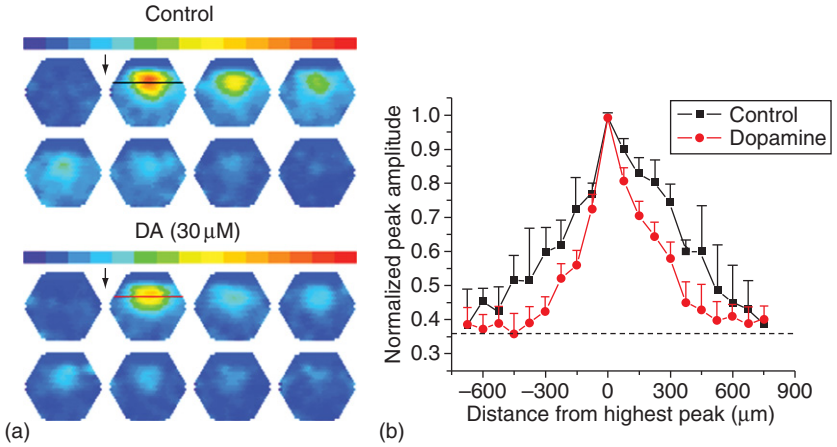


Figure 3 Dopamine decreases spatiotemporal spread of activity evoked by intracortical stimulation in prefrontal cortex under conditions of normal synaptic transmission. (a) Pseudocolor maps of activity evoked in a prefrontal cortical slice (pial surface up) by a single intracortical stimulation (↓, time of stimulation) before and 10 min after application of dopamine (30 μM) in bath. Fixed pseudocolor scale was used for all frames. A frame interval of 15 ms was used. (b) Normalized peak dye signal amplitudes measured along a line drawn across the diode array (as shown in (a)) plotted as a function of distance from the diode showing highest peak dye signal. Dye signals were normalized to the highest peak dye signal. Error bars indicate SE ($n = 9$). Dashed line indicates the level of background noise. Reproduced from Bandyopadhyay S and Hablitz JJ (2007) Dopaminergic modulation of local network activity in rat prefrontal cortex. *Journal of Neurophysiology* 97: 4120–4128, with permission from American Physiological Society.

dopamine receptor subtypes, the time to develop seizures and/or the frequency and severity of established seizures could be examined. A variety of models, including those involving both hippocampus and neocortex, should be considered, because given the heterogeneity of dopamine effects, differential outcomes might occur.

Dopamine is but one modulatory system in the neocortex. Innervation by serotonergic and noradrenergic fibers is also prominent. Considerable controversy exists concerning whether antidepressant drugs, which affect these modulatory systems, are anticonvulsant or proconvulsant. Application of techniques outlined above for study of dopamine could also be profitably applied to serotonergic and noradrenergic systems, leading to a more complete understanding of the role of modulatory systems in epilepsy.

See also: **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; The Substantia Nigra in the Control of Seizures.

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Impact of Norepinephrine on Epileptogenesis

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Introduction

The impact of neurotransmitters, neuropeptides, and other endogenous chemicals on the development and expression of epileptic seizures is of great clinical and experimental/theoretical interest. Indeed, as convulsive seizures are recruited in the brain, it is quite likely that nearly all neural networks in most structures are engaged differentially in the seizure, depending upon many factors, including genetic background. In this article, I present some of the evidence concerning the involvement of norepinephrine (NE) in epilepsy, where its major impact appears to be on epileptogenesis – i.e., the development of seizures, rather than on modulation of the chronic condition of epilepsy – i.e., the epileptic state. If epilepsy is the pathological offspring of otherwise normal brain mechanisms, as has been suggested by others like Graham Goddard, the normal mechanisms that are likely usurped during epileptogenesis are probably involved in the neuroplasticity of learning; further, the network changes that subsequently represent the epileptic state are likely realized through the usurped mechanisms of memory, where the epileptic state represents the engram of the neuroplastic, epileptogenic process.

Background

The role of NE in the expression of seizures has a long and distinguished history. Although relatively less work has been done recently in this regard, NE historically has

dopamine receptor subtypes, the time to develop seizures and/or the frequency and severity of established seizures could be examined. A variety of models, including those involving both hippocampus and neocortex, should be considered, because given the heterogeneity of dopamine effects, differential outcomes might occur.

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Introduction

The impact of neurotransmitters, neuropeptides, and other endogenous chemicals on the development and expression of epileptic seizures is of great clinical and experimental/theoretical interest. Indeed, as convulsive seizures are recruited in the brain, it is quite likely that nearly all neural networks in most structures are engaged differentially in the seizure, depending upon many factors, including genetic background. In this article, I present some of the evidence concerning the involvement of norepinephrine (NE) in epilepsy, where its major impact appears to be on epileptogenesis – i.e., the development of seizures, rather than on modulation of the chronic condition of epilepsy – i.e., the epileptic state. If epilepsy is the pathological offspring of otherwise normal brain mechanisms, as has been suggested by others like Graham Goddard, the normal mechanisms that are likely usurped during epileptogenesis are probably involved in the neuroplasticity of learning; further, the network changes that subsequently represent the epileptic state are likely realized through the usurped mechanisms of memory, where the epileptic state represents the engram of the neuroplastic, epileptogenic process.

Background

The role of NE in the expression of seizures has a long and distinguished history. Although relatively less work has been done recently in this regard, NE historically has

shown one of the most profound suppressive effects ever recorded on the development of epilepsy. Thus, the neuroplasticity associated with epileptogenesis may be associated with the period in which NE exerts the majority of its antiepileptic effect. Although NE exerts its antiepileptic effect in a variety of experimental paradigms, including systemic pentylenetetrazol and electroshock, its most robust action has been recorded in the kindling paradigm.

Originally, Racine's group demonstrated that suppression of catecholamine activity facilitated the development of amygdala kindling. In their experiment, they administered (systemically) reserpine (a nonselective catecholamine depleting agent) and 6-hydroxydopamine (6-OHDA; a nonspecific monoamine neurotoxin) that depleted both NE and dopamine. Subsequently, they showed that the effects of this 6-OHDA treatment depended on the loss of NE-containing neurons; if 6-OHDA administration was preceded by systemic administration of desmethylimipramine (DMI) (a NE reuptake inhibitor that selectively protects NE neurons from 6-OHDA), 6-OHDA killed only DA neurons. These NE protected rats now kindled similarly to saline controls. In a reciprocal fashion, increasing NE availability with antecedent systemic DMI injections alone or by locus coeruleus (LC) stimulation significantly delayed the kindling process.

The involvement of NE in epileptogenesis was also supported by the demonstration that transplants of fetal LC cells into the hippocampus or amygdala-piriform area of 6-OHDA-pretreated rats significantly slowed kindling from the hippocampus and amygdala, respectively (compared to 6-OHDA-pretreated rats without fetal LC grafts). The grafts produced robust sprouting of NE terminals in the area of the transplant, which spontaneously released NE and clearly affected local epileptogenesis. Importantly, when the grafts were implanted into previously kindled rats, they had no discernible effect on the triggering or the form (i.e., the expression profiles, including latencies and durations of convulsive events) of the kindled seizures. Thus, as shown with other manipulations, NE applied after kindling had little impact on the triggering or expression of already kindled limbic seizures.

Methods

In our laboratory, we have questioned NE involvement in epileptogenesis and the epileptic state in several different ways. First, we have examined the effect of NE on local circuitry in the limbic system that is known to be critically important in the convulsive expression of seizures. This circuitry is manifested heavily in the parahippocampal cortices, especially the piriform, perirhinal, insular, and entorhinal areas. The convulsive expression can be directly driven by electrical stimulation of perirhinal and insular neurons that project robustly to the frontal motor fields, where the convulsive motor event seems to originate. Second, we selectively bred rats to be natively seizure prone or seizure resistant to the kindling process. With these rats, we have asked how their NE system responds to kindling and other natural stressors, and to NE manipulations that affect epileptogenesis and its expression. Third, we have performed microdialysis studies to measure the release of NE and other transmitters in the amygdala during the early stages of kindling epileptogenesis, using different stimulation protocols (i.e., distributed or traditional once-daily electrical kindling versus massed stimulation kindling procedures).

Recent Results

In developing a coronal slice preparation of the amygdala/piriform/perirhinal area, we showed that this temporal lobe area is intrinsically excitable and readily shows spontaneous burst discharges in vitro after being excised from either amygdala or hippocampal kindled rats. The in vitro perirhinal/insular area taken from naïve rats showed small burst discharges without kindling, but antecedent kindling augmented the bursting, promoting increased spontaneous discharge frequencies and durations in the network. The burst activity in naïve, control slices could be significantly dampened by perfusion of the slices with NE. The effect of NE on kindled slices, however, was much less robust – much like the results obtained from intact kindled animals, where NE had lost its suppressive efficacy.

Selective NE agonists and antagonists to slices from control tissue indicated that the $\alpha 1$ NE agonist, L-phenylephrine, had little effect on the burst responses, but the $\alpha 2$ agonist, clonidine, duplicated the suppressive effects of NE itself. In contrast, the β agonist, isoproteronol, augmented burst responses. Clearly, in this in vitro preparation with manipulation of NE receptor subtypes, NE itself has the potential to either promote suppression or enhancement of triggered or spontaneous burst events, depending upon the compliment of receptor subtypes it activates and the networks in which they reside. Since these different receptors have differential affinities for NE, we have hypothesized that these differential effects of NE might be a result of dose-dependent actions, where low doses of NE could activate the network (assuming the β receptors have a greater affinity), while higher doses (acting on the postsynaptic $\alpha 2$ receptors) could suppress it; this hypothesis has not yet been tested.

Extrapolating this hypothesis to the whole animal, one might predict that low levels of NE (conditions of low arousal) would potentiate responsiveness to stimulation,

while higher levels of NE (during higher arousal) would 'channelize' responsiveness (information) by suppressing competitive but less intensely activated networks, and focusing activity/attention. The suppressive postsynaptic action of the α2 agonist can be seen in whole animals deprived of their presynaptic NE machinery, where clonidine suppressed the development of amygdala kindling in 6-OHDA-pretreated rats in a manner similar to intact controls.

When outbred rats from a Wistar and Long-Evans hooded cross were selectively bred to be seizure prone or seizure resistant to amygdala kindling (based on their Fast or Slow rates of kindling development to stage-5 convulsive seizures), a number of important observations were made concerning NE and associated behaviors. The fast kindling strain in their naïve state showed higher postmortem levels of NE activity (3-methoxy-4-hydroxyphenolglycol, MHPG) in several limbic structures compared with the Slow strain, in the presence of various stressors or aversive conditioning procedures. This result was corroborated in microdalysis experiments, where the levels of NE in the amygdala/piriform area in Fast rats rose to higher levels than in Slow rats when the animals were 'challenged' by amygdala kindling procedures. The unique importance of the NE system to Fast rats was further suggested when Fast and Slow rats were subjected to intraventricular 6-OHDA administration, as described earlier. The amygdala kindling rates of both Fast and Slow rat groups significantly decreased following depletion of NE by 6-OHDA; however, the NE depletion was catastrophic for the behavior of Fast rats, where most Fast rats experienced full stage-5 seizures on their first kindling stimulation compared with the much more resistant kindling profiles in the 6-OHDA-pretreated Slow rats (Mean ± SEM kindling rates: Fast controls = 7.9 ± 1.1; Fast 6-OHDA = 1.2 ± 0.1; Slow controls = 32.6 ± 3.8; Slow 6-OHDA = 8.0 ± 1.2; $N = 8$ in all groups). Clearly, with a similar reduction of NE levels in Fast and Slow rats (94–97% reduction compared with their controls), Slow rats had other effective inhibitory mechanisms in place against epileptogenesis that were not evident in Fast rats. Our studies suggest that the residual inhibition in Slow rats, that attenuated their amygdala kindling rates compared with Fast rats after the loss of NE, was likely to be GABAergic in nature.

Using a different kindling paradigm, additional information about NE involvement in epileptogenesis can be obtained. If one mass stimulates (MS treatment) the amygdala (with interstimulation intervals, ISI, of 10 min or less) above threshold for triggering afterdischarges (ADs), very little kindling occurs in outbred rats (e.g., Long-Evans hooded). In contrast, such short ISIs tend to facilitate or promote kindling from the hippocampus. Clearly, different neural networks respond differentially when challenged in this manner. When stimulating the amygdala in rats given the α2 NE receptor blocker, yohimbine, we found that a significant increase in the expression of generalized seizures was triggered throughout the 50 stimulation experiments (a 5-min ISI). In contrast, the β blocker propanolol marginally reduced the triggered seizure activity, by shortening the evoked ADs (so that there was no concomitant behavioral seizure expression). When a similar MS treatment was applied to naïve Fast and Slow rats, paradoxically perhaps, Fast rats had a greater release of NE in their amygdala/piriform area compared with Slow rats (as seen with microdialysis), yet they also exhibited intermittently several strong generalized seizures. There were no such enhanced responses in Slow rats. Clearly, the greater focal release of NE in Fast rats was not able to suppress the intermittently stronger seizure response (compared with Slow rats); however, this increased NE release was associated with a phasic elevation of AD thresholds that rarely was seen in the Slow rats or outbred controls.

Pharmacological manipulation of the NE system of Fast and Slow rats during MS, and its impact on subsequent daily amygdala kindling, was also informative. The α2 antagonist, idazoxan, greatly increased the AD durations of Slow rats during the MS procedure, and several of these discharges were accompanied by generalized seizures; yet idazoxan had very little effect on Fast rats. In contrast, an α2 agonist, guanfacine, facilitated the appearance of generalized convulsive seizures during MS in Fast rats, with little effect on Slow rats. The enhanced responsiveness of Slow rats to idazoxan during MS treatment supports the earlier demonstration, in distributed stimulation paradigms, that activation of the postsynaptic α2 NE receptors provides considerable suppressive effect on the kindling epileptogenesis in outbred rats. This action was clearly the case during MS treatment in Slow rats, but not in Fast rats. By contrast, Fast rats during the MS treatment showed little local amygdala sensitivity to either of the two α2 NE manipulations, but did show an increase in the number of recruited generalized convulsive seizures in the presence of guanfacine. These observations suggest that the various NE receptor subtypes, their distribution, pre- or postsynaptic expression, or efficacy in several brain areas are different between Fast and Slow rats.

Data from such pharmacological studies might involve activation of pre-versus postsynaptic α2 NE receptors to create these differential effects on seizure behavior in the two strains; however, this possibility must be interpreted with caution, as variations in methodology may often lead to conflicting results. For example, differences in the distribution of NE receptors between species, strain, and brain regions are possible; further, different receptor subtypes are likely to influence the selectivity and binding properties for various agonists and antagonists, all of which may depend on dosage of the drug, as suggested earlier. Because different α2 agonists can produce opposite responses (proconvulsant and anticonvulsant) in the

same or different animals, Szot et al. have argued, based on their data with clonidine and guanfacine, that these two agonists have different affinities for pre-versus postsynaptic α_2A receptors; further, other α2 phenotypes exist (e.g., α2C). In fact, we have reported significant elevations of α2C mRNA expression in naïve, unkindled Slow rats compared with Fast rats in several brain regions, including nucleus accumbens, dentate gyrus, cerebellum, cingulate gyrus, and neocortex layer II.

Future Directions

At the present time, the involvement of the α2C receptor in NE activity is unknown, as is the extent of the effect that kindling has on mRNA expression in Fast and Slow strains; analyses of these issues should provide an interesting avenue for future investigation. Previous studies have shown that α2A receptors are approximately 10× more abundant than α2C receptors in many areas of the brain, and likely responsible for the rapid inhibition of NE release. The α2C receptor subtype is interesting, as it is located both in the intracellular compartment and on the plasma membrane but with the vast majority of receptors found intracellularly. Although both α2A and α2C receptors are present pre- and postsynaptically, MS of the amygdala may potentially alter, differentially, the expression and/or localization of receptors in Fast and Slow rats. In the present study, differences in pre-versus postsynaptic properties in Fast versus Slow rats could easily be the case, especially since guanfacine did not affect focal AD characteristics (like AD thresholds and durations) in the amygdala of either strain, but facilitated the occasional recruitment of the convulsive motor network in Fast rats. This result was surprising, since several studies have reported guanfacine to be anticonvulsant against a number of seizure models in adult outbred animals; however, it would explain Fast rat behavior if guanfacine was only effective presynaptically in these animals, and only before kindling.

Although we had postulated that idazoxan might have a preferential affinity for postsynaptic α2 receptors, it can also block presynaptic α2 autoreceptors and additionally bind to α1 and β receptors. Further, numerous reports have shown that idazoxan binds at high affinity to non-adrenoceptor idazoxan binding sites. It has been suggested that occupation of these sites by idazoxan may counteract the proconvulsant effects of postsynaptic α2 adrenoceptor blockade. If this is the case, it is possible that MS of the amygdala may have increased the number of these non-NE idazoxan binding sites in Fast rats compared with Slow rats. Higher NE levels in Fast rats during MS would facilitate binding of idazoxan to these other receptors, and depending upon their location and function, could impact other neurotransmitter systems and the release of other inhibitory transmitters such as GABA or serotonin.

Finally, the fact that chronic activation of postsynaptic α_2 noradrenergic receptors via distributed kindling protocols or by drug administration can presumably regulate NE receptors, it is possible that the first few stimulations of the MS protocol effected such a change in the amygdala area in Fast but not Slow rats, rendering their amygdala area insensitive to the NE manipulations. If such a downregulation occurred largely in the local amygdala/neocortical network, application of the systemic α2 agonist, guanfacine, should have reached the LC, and with presynaptic agonistic efficacy, reduced forebrain NE, making Fast rats more seizure prone. This result was, indeed, the behavioral outcome of the treatment. By contrast, Slow rats maintained considerable sensitivity to the α2 antagonist, idazoxan, throughout the MS protocol, as well as through the completion of the distributed kindling phase that followed the MS treatment, suggesting that they might be less subject to changes in NE receptor regulation in the face of frequent activation.

In the 2 weeks following MS treatment, all rats had their AD thresholds checked once weekly. Fast rats in all treatment groups showed very progressive and large increases in their AD durations compared with Slow rats that exhibited relatively short, stable discharges over that period. Interestingly, previously guanfacine-treated Fast rats showed the greatest AD growth during MS treatment, and were the fastest kindlers, requiring the fewest stimulations to develop their first stage-5 seizure after the MS treatment. It was also found that both idazoxan and guanfacine administered during the MS treatment slightly facilitated the daily distributed kindling rates of Slow rats compared with saline controls, but perhaps for different reasons.

The correlative data indicate that rats with the greatest number of long ADs during the MS treatment subsequently kindled the fastest (i.e., experienced the most positive transfer between the two protocols). It appeared, therefore, that if one can create long ADs during the MS treatment, independent of other considerations, the long ADs will recruit broadly throughout the network and enduringly alter synaptic efficacy. The network modifications affected by those long discharges, when accessed subsequently with daily kindling, result in rapid, spatially broad recruitment and faster generalization. This outcome leads quickly to reliable stage-5 generalized seizure expression. Short AD durations during MS treatment did not seem to affect such changes. Perhaps this effect of prolonged AD helps to explain why the long seizures of limbic status epilepticus so readily and reliably promote spontaneous generalized convulsive behavior.

Once an outbred rat is fully kindled, NE manipulations historically have had little effect on the kindled state. Thus, it was surprising that idazoxan resulted in an increase in the duration of motor convulsions of fully

kindled Slow rats, but was without a significant concomitant increase in their associated AD durations. This result suggested that the α2 noradrenergic receptors in the recruited motor network were still capable of modulating seizure expression after kindling (i.e., could be rendered less effective by the antagonist). Noteworthy, idazoxan did not affect the seizure profile of kindled Fast rats, a result that was consistent with earlier lack of response to idazoxan before kindling during the MS treatment. However, guanfacine, which failed to significantly affect the naïve Slow rats during MS treatment, now increased the duration of their kindled amygdala AD without influencing the concomitant convulsion. It is not obvious how to explain this result, unless amygdala kindling in the Slow rats altered the expression of α2 receptors or their subtypes in that local area, where they interface with other transmitter systems (e.g., GABA) to altered GABA release. Guanfacine, which affected the expression of convulsive seizures in Fast rats during the MS treatment, was without effect once Fast rats were kindled. Thus, the seizure behavior of Fast rats after amygdala kindling is consistent with the notion that kindling makes the rat insensitive to NE treatments, similar to outbred rats. This outcome did not occur in the Slow rats that maintained sensitivity (albeit slightly altered) to α2 manipulations after amygdala kindling.

Clearly, the expression of α2 NE receptors in the Fast and Slow rats, and their alteration with MS treatment and subsequent distributed kindling, needs to be explored in detail to more fully explain the unique patterns of results described here. The binding studies have indicated that Fast and Slow rats have similar distributions and labeling for the NE α2A receptor. Thus, activation of the α2A receptor might not be critical in differentiating the Fast versus Slow seizure phenotypes. Certainly, the α2 C receptor mRNA expression is quite different between the two strains. In general, Fast rats had significantly lower levels of α2 C expression than Slow rats in several limbic and motor structures. How NE affects that receptor subtype, and its consequences for excitability of the network, is unknown but should provide a fertile area for future research. Most importantly in that context, the two different strains should be viewed as modeling two different 'people' who have been exposed to an event (e.g., an injury) that could lead to epileptogenesis. This prelude begs the research question concerning differential treatment of those 'individuals,' involving the NE systems and its receptor subtypes, which might be appropriate for those two different natural predilections for development of the disorder. Certainly, manipulations that appear to be good for one strain are not necessarily good for the other. Therapeutically, such information should be quite valuable.

See also: **Models**: Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; Role of the Noradrenergic System in Seizure Regulation.

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Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling

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Introduction

Epilepsy is a multifactorial disease with debilitating effects. Its quintessence, the synchronization of neuronal networks, is generated at least in part through the inappropriate release of, or response to, neurotransmitters (NT). This article focuses on the molecules that control presynaptic NT release. We seek to understand the molecular mechanisms accounting for the heightened release of NT seen in epilepsy. Identification of the pathways and associated regulatory proteins involved in this process may illuminate potentially useful therapeutic targets to control NT release, and perhaps to mitigate or even abort seizure activity. Given the advances in genetic methods and small molecule inhibitor design, a mechanistic understanding of NT release is required to focus the search on new classes of anticonvulsant or antiepileptogenic agents.

Background

Kindling and the Dysregulation of NT Release

Kindling is a useful animal model of epilepsy and epileptogenesis. It is a progressive process, in response to repeated subconvulsive electrical or chemical stimulation, yielding a permanent intensification of epileptiform afterdischarges that culminates in generalized seizures. Many kindling studies have focused on the deregulation of NT release, particularly glutamate, as a mechanism of epileptogenesis. Several studies have used microdialysis techniques to show that increased glutamate release precedes seizures. We have shown that entorhinal kindling induces a permanent increase in K^+-evoked, Ca^{2+}-dependent release of glutamate from the hippocampus. Similar results are seen in hippocampal slices after Schaffer collateral-commissural kindling, in hippocampal and amygdalar slices of amygdalar-kindled rats, in cerebral cortical synaptosomes, and in hippocampus after septal kindling. Increased excitatory NT release has been measured in several different systems, and although the underlying molecular mechanisms accounting for it are still undefined, it is clear that increased release is a chronic change that once established is permanent in kindled animals.

Certainly, epileptic convulsions can result from defective release of inhibitory NTs; however, it is not clear whether long-term changes of GABA release play a role in epileptogenesis. Conversely, GABA systems themselves can be modified by seizure activity; these changes may provide compensatory augmentation or even an epilepsy-enhancing attenuation (e.g., with loss of inhibitory neurons in association with mesial temporal sclerosis). In our studies, there was no increase of K^+-evoked Ca^{2+}-dependent GABA release in hippocampus following entorhinal kindling; similar findings have been reported in amygdala following amygdala kindling. In contrast, others have shown increased K^+-evoked GABA release in hippocampal slices of hippocampal-kindled animals. Furthermore, there are microdialysis studies of human epilepsy patients showing increased GABA release with stimulation. These observations suggest that during epileptogenesis, alterations in GABA release are likely compensatory mechanisms; however, they could reflect molecular changes in the secretory machinery that would be subject to targeted modification.

Basic Machinery of NT Release

Neurotransmitters are stored in synaptic vesicles (SV) and their extracellular release is facilitated by the fusion of the SV with the presynaptic plasma membrane. SVs are cycled in the presynaptic area, where they are either held in a reserve position, poised for release (ready release pool), or recycling from the plasma membrane following fusion. The ready release pool is thought to represent a stage in which the final step of membrane fusion is fully engaged but not consummated. In fact, the two membranes (synaptic vesicle and active zone membranes) are thought to exist in a hemi-fused state in which the cytoplasmic faces of the two bilayers have already mixed. Many of the proteins, now known to affect NT release, appear to affect the formation or stability of the ready release pool.

Membrane fusion is mediated by the soluble *N*-ethylmaleimide sensitive factor (NSF) attachment protein (SNAP) receptors or SNAREs. These integral membrane proteins are the minimal elements required for membrane fusion. In neurons, there are two classes of SNAREs that can be distinguished based on their localization. The t-SNAREs, or target membrane SNAREs, are found in the plasma membrane and fall into two

categories, syntaxins and SNAP-25-like. The vesicle SNAREs, (v-SNAREs, also known as vesicle associated membrane proteins (VAMPs)) are localized to the SV membranes. Each SNARE contains at least one SNARE motif (SNAP-25 has two), which contains heptad repeats in which the A and D position are hydrophobic amino acids. Repeats from the three SNAREs form a parallel, coiled-coil that buries these hydrophobic residues. Such a complex is known as the 7S SNARE complex due to its hydrodynamic properties in centrifugation experiments. This structure is quite stable, requiring $>75\,^{\circ}C$ to denature (this property is exploited in our assays for SNARE complex formation). Such stability is thought to contribute thermodynamically to the membrane fusion process when the SNARE complex is in a trans-membrane configuration. Disassembly of the postfusion, *cis*-SNARE complex requires the action of the ATP-dependent chaperone, NSF, and is essential for further NT release.

Regulation of SNARE Interactions

The trans-membrane SNARE complex at the apposition of the synaptic vesicle and active zone membranes is the basic element required for membrane fusion. The stability of the SNARE complex, a potential source of energy for membrane fusion, poses mechanistic problems both for SNARE complex disassembly (which is required for recycling SNAREs) and for complex assembly (which is required for promoting fusion events). As alluded to above, the ATPase/chaperone NSF and its adaptor protein, α-SNAP, are required for disassembly and the recycling of the spent SNARE complexes in the *cis* configuration. NSF participation in this disassembly appears to be essential, because loss-of-function mutants of NSF cause an activation-dependent loss of NT release in *Drosophila*. Several reports suggest that NSF activity may be regulated via posttranslational modifications such as nitrosylation, serine/threonine, or tyrosine phosphorylation. Other studies have suggested that NSF RNA and protein levels are affected during epileptogenesis. At this time, it is unclear if/how control of NSF activity affects epilepsy.

Assembly of the *trans*-SNARE complex represents a key step through which NT release can be regulated. The function of most SNARE regulators is to bind the individual SNARE proteins, thereby controlling their access to each other. Thus, the SNARE regulators act as chaperones and/or matchmakers, affecting where and when SNAREs can bind to facilitate membrane fusion. From the analysis of the known effectors, controlling syntaxins appears to be a pivotal step for controlling membrane fusion – as suggested by the various conformational changes in syntaxin that occur as it becomes competent to interact with the other SNAREs. In its 'closed' conformation, the SNARE motif in syntaxin 1 is thought to be masked by the folding back and binding of the N-terminal H_{abc} domain. In syntaxin's 'open' conformation, its SNARE motif is exposed and thus available to interaction with the SNARE motifs of SNAP-25 and VAMP-2 to form the SNARE complex for membrane fusion.

Munc18

One of the first identified syntaxin regulators was the nSec1/Munc18 family. Munc18s have high affinity for syntaxins and were first purified by affinity chromatography on immobilized GST-syntaxin. Three Munc18 isoforms are present in mammalian tissues – a, b, and c – and each shows specificity in its syntaxin binding. In neurons, the most relevant isoform is Munc18a which interacts with syntaxin 1. The role of this Munc18 family in regulating syntaxin is confusing because of the varied effects of overexpression and deletion. Overexpression studies and in vitro binding experiments suggest that Munc18s are negative regulators, affecting syntaxin binding to other SNAREs. However, this view is inconsistent with the phenotypes of Munc18a$^{-/-}$ mice, or conditional mutants in yeast (Sec1p,) and *Caenorhabditis elegans* (unc18p), where Munc18s are required for secretion. One explanation for these latter results is that Munc18s are regulated chaperones required to maintain syntaxins in an 'activated' conformation at a specific site. Thus, overexpression may be inhibitory, because more Munc18 disrupts the dynamic balance between syntaxin binding and release, which is required for exocytosis. What is clear from all the data is that Munc18 must 'pass' syntaxins to their cognate SNARE partners to propagate membrane fusion. While the terms 'release or pass' are used, it may be more a conformational change in the interaction between Munc18 and syntaxin that denotes Munc18 function.

As Munc18s have multiple kinase recognition sites, phosphorylation could trigger syntaxin 'release.' This interpretation is consistent with in vivo studies showing that phosphorylation of Munc18a (at Ser313) decreases its affinity for syntaxin 1, but does not completely inhibit binding. Based on these observations, Munc18 phosphorylation could be a priming step that promotes 'release' of syntaxins. Such a hypothesis is supported by the recent findings which suggest that PKC phosphorylation of Munc18a is critical for vesicle priming at a step upstream of Munc13–1.

Munc13

The Munc13 family has at least four isoforms (1–4). Munc13 proteins show a bipartite structure, with a diverse N-terminal region and a more conserved C-terminal region. The C-terminus contains two C2 domains and two conserved Munc13 Homology Domains (MHD). The diverse N-terminus can contain a third C2 domain, one C1 domain and a calmodulin binding site. The C2

domains are homologous to the calcium-dependent phospholipid binding domains of PKC. The C1 domain is significant because it is homologous to the diacylglycerol (DAG) binding domain of PKC. While the N-terminus contains domains that might be responsive to signals, the C-terminal region contains some of the key interaction domains. The MHD contains a DOC2 interaction domain (DID) that is important for binding DOC2. Overexpression of this domain inhibits secretion from PC12 cells. This same region is important for binding to syntaxins, specifically, the N-terminal helices of syntaxin (H_{abc}) that do not form the coiled-coil structure of the SNARE complex.

Munc13–1 is required for maintenance of the ready release pool of synaptic vesicles in neurons. It acts as a priming factor accelerating the transfer of docked, unprimed vesicles to the ready releasable pool, and therefore increasing their fusion competence. Calmodulin (CaM) binding to Munc13–1 is an important component of this priming, and loss of CaM binding slows the transfer rate. The C1 domain is also critical. Knock-in mice expressing a mutant of Munc13–1 (H567K, which fails to bind DAG or phorbol esters) had a lethal phenotype similar to the Munc13–1 knockouts. Explanted neurons from these animals failed to respond to phorbol esters, suggesting that Munc13–1 is, along with PKC, a major site of phorbol esters' enhancing effects on neurotransmission.

It should be pointed out that DOC2s and syntaxins share a similar binding site on Munc13, and Munc13–1 and Munc18a share a common binding site on syntaxin 1. The overlapping binding sites are of interest, as recent reports suggest dynamic in vivo interactions between syntaxins, Munc13s, and Munc18s. Overexpression of Munc13–1 suppressed the inhibitory effect of Munc18a expression on syntaxin activity; this action also led to an increased association of SNAP-25 with the plasma membrane, consistent with an increase in SNARE complex formation. Expression of an 'open' conformation mutant of syntaxin 1 (that does not bind Munc18) suppressed the deletion of Munc13 in *C. elegans*. These observations show that these proteins – syntaxin, Munc13, and Munc18 (and perhaps DOC2) – are involved in a pathway of protein–protein interactions which could promote SNARE pairing and perhaps membrane fusion.

Tomosyn

Tomosyn is a 130-kDa protein originally purified from brain. Structurally, tomosyn contains 14 WD40 repeats arranged in two 7-bladed propeller domains and a C-terminal domain that mimics the SNARE motif of v-SNAREs. This motif allows tomosyn to interact with t-SNARE heterodimers to form a heterotrimer very similar to the SNARE complex. The complex can be disassembled by SNAP and NSF, suggesting another mechanism by which NSF could control secretion. Functionally, tomosyn appears to affect vesicle priming prior to membrane fusion; however, the precise mechanism is confusing. As with Munc18t's, tomosyn is needed for NT release in neurons and secretion in yeast, but overexpression inhibits release – suggesting that tomosyn and Munc13 play antagonistic roles, the former exerting negative and the latter positive effects on vesicle priming. In a model proposed by McEwen et al., tomosyn is a place-holder for the v-SNARE and is displaced by activated Munc13. Thus, NT release is controlled by balancing tomosyn binding of the t-SNARE heterodimer, and Munc13 activation of the SNAREs for membrane fusion.

SV2

Although the specific molecular role(s) of the SV2s (A, B, or C) are debated, they appear to be a key control element for neurotransmitter and neuroendocrine release. In hippocampal slices, action potential-dependent release of GABA (operationally defining the 'ready release' pool of vesicles) is reduced in $SV2A^{-/-}$ mice. In these knockouts, the frequency and amplitude of spontaneous inhibitory postsynaptic currents (IPSC) is decreased, whereas miniature IPSCs measured in the presence of tetrodotoxin are no different from wild type mice. This observation suggests a role for SV2A in controlling assembly of SNARE complexes, but not in membrane fusion. Consistent with this view, analyses of brain tissue from $SV2A^{-/-}$ animals show a 50% decrease in SNARE complexes. These data suggest that SV2A is important for maintaining SNARE complexes but is perhaps not essential for complex formation.

Lynch *et al.* demonstrated that the antiepileptic drug, levetiracetam (LEV) binds stereospecifically, saturably, and reversibly to SV2A. These data suggest that LEV's anti-epileptic effects may be through the modulation of SV2A. LEV does not acutely affect the electrophysiology of normal tissue, and recent data show that prolonged treatment with LEV is required before presynaptic neurotransmission defects can be observed. These results suggest that LEV may affect some aspect of SV2 synthesis or transit to the synapse, but not its acute function. Further analysis will be needed to address this point.

Methods

Kindling

Kindling can be induced by electrical or chemical stimulation of many brain sites, especially in limbic areas (e.g., amygdala, hippocampus, and entorhinal cortex). The development of limbic kindling is characterized by electrographic discharges and behavioral seizure stages 1–5. An animal is considered fully kindled once enhanced sensitivity to an initial subthreshold stimulus has developed, as evidenced by a full (Racine) stage 5 seizure

(rearing and falling). Kindling is a particularly valuable technique to study chronic epilepsy because the site of stimulation is known, and no other disturbances are introduced (in contrast to status epilepticus models which are associated with widespread unilateral or bilateral limbic damage caused by cell death). Analysis of the electrophysiology of kindling, including observations that continued stimulation (i.e., the over-kindled state) leads to the occurrence of spontaneous seizures, suggests that kindling represents a permanent alteration which parallels changes seen in some human epilepsies.

Quantitative Western Blotting

The ability to better quantify proteins by western blotting has come with the advent of new detection systems, specifically those based on fluorescence imaging. Such readouts offer broader linear ranges and the ability to probe multiple proteins on the same membrane. One challenge in such analyses was to overcome the potential for animal-to-animal variation. We have solved this problem by using ratio measurements, comparing ipsilateral and contralateral hemispheres; in this way, each animal serves as its own control. We have validated this methods by showing that in naïve and surgical-control animals, the ipsilateral/contralateral ratio of SNARE complexes is consistently close to unity. Deviation from unity consistently appears as an increase in ipsilateral complexes in kindled animals. As would be expected, it does not occur with electroshock seizures; we have not measured this ratio in any other models of epileptogenesis.

Important Results to Date

Asymmetric Accumulation of SNARE Complexes

We have examined the state of the SNARE proteins and several of the SNARE regulators in hippocampi from kindled animals. Initial studies showed an asymmetric accumulation of SNARE complexes in ipsilateral hippocampal synaptosomes prepared from animals kindled by entorhinal stimulation. Control experiments suggest that this ispsilateral buildup of SNARE complexes is a marker of some basic biochemical change occurring during kindling and is indicative of epileptogenesis (see Figure 1(a) and below). Asymmetric accumulation cannot be induced by electroconvulsive shock nor is it seen in synaptosomes prepared from tissues outside of the limbic system (e.g., cerebellum or occipital cortex). This asymmetry can be induced by stimulating at several different sites, including entorhinal cortex, septal region, and amygdala; it develops in the ipsilateral hippocampus regardless of whether the right or left hemisphere is stimulated. As with kindling, asymmetric SNARE complex accumulation requires time to evolve and only occurs with stimulation within a frequency range that promotes kindling. The accumulation occurs gradually during kindling, mirroring the progression of Racine behavioral stages (Figure 1(a)), but it is persistent and can still be detected up to one year after the animal has attained a minimally kindled state (defined as two successive stage 5 seizures). It appears that a certain minimal level of kindling induction is necessary to induce permanence of the SNARE complexes asymmetry. In animals advancing up to, but not beyond Stage 3, the asymmetry begins to develop; if they receive no further stimuli, by

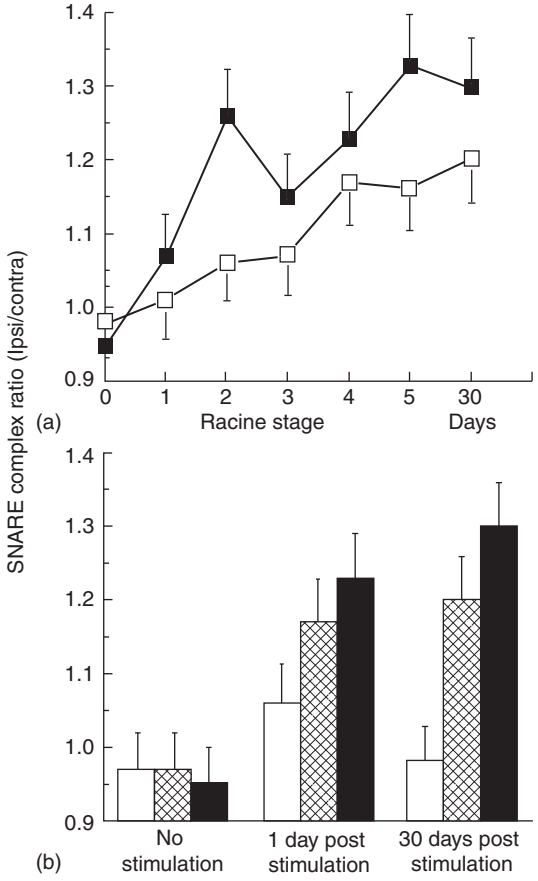


Figure 1 Asymmetric SNARE complex accumulation correlates with kindling stage. (a) Animals were electrically kindled by amygdalar (open squares) or entorhinal cortical (closed squares) stimulation until they exhibited behavior consistent with the indicated Racine stages. The next day, hippocampi were harvested from each animal and the ipsilateral/contralateral ratios of SNARE complexes were measured. One set of animals was fully kindled and hippocampi were harvested after 1 month (30 days). (b) Four cohorts of animals were held as naïve (no stimulation), kindled to stage 2 by amygdalar (open bars), or kindled to stage 4 by either amygdalar (hatched bars) or entorhinal cortical (solid bars) stimulation. Hippocampi were harvested from the animals either 1 day post stimulation or 1 month post stimulation and the ipsilateral/contralateral ratios of SNARE complexes were measured.

one month after the last stimulation hippocampal SNARE complexes symmetry is reestablished (**Figure 1(b)**).

Once attained, the asymmetric accumulation of SNARE complexes is the most stable biochemical marker of kindling-induced epileptogenesis that we have been able to find in the literature. It is not clear whether *trans*- or *cis*-SNARE complexes accumulate. If they are *trans*-SNARE complexes, it represents an increase in engagement of the membrane fusion machinery and our observations might be consistent with a focal enhancement of NT release. Accumulation of *cis*-SNARE complexes, in contrast,might signal defective recycling machinery – although this interpretation would not be entirely consistent with the observed increases in NT release, particularly glutamate, measured in hippocampi from patients undergoing epilepsy surgery and in several seizure models (including kindling).

At this stage of our investigations, it is also unclear whether the accumulation of SNARE complexes is associated with the cause of the kindled state or represents a *sequella* of the process. The control experiments described above demonstrate a tight correlation between kindling and asymmetric complex accumulation, but as discussed below, at least one anti-epilpetic drug can separate accumulation from kindling behavior. Further analysis of this association, for example, with transgenic mice expressing reduced SNARE levels or defective SNARE regulation, may prove to be a useful strategy to lower the availability of SNAREs and thus decrease the formation of SNARE complexes. Determining whether these conditions affect kindling progression might at least address whether complex accumulation is causative, responsive, or simply correlative. Either of the first two situations would indicate a locus for modification with agents useful to arrest epileptogenesis or quell epilepsy.

Effects on Other Elements of the Neuronal Secretory Machinery

Examination of several known elements of the neurosecretory machinery (α-SNAP, Munc18a, Munc13–1, complexin 1 and 2, and synaptotagmin 1) has generally shown that their protein levels do not undergo significant alteration during kindling epileptogenesis and upon attaining a fully kindled state. Three apparent exceptions are NSF, SV2, and tomosyn. NSF levels decrease with amygdala and septal kindling; the levels also appear to decline in entorhinally kindled animals (although that decrease was demonstrated only as a trend). A drop in NSF mRNA has also been reported in kainic acid-induced epilepsy. In our laboratory, the decrease of hippocampal NSF protein appears permanent and correlates with the altered asymmetry of SNARE complexes measured 1 year after amygdala kindling. Decreases in NSF could account for the accumulation of SNARE complexes, especially if such complexes are *cis* and thus represent postfusion events. In contrast to NSF, tomosyn and SV2 levels increase during amygdala kindling. In our studies, tomosyn increased early in the kindling process and then drifted back to normal levels as kindling progressed. The significance of this effect is not yet clear, although one could speculate that tomosyn might compete with v-SNAREs for t-SNARE binding; an increase in tomosyn could then lessen SNARE complexes assembly. SV2 levels began to rise early in the kindling process and subsequently remained elevated at 1 month following completion of kindling and cessation of further stimulation. The molecular ramifications of these increases are less clear than the changes for NSF, but given the role that these two proteins play in affecting secretion efficacy, one may propose that increased SV2 and tomosyn could affect either the formation of *trans*- or *cis*-SNARE complexes, generating several potentially testable scenarios. For example, if the kindling-induced increase of tomosyn is affecting formation of *trans* complexes at predominantly excitatory synapses in hippocampus, then the rise may represent a transient and unsuccessful compensatory response.

Effects of Levetiracetam

Levetiracetam (LEV) is an antiepileptic drug that specifically binds to SV2 (discussed above). Based on those data, it has been assumed that LEV's action is dependent on that interaction, although this assumption has not been definitively proved. Treatment of rats with LEV delays the progression of amygdala kindling, but does not prevent kindling if stimulations are continued. In our studies, LEV treatment dampens the asymmetric accumulation of SNARE complexes, even in animals taken to a fully kindled state. Thus, LEV treatment appears to separate the association of asymmetric SNARE complex accumulation and kindling progression. However, this effect is perhaps analogous to the results presented by Constantin *et al.*, who showed that intrahippocampal injection of botulinum toxin E (known to cleave the SNAP-25 t-SNARE) caused a delay in the development of hippocampal kindling. Such treatments would be expected to lower SNARE complex levels (not examined by Constatin *et al.*) as observed in LEV treated rats. However, both LEV and toxin treated animals did reach a fully kindled state with repeated stimulations.

LEV treatment also dampened the kindling-induced increase in SV2. Because the $SV2A^{-/-}$ mice show a decrease in global SNARE complex levels, perhaps LEV's effects are due to some alteration in SV2A function which directly affects complexes during kindling. Such an effect would only be relevant during epileptogenesis, as LEV appears to affect neither the normal function of neurons nor their SNARE complex levels.

Future Goals

At least three issues will require resolution before these data can be forged into a viable therapeutic approach to mollify epilepsy and to potentially protect against epileptogenesis. First, it will be necessary to determine in which cell types what aspects of the neurotransmitter release machinery become dysfunctional during epileptogenesis and persist with the epileptic state. Is the SNARE complex asymmetry distributed throughout the hippocampal formation, or is it confined to dentate gyrus, to a subdivision of the hippocampus proper (e.g., CA3), or is it seen in other structures? Is it confined to a certain cell type, specifically excitatory dentate granule or hippocampal pyramidal neurons, or inhibitory interneurons? If, for example, the SNARE complex accumulation localizes to glutamatergic neurons, then (based on the extensive literature of enhanced hippocampal glutamate release with kindling) one would propose testable mechanisms associated with accumulation of *trans* complexes. Do the regional and cellular localizations of altered NSF, SV2, and tomosyn comap with each other or with the SNARE complexes asymmetry? If they do not comap, are their changes consistent with a unified hypothesis consistent with epileptogenesis – that is, a synchronized hyperexcitable state?

Second, what molecular processes are functioning outside the bounds required for maintaining normal neuronal function? Certainly, resolution of the first issue will help in addressing this issue about mechanisms, by allowing more specific targeting. Do our observations reflect a pre or posttranslational process? One approach our laboratory has used to answer this question utilizes the hippocampal 'zipper' slice. This method allows for electrophysiologic characterization of individual excitatory neurons followed by single cell m-RNA RT-PCR analysis. Such a study will not only help determine if changes are occurring at the genetic level, but also whether such potential changes in NSF, SV2, and tomosyn are confined to a cell type or are dispersed (either regionally or by neuronal type). An additional approach to mechanism is the use of mutants that over- or underexpress various elements of the neurosecretory machinery. Our laboratory is currently evaluating amygdala-kindled viable heterozygotes that show a significant reduction in the levels of one or another of several proteins including synaptobrevin/VAMP-2, SNAP-25, and synaptotagmin I. Finally, our observation of altered protein levels may reflect our choice of antibody and whether posttranslational modification has merely altered detection ability. If there appears to be no evidence for pretranslational alterations, then further use of more refined, state-specific monoclonal antibodies may be used to explore this question. Again, determination of regional localization and cell type will make this quest more manageable.

The third issue for devising therapies directed toward the NT secretory machinery is the need to craft small molecule inhibitors or effectors which can affect the process. In this way, levetiracetam proves to be an interesting example because it has no adverse effect on normal neurons but dampens the process of epileptogenesis. Other newer anticonvulsants such as topirimate that have shown antiepileptogenicity in the kindling model are also potential probes. Methods for developing and using such probes, both to better understand basic mechanisms and for use as potential therapeutic agents, are discussed in more detail in another article of this Encyclopedia.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy; **GABA**: Pathophysiology of Status Epilepticus; **Models**: Characteristics and Mechanisms of Kindling; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling.

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Role of Noradrenergic System in Seizure Regulation

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Introduction

There is a vast literature, extending over five decades, supporting an anticonvulsant role for norepinephrine (NE) in essentially all animal models of epilepsy. Despite the preponderance of evidence regarding a powerful NE anticonvulsant effect, there has been little effort to augment noradrenergic signaling as antiepileptic or seizure suppressing therapy. The reluctance to pursue adrenergic agents in this context is based partly on the perception (mistaken) that antidepressants that increase NE extracellular concentration have proconvulsant effects. In addition, the experimental data have been quite complex, with seemingly contradictory reports about pro- and anticonvulsant effects of various noradrenergic agents. Clarification of the specific receptor subtypes mediating antiepileptic (and potentially, antiepileptogenic) effects, and their localizations in different brain regions, would help to guide potential NE-based therapeutic strategies.

Our laboratories have a long standing goal to gain more complete understanding of the processes whereby central noradrenergic and serotonergic systems regulate seizure predisposition, including seizure susceptibility and severity. To achieve this objective, we have carried out numerous studies in rodent models of epilepsy. Most of these studies have been carried out using the Genetically Epilepsy-Prone Rat (GEPR), a unique seizure-prone rat that is innately deficient in CNS norepinephrine (NE) and serotonin (5-HT). *Why focus on these slower acting modulatory neurotransmitters* that signal through G-protein-coupled receptors (GCPRs) when we know that the faster acting amino acid neurotransmitters (GABA, glutamate) have profound regulatory effects on seizures (to the extent that they can start or stop a seizure even in a normal brain)? The answer to this question lies in the fact that the consequences of manipulating the synaptic effects of GABA or glutamate can be so dramatic, in terms of altering behavior, that the side effects/toxicity of drugs that alter these chemicals have – in some cases – turned out to be unacceptable. Nevertheless, we do have antiepileptic drugs on the market that enhance the inhibitory action of GABA (reducing the influences of glutamate has been more difficult to achieve without unacceptable side effects).

The monoamine neurotransmitters, however, have been shown to exert anticonvulsant effects in almost every animal model of seizures so far examined. Moreover, there is some evidence that NE may be more than seizure suppressant; it may also be antiepileptogenic. Despite a vast literature supporting NE's suppressive effect on convulsive seizures in many different models, there are many unanswered questions. For example, if the noradrenergic system in the brain is so effective at suppressing seizure activity, why do we not have more treatments of epilepsy that act primarily by augmenting this system? What is the cellular/molecular mechanism responsible for the seizure suppressant effects of NE? Does the pathophysiogical basis of some forms of epilepsy involve dysregulation of the noradrenergic system? While we cannot provide answers to all of these questions, we hope to provide the reader with some insight into what is known about them, and suggest some ways in which future research may reveal more answers.

Background and Recent Results

In this section, we examine the evidence for an anticonvulsant role of NE in experimental models of epilepsy where the seizures are restricted largely to a particular neuronal network. We have previously shown that convulsive behavior in rodents can be described as being driven by either the *forebrain circuitry* (limbic/cortical) or by a *brainstem seizure network*. The seizure behavior in a rodent experiencing a forebrain seizure is distinct from that of a rodent experiencing a brainstem driven seizure. The former is typically characterized and quantified by a scoring system described by Racine, while the latter is characterized and quantified by the scoring system described by Jobe and coworkers. While the behavioral components of forebrain and brainstem seizures have been best characterized in rodents, they can be seen in essentially all species, including humans. As we have indicated earlier, the noradrenergic system is a slower signaling system than the amino acids systems, but it has a widespread influence on brain function through two major projections that have been well mapped in brain: (1) the locus ceruleus (LC) system and (2) the lateral tegmental system. Both systems provide innervation to limbic forebrain as well as the brainstem, and the cell bodies of both systems are localized to the brainstem reticular formation. While both of these neuronal systems

seem to have effects on all types of seizures, the noradrenergic neurons of the LC have been more widely studied.

Forebrain Seizures

Seizures driven by forebrain circuitry that involves a limbic network serve as models of temporal lobe epilepsy in humans. Since rodents have been more extensively studied than any other species experimentally, we focus our remarks on studies involving rats or mice. Limbic structures such as the amygdala, hippocampus, and piriform cortex have a rich noradrenergic innervation.

Effect of a Noradrenergic Deficit

A reduction in the synaptic concentration of NE by lesions or pharmacological depletion has consistently resulted in a reduction in threshold for limbic seizures (e.g., decreased minimal electroshock threshold using corneal electrodes), or an increase in seizure severity (e.g., in response to low dose pentylenetetrazol (PTZ), pilocarpine, kainate). Moreover, lesions of ascending noradrenergic pathways with 6-hydroxydopamine (6-OHDA) facilitate the rate of electrically induced amygdala-kindling. Infusions of GABA antagonists or glutamate agonists into the deep prepiriform cortex ('area tempestas') of rats evoke limbic motor seizures lasting several minutes; the depletion of NE with DSP-4 (which damages LC neurons) converts these relatively short lived limbic seizures into status epilepticus.

Genetic studies in mice with mutations or deletions of key NE synthetic enzymes have also been shown to increase the susceptibility of such animals to PTZ or flurothyl-induced seizures. And genetic strains of rats deficient in brain NE, such as the GEPR, have a reduced threshold for minimal electroshock, chemically induced limbic motor seizures (e.g., flurothyl), and electrical kindling of amygdala or angular bundle. We discuss the GEPR in more detail in the following sections, in connection with the role of NE in brainstem seizures (see the later sections); it is worth noting, however, that it represents a unique model with enhanced predisposition for both forebrain and brainstem seizures. Our studies show that seizure predisposition in both forebrain and brainstem seizure networks is, in part, determined by the noradrenergic deficiency.

Effect of Noradrenergic Augmentation

The observations of the proconvulsant properties of deficiencies in noradrenergic transmission described earlier are reinforced by studies demonstrating anticonvulsant effects following treatments that increase signaling through noradrenergic receptors. For example, electrical stimulation of the LC retards amygdala kindling and reduces the severity of pentylenetetrazol (PTZ)-induced seizures, as well as seizures induced by injection of penicillin into the hippocampus. Grafting of fetal LC cells into the hippocampus of rats that have a reduced seizure threshold, following sustained damage to the LC, helps restore the seizure threshold to normal. Pharmacological treatments that elevate the synaptic concentration of NE or activate adrenoceptors produce anticonvulsant effects (see the later sections). A genetic mutation designed to increase the synaptic concentration of NE in mice, such as that seen in norepinephrine-transporter (NET) knock-out mice (NET-KO), has also been shown to provide protection against cocaine-, kainate-, and PTZ-induced seizures.

Interestingly, unconventional treatments known to suppress seizures in human patients and to protect various animal models from epileptic stimuli have recently been shown to require NE for their anticonvulsant effect. For example, we have found that vagus nerve stimulation fails to protect rats against PTZ and maximal electroshock seizures following lesions of the LC. Others have found that the ketogenic diet is no longer effective against flurothyl-induced seizures in mice that are lacking NE due to a genetic deletion of the dopamine-β-hydroxylase gene.

Receptor Subtypes Involved

Interpretation of the studies that have examined the role of various subtypes of adrenoceptors in the seizure suppressing effects of NE is not always easy. Variations in receptor subtype, distribution, and function – and the lack of complete specificity of the agonists and antagonists – make it difficult to interpret a literature that often has seemingly conflicting findings. Most of the conflict appears to arise from the lack of pharmacological specificity and the presence of autoreceptors on noradrenergic neurons. The most consistent finding regarding forebrain seizures is that the antiepileptic effects of NE on limbic seizures appear to be mediated through alpha-2 receptors. Certainly, the *development* of seizures kindled from the amygdala are inhibited by alpha-2 agonists and facilitated by alpha-2 antagonists. Further support for an anticonvulsant effect mediated by the alpha-2 receptor was obtained by making a point mutation in the alpha-2A receptor; such mice develop amygdala kindled seizures much faster than wildtype mice.

While both pro- and anticonvulsant effects of clonidine have been reported using chemoconvulsant-induced seizures in rodents, some of the conflicting findings appear to arise from the fact that alpha-2A receptors are found presynaptically (autoreceptors) and postsynaptically. Studies using receptor knockout and synthetic enzyme knockout mice have suggested that the *presynaptic* alpha-2A receptor is responsible for the proconvulsant effects of clonidine, while the *postsynaptic* receptors are

responsible for the anticonvulsant effects. Although there is some pharmacological evidence in favor of an anticonvulsant role for alpha-1 and beta adrenoceptors, more work will be needed before definitive conclusions are drawn.

Human Studies

Most seizures in humans with epilepsy are of forebrain origin (e.g., temporal lobe epilepsy). Can we suppress such seizures by enhancing noradrenergic signaling? The answer appears to be yes, but some of the evidence is indirect and must be deduced from effects of anticonvulsants or antidepressants on NE signaling. One of the more direct pieces of evidence comes from a study showing a deficit in alpha-1 adrenoceptor density in the temporal lobe taken from patients who were undergoing a temporal lobectomy. This finding is consistent with a recent study showing that alpha-1 adrenoceptor antagonists have proconvulsant effects in patients with medial temporal lobe epilepsy. Some antiepileptic drugs, such as valproate, are known to increase the activity of tyrosine hydroxylase and increase extracellular levels of NE in animals. However, the extent to which the effects of valproate and carbamazepine on NE signaling contribute to their other substantial anticonvulsant effects (e.g., blocking sodium channels) is not known. Nevertheless, a substantial number of animal studies have demonstrated that drugs which enhance NE signaling augment the antiseizure effects of standard anticonvulsant drugs used clinically. Thus, it is likely that enhancing noradrenergic signaling in treatment-resistant patients receiving other antiepileptic agents will be beneficial. Indeed, current data support the hypothesis that this mechanism is the basis for the beneficial effects of vagus nerve stimulation in patients with intractable epilepsy.

Additional evidence comes from the literature on tricyclic and other antidepressants that elevate extracellular levels of NE. While the tricyclic antidepressants are proconvulsant at supratherapeutic doses, they are universally anticonvulsant in both forebrain and brainstem seizure models. Several studies in humans have shown that antidepressant doses of antidepressants suppress seizures in humans. Indeed, the proconvulsant effect of these drugs appears to occur only at supratherapeutic doses and involve biological systems other than the monoamines.

Brainstem Seizures

Brainstem seizures manifest as generalized tonic–clonic seizures (GTCS) in humans and other mammalian species. All elements of GTCS in humans with epilepsy – including initiation, continuation and termination – are closely approximated by GTCS in GEPR-10s (rat model) and in the epileptic *Papio papio* (baboon model). In the GEPR-10 model, the brainstem seizure is reflected in tonic hindlimb and forelimb extension, but running-bouncing clonus alone or in combination with fore- and hindlimb extension is reflected in less severe brainstem seizures observed in GEPR-3s or GEPR-9s (see the later sections) as well as in other rodent brainstem seizure models.

Effect of Noradrenergic Deficits

Treatments that interfere with NE signaling clearly facilitate brainstem seizures. Indeed, loss of NE can convert forebrain seizures into brainstem seizures. The best animal models for studying brainstem seizures are the maximal electroshock seizure evoked by ear-clip electrodes and sound-induced seizures in genetically susceptible animals (e.g., in GEPRs). The GEPRs are innately deficient in NE and this deficiency has been shown to be a determinant of their seizure-prone state. Lesioning of the locus ceruleus with DSP4 clearly enhances the severity of maximal electroshock-induced seizures, as indicated by the increased extensor-to-flexor ratio. The loss of NE in GEPRs leads to an enhancement in seizure severity whether produced by reserpine, a nonselective drug, or by lesions that are more selective for the locus ceruleus (e.g., infusions of 6-OHDA or DSP-4). When exposed to a 100 db sound, the GEPR-3 displays a less severe form of brainstem seizure (i.e., no tonic extension), while the GEPR-9 displays a maximal tonic–clonic seizure. Impairment in NE signaling in the brain of a GEPR-3 can convert its seizure to that of a GEPR-9. Lesions of the LC also increased seizure severity in other strains of rat that are genetically susceptible to sound-induced brainstem seizures. A large number of pharmacological agents have been used (alone or in combination) to more specifically examine noradrenergic signaling in the GEPR; these studies have demonstrated that the loss of NE leads to a facilitation of the tonic (brainstem) seizure.

Effect of Noradrenergic Augmentation

Treatments that increase NE signaling have been shown to protect rats from the brainstem seizure phenotype. However, most pharmacological treatments designed to selectively increase NE neurotransmission (e.g., reuptake inhibitors of NE, amphetamine to increase release) are not entirely selective for NE, making conclusions more tentative. The administration of NE and several noradrenergic agonists directly into the brain of GEPRs has further demonstrated the seizure inhibitory role of NE. Studies designed to identify the noradrenergic terminal field(s) responsible for the anticonvulsant effects of NE on brainstem seizures in GEPR-3s showed that the critical regions are contained in the midbrain exclusive of the

inferior colliculus. Even stronger evidence for the seizure suppressing effects of NE on brainstem seizures was obtained by showing that desipramine (a selective NE uptake blocker) and yohimbine (an alpha-2 adrenergic antagonist) are anticonvulsant in GEPR-9s. Moreover, the time-course for their anticonvulsant effects was highly correlated with the increase in extracellular brain NE (measured by microdialysis) produced by these drugs. The depletion of norepinephrine stores negates the anticonvulsive effects of desipramine.

More recently, Jobe and colleagues have shown that microinfusion of selective NE reuptake blockers into the superior colliculus (a midbrain site) of GEPRs suppresses (does-dependently) brainstem-driven tonic–clonic seizures. Moreover, the alpha-1 agonists phenylephrine and methoxamine infused into the superior colliculus produced similar effects, and these effects were blocked by the alpha-1 antagonist prazosin. Such findings are in agreement with NE pathway lesion studies and support the concept that the superior colliculus is the midbrain site where NE exerts its seizure suppressing effect on tonic seizures. These studies also show that this effect is mediated, at least in part, by the alpha-1 adrenoceptor. The importance of the superior colliculus as the site of action of NE on brainstem seizures has also been reported by another group.

Receptor Subtypes Involved

Studies designed to identify which adrenergic receptor subtype is responsible for the brainstem seizure inhibitory effects of NE have suggested that more than one subtype is involved. Intracerebroventricular injections of alpha (phenylephrine), beta-1 (dobutamine), and beta-2 (terbutaline) agonists can suppress sound-induced seizures in GEPRs, while an alpha-2 agonist cannot. More recent studies in GEPR-9s show that the seizure suppressing effects of alpha-1 receptors is produced in the superior colliculus (as indicated earlier). Whether the other adrenoceptors produce their effects in this same brain region has not yet been examined.

Human Studies

Generalized tonic–clonic seizures (grand mal) are essentially brainstem-driven convulsive seizures in humans. Antidepressants that inhibit NE reuptake are anticonvulsant against tonic–clonic seizures in humans. This effect is expected, given the data showing that NE is so dramatically effective against tonic extension in both the maximal electroshock and in tonic–clonic seizures in GEPRs. Since many of these tonic–clonic seizures are secondarily generalized, it is expected that the effects of NE on forebrain seizures (e.g., temporal lobe seizures), as described earlier, would also suppress the transition from limbic/cortical to brainstem seizures and be beneficial in suppressing tonic–clonic seizures. In fact, there is evidence that monoamine enhancing antidepressants can suppress seizures in patients with secondarily generalized convulsive seizures.

Methods

Whole Animal Integrative Studies

We have employed an integrative system approach, which we feel is best suited for translational research. In our laboratory we use genetically epilepsy-prone rats (GEPRs) because they provide an ideal animal model for studying epilepsy and its comorbidities. Our rationale is based on multiple factors:

1. GEPRs have an innate predisposition for forebrain and brainstem seizures, and therefore are able to model the major convulsive epileptic disorders seen in human patients.
2. Details of brain function can be determined through the use of excellent stereotaxic atlases of the rat brain. When used with the GEPRs, these atlases provide opportunities for targeting discrete brain regions for functional and anatomical analyses.
3. The extensive and detailed neuroanatomy of the noradrenergic and serotonergic systems are well known in the rat brain and are readily adaptable to GEPR studies.
4. We have developed methods whereby the behavioral (convulsive) and electrographic characteristics of the seizures and comorbid disorders can be studied with the GEPR.
5. As a tool for the identification of antiepileptic drugs, GEPR paradigms clearly separated clinically useful antiepileptic drugs based on their effectiveness in absence versus GTCS indicating that it is a model with predictive value.
6. The GEPR model has proved useful in correcting longstanding mistaken conclusions regarding the convulsant liabilities of antidepressant drugs, drugs that are otherwise essential to the treatment of affective disorders and other psychiatric illnesses.

Localization of Noradrenergic Anticonvulsant Effect

We have used the systemic administration of DSP-4 to lesion selectively the locus ceruleus noradrenergic system; we have shown that activation of these neurons suppress both forebrain and brainstem seizures in Sprague–Dawley rats subjected to corneal electroshock (forebrain seizures) or maximal electroshock (ear-clip electrodes).

Discrete microinjections of the catecholamine neurotoxin 6-OHDA into specific ascending or descending noradrenergic pathways have been used to assess the influence of the forebrain, brainstem, cerebellum, and spinal cord norepinephrine on brainstem seizures in GEPR-3s. In a typical experiment, animals are anesthetized with chloral hydrate 400 mg kg^{-1}, placed in a stereotaxic apparatus; after exposing the skull and drilling a small hole, animals are infused with 4 μg per 2 μl of 6-OHDA. Two weeks later they are subjected to sound-induced seizures and their behavior is assessed for evidence of changes in severity. EEG electrodes can also be implanted, at the time neurotoxin lesions are made, for electrographic assessment of seizure severity. Similar studies, using the serotonin neurotoxin 5,7-dihydrotryptamine, have been performed by our laboratories to determine the site of action of 5-HT in GEPRs. The superior colliculus has also been identified as an important site for mediating the anticonvulsant effect of 5-HT on brainstem-driven (tonic–clonic) seizures.

Microinjection Studies

Stereotaxic implantation of guide cannulae (Plastics One Inc.), followed one week later by infusion of adrenergic drugs (agonist or antagonist), provide another approach to localize the site of anticonvulsant action of NE. In microinjection studies it is important to test nearby (1–2 mm away) drug infusions to determine that an effect is specific for a particular structure. Recent studies in our lab have revealed that infusion of NE into brainstem nucleus pontis oralis (RPO), a nucleus that is important in the brainstem seizure network, does reduce the duration of tonic hindlimb extension in the maximal electroshock test, but does not abolish this response. Microinjection of NE alone, as well as injections of alpha and beta agonists into this region of the brainstem reticular formation, failed to alter the tonic seizure in GEPRs. Such findings are consistent with the superior colliculus as a site of action of NE in brainstem seizures (see earlier). Infusions of NE or other adrenoceptor agonists selective for alpha or beta receptors into the inferior colliculus also failed to attenuate tonic seizures in GEPRs. Such findings are consistent also with the studies mentioned earlier showing that the superior colliculus is the site at which the noradrenergic system exerts seizure attenuating effects on brainstem seizures.

Microdialysis Studies

We have extensively employed microdialysis to measure extracellular levels of norepinephrine and serotonin in GEPRs receiving anticonvulsant drug treatment, and also as a way of administering drugs site specifically within the brain (e.g., to superior colliculus). Animals are implanted with guide cannulae stereotaxically, and allowed to recover. Prior to sample collection, the microdialysis probe is inserted into the guide and baseline levels of extracellular NE are measured for several hours. Animals are then subjected to drug treatment and seizure initiation by a 100 db sound stimulus.

Fos Immunohistochemistry

We have employed fos immunohistochemistry to map seizure circuitry in rats subjected to various types of seizures (both forebrain and brainstem). Rats were subjected to seizure testing and two hours later were perfused with PBS and paraformaldehyde, followed by sectioning of brain and exposure to antibodies directed at fos. Fos is a marker for increased neuronal activation, and cells that are activated by the seizure display enhanced fos staining. Of interest to the noradrenergic hypothesis of seizure suppression is that irrespective of the model examined (PTZ, electroshock, audiogenic) or the rat strain (Sprague–Dawley or GEPR), the locus ceruleus was activated in all seizure models. Other laboratories have also reported LC activation using fos following other types of seizures, including secondarily generalized limbic motor seizures initiated from the deep prepiriform cortex.

Vagus Nerve

Vagus nerve stimulation (VNS) has been used since the late 1990s to treat intractable seizures in patients with epilepsy. As indicated earlier, we have previously found that the anticonvulsant effects of VNS are essentially abolished following 6-OHDA lesions or lidocaine inactivation of the locus ceruleus. More recently, we have shown, using microdialysis, that VNS (0.5 and 1.0 mA) increases extracellular NE in the hippocampus, and that 1.0 mA stimulation increases NE in the cerebral cortex of awake freely moving rats. Two other laboratories have made similar findings. Thus, it seems possible that VNS provides a means of producing locus ceruleus activation without producing peripheral side effects seen with the numerous drugs that are available to enhance noradrenergic signaling. There is also electrophysiological evidence that VNS increases the firing rate of LC as well as raphe neurons, suggesting that the VNS increases the extracellular concentration of both NE and 5-HT. Such findings appear to explain the effectiveness of VNS in the treatment of intractable depression. There is now substantial evidence that depression and epilepsy are comorbid diseases, with perhaps a common pathophysiology (i.e., deficits in NE and/or 5-HT).

Future Directions

Despite the fact that the seizure suppressing effects of NE have been repeatedly demonstrated in animal models of epilepsy, the cellular/molecular mechanism by which NE produces this effect remains unknown. However, new evidence has shown that NE can reduce electrical coupling between GABAergic interneurons in the hippocampal slice, via a beta adrenergic receptor coupled to PKA phosphorylation. This mechanism provides a means for preventing the hypersynchronous activity that is essential for seizures. Whether this intriguing mechanism is responsible for the seizure suppressing effects of NE remains to be determined. However, it would seem unlikely that this is the only mechanism, since NE has been shown to also act through alpha adrenoceptors to suppress seizures. Perhaps there are different mechanisms in different parts of the brain.

Genetic studies should be carried out to identify the gene(s) responsible for the NE deficit in GEPRs, and studies should be carried out in humans afflicted with both depression and epilepsy to determine if there are abnormalities in genes involved in development or functional maintenance of the noradrenergic neurons.

More effort should be directed at clinically augmenting central noradrenergic signaling, without a concomitant increase in peripheral sympathetic autonomic signaling, such as seems possible via vagus nerve stimulation. Other approaches besides VNS should be examined – for example, via transcranial magnetic stimulation or deep brain stimulation. There is one report of locus ceruleus stimulation producing antiepileptic effects in humans. Could adrenoceptor agonists be infused into the lateral ventricles to maximize central and reduce peripheral effects? Would the benefits of deep brain stimulation of noradrenergic pathways outweigh the risks in patients with intractable epilepsy?

Another area that should be carefully assessed in future studies is the role of NE in epileptogenesis. Do the noradrenergic neurons of the locus ceruleus have the ability to retard the development of epilepsy? In this regard, could activation of the locus coeruleus system prevent the development of seizures associated with traumatic brain injury? Clearly, this is an area of great importance that should receive attention in future studies.

See also: **Models**: Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; **Psychiatric Symptoms**: Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Synaptic Activities and Transmitter Function: Receptor Trafficking

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Introduction

Receptor trafficking is a well-established process affecting synaptic plasticity as part of normal function and development in the central nervous system. It plays an important role in such normal processes as experience, activity-dependent plasticity, and LTP, and can occur rapidly – on the order of minutes or hours. As a part of the dynamic regulation of synaptic strength, receptor trafficking also may mediate a homeostatic process of 'synaptic scaling', important in maintaining balanced inputs to neurons in a circuit.

Although the importance of NMDA and AMPA receptor trafficking is well-established in the nervous system, much less is known regarding trafficking at inhibitory synapses. Even less is known regarding the role of trafficking associated with neurological disorders such as epilepsy and status epilepticus (SE).

When seizure activity continues for prolonged periods, we refer to that condition as 'status epilepticus.' By 30 min of such activity, rapid changes at both inhibitory and excitatory synapses occur, and seizures become self-sustaining and pharmacoresistant. Postsynaptic receptor trafficking during SE contributes to this pathophysiology and provides a mechanism for the initiation, as well as the maintenance, of SE.

Study of receptor trafficking, at both inhibitory and excitatory synapses, can provide insights into the mechanisms underlying alterations of synaptic strengths, and the subsequent imbalance between excitation and inhibition. In the hippocampus, that imbalance is thought to lead to circuit pathology that gives rise to seizures and SE. Thus, it is important to determine if aberrant plasticity at synapses contributes to the disease process – and if so, what mechanisms regulate this process?

As mentioned earlier, most studies of receptor trafficking have assessed the upregulation of AMPA receptors at synapses (within the context of LTP). Since seizure activity rapidly propagates through circuits to affect both excitatory and inhibitory pathways, SE provides a useful model to study simultaneous changes at inhibitory and excitatory synapses, and a context for exploring how opposed inhibitory and excitatory pathways interact and regulate each other. For example, activation of NMDA receptors can lead to internalization of $GABA_A$ receptors through LTD, and blockade of NMDA receptors preserves the efficacy of benzodiazepines during SE.

Regulation between inhibitory and excitatory pathways that is important for normal hippocampal function may go awry with pathological states such as SE. During SE, there is a dramatic loss of inhibition, offering investigators an opportunity to evaluate various mechanisms involved in downregulation of inhibition at GABAergic synapses – including the possibility of $GABA_A$ receptor trafficking. $GABA_A$ receptor internalization has been shown to be a consequence of agonist/ligand exposure in several studies; similarly, excessive GABA release – with receptor exposure and desensitization – may precede $GABA_A$ receptor internalization during SE and contribute to a loss of inhibition.

Another important consequence of synaptic plasticity during SE involves the development of a chronic epileptic state. Approximately 60% of animals develop chronic recurrent seizures after SE. The acute changes observed at synapses during SE may help to reveal the mechanisms that contribute to the development of this chronic epileptogenicity, and thus to epilepsy in general. In particular, trafficking of NMDA receptors towards the cell surface during SE may initiate epileptogenic plasticity and/or excitotoxicity, with alterations of circuit architecture and selective cell loss.

Background

A proper balance between excitation and inhibition is necessary for normal functioning in the brain, and pathophysiological states, such as SE, likely result from a disruption of that balance. As the primary inhibitory and excitatory transmitters in the brain are GABA and glutamate, respectively, many animal models of SE use pharmacological agents that act on GABA or glutamate systems in order to upset that balance. For example, SE is elicited by either blockade of ionotropic $GABA_A$ receptors (e.g., bicucculline, picrotoxin, penicillin, pentylenetetrazol) or activation of ionotropic glutamatergic receptors (e.g., kainic acid, domoic acid).

Failure of inhibition occurs early during SE. Even brief periods (1–5 min) of convulsion-mimicking electrical stimulation of perforant path inputs to the hippocampus cause loss of paired-pulse inhibition; this loss may last 45 min or more, in both in vivo and in vitro models. As a result of this loss of inhibition, there is a further lowering of seizure threshold, thus establishing a self-perpetuating

feedback cycle during which seizures become self-sustaining and SE is established. That is, removal of the initial trigger for seizure activity will not arrest SE, and seizures will continue unabated. In parallel with the transition to self-sustaining SE, there is a loss of responsiveness to GABAergic drugs. While benzodiazepine treatment within 10 min of SE onset can arrest seizure activity, treatment after 45 min has little or no effect in animal models – or in studies of patients.

Alterations in the composition and characteristics of postsynaptic $GABA_A$ receptors contribute to this failure of synaptic inhibition during SE. $GABA_A$ receptors are pentomeric structures, generally consisting of two α subunits, two β subunits, and either a γ or δ subunit. At synapses, $GABA_A$ receptors predominantly contain $\gamma 2$ subunits; δ-subunit containing receptors are found at extrasynaptic/perisynaptic sites. Receptors containing $\gamma 2$ subunits have attributes that include pronounced desensitization, sensitivity to benzodiazepines, and the ability to directly interact with the AP-2 proteins involved with clathrin-dependent endocytosis. In contrast, receptors containing δ subunits show little desensitization, are benzodiazepine insensitive, and do not have consensus sequences for interaction with AP-2 proteins/clathrin. As a result of the properties of synaptic $GABA_A$ receptors, GABA exposure can cause $GABA_A$ receptor desensitization and internalization. Our results suggest that trafficking of extrasynaptic δ-subunit containing receptors does not occur during SE. However, ovarian cycle-dependent decreases in cell surface expression of these extrasynaptic $GABA_A$ receptors are associated with an increase in seizure susceptibility, and may be relevant to catamennhial epilepsy.

Postsynaptic glutamatergic receptors consist of NMDA and non-NMDA (AMPA, KA) types. The NMDA receptors have slower kinetics and greater Ca^{2+} permeability than the non-NMDA receptors. Both types coexist at the same synapses. Trafficking of non-NMDA receptors, AMPA receptors in particular, has been well characterized in the context of LTP, where activation of NMDA receptors leads to a rapid delivery of AMPA receptors to synaptic sites.

Unlike synaptic inhibition, where rapid degradation of the synaptic $GABA_A$ receptors renders SE resistant to GABA-enhancing agents, synaptic excitation mediated by NMDA receptors remains susceptible to pharmacological blockade; NMDA antagonists can arrest seizures hours after the onset of SE. This observation suggests that a rapid loss of GABAergic inhibition is important in the initiation of SE, while the persistence of robust glutamatergic excitation is responsible for the maintenance of SE. In addition, glutamatergic excitation/NMDA activation may have important effects on the later sequelae of SE, since NMDA blockade is able to prevent the development of spontaneous recurrent seizures. Interestingly, trafficking of NMDA receptors initially appears to involve their delivery to extrasynaptic sites (i.e., prior to their movement into synapses); as extrasynaptic receptors are implicated in excitotoxic cell death, NMDA blockade during SE may provide neuroprotection.

Methods

Electrophysiological Recordings

Wistar rats, 4-7 weeks old received lithium (3 mEq kg^{-1} intraperitoneal; night before) and methylscopolamine (1 mg kg^{-1} subcutaneous) 30 min before either pilocarpine (40 mg kg^{-1} intraperitoneal) or normal saline. After $\sim$1 h from the onset of stage 5 seizures, rats are anesthetized with halothane, decapitated, and the brains placed in ice-cold artificial cerebrospinal fluid (ACSF). Brain slices are cut (350 μm coronal sections) and perfused at 1.5 ml min^{-1} with 33–35 °C ACSF. Whole cell patch clamp recordings from somata of visualized neurons are made using borosilicate microelectrodes. Inhibitiory postsynaptic currents (IPSCs) are isolated using selective pharmacological blockade of both NMDA and non-NMDA excitatory currents with kynurenic acid (2 mM). Alternatively, picrotoxin (30 μM) is used to block $GABA_A$ inhibitory currents, either with DNQX (10 μM) with 0 Mg^{2+} in the ACSF or with APV (50 μM) to isolate NMDA and non-NMDA EPSCs, respectively.

Immunocytochemistry

Serial formaldehyde-fixed 40 μm coronal sections are incubated overnight in mouse antibody to either the $GABA_A$ receptor $\gamma 2$ or the NMDA receptor NR1 subunits and rabbit antisynaptophysin antiserum. The sections are then incubated for 1 h in FITC-labeled goat antirabbit IgG and Cy^3-labeled donkey antimouse IgG diluted in PBS containing 1% goat serum, horse serum, and 0.3% Triton X-100. Quantification of immunofluorescent receptors is acquired at 100× with a confocal microscope. Colocalization is determined by examination of the overlaid synaptophysin and receptor-stained images.

Computer Analysis

The synapse is an elaborate structure with presynaptic and postsynaptic components that add complexity that is difficult to resolve and interpret without computer and mathematical analysis. Such analysis can help identify receptor desensitization/trafficking at synapses. Mean and variance traces generated from superimposed postsynaptic currents recorded from a dentate gyrus granule cell are analyzed using computer optimization programs

that estimate the number of postsynaptic NMDA receptors (N), the single channel conductance (i), and the channel open probability (P_{open}). For channels with open probability P_{open}, the binomial distribution for the population of channels at a synapse generating postsynaptic currents has a mean current (I)

$$I = NP_{open}i \quad [1]$$

and variance (σ^2)

$$\sigma^2 = NP_{open}(1 - P_{open})i^2 \quad [2]$$

P_{open} will depend on several factors, including the concentration of GABA or glutamate in the synaptic cleft and the kinetics of the individual postsynaptic receptors. Substituting eqn. [1] in eqn. [2] determines the parabolic equation,

$$\sigma^2 = iI - I^2/N \quad [3]$$

and eliminates P_{open} as a variable, allowing N and i determination from optimized fits of variance versus mean plots.

The basic assumptions of a more elaborate synaptic model are: (1) individual $GABA_A$ (or NMDA) receptors open and close randomly, with an open probability determined by the synaptic cleft [GABA] (or [glutamate]); (2) an m-state receptor kinetic model (including closed, ligand-binding, open, and desensitized states) can characterize the relation between [GABA] (or [glutamate]) and the receptor channel open probabilities; (3) vesicle release of transmitter into the cleft can be modeled as a pulse with exponential decay; and (4) postsynaptic currents represent the linear sum of the individual postsynaptic receptor current responses.

A computer model of postsynaptic currents is generated by matrix multiplication of $\mathbf{A}$ (where $\mathbf{A}$ is $m \times m$ for the m number of receptor states; the particular value of the matrix indexed by j and k, $\mathbf{A}_{jk}$, is the probability that a receptor in state k will transition to state j during a specified time increment of iteration $\sim$ 0.01 ms) by $\mathbf{x}_n$ (m $\times$ 1; containing the probability of each state at the current time), thereby generating the time-updated probability vector, $\mathbf{x}_{n+1}$, to be substituted for $\mathbf{x}_n$ in subsequent iterations ($\mathbf{A} \times \mathbf{x}_n = \mathbf{x}_{n+1}$) across time. P_{open} is the sum of the probabilities from the open states of $\mathbf{x}_n$, and P_{desens} the sum of the probabilities for desensitized states of $\mathbf{x}_n$.

Results

After 1 h of SE, there is a 27% reduction in the mean mIPSC absolute amplitude (from 53 pA for control animals to 39 pA for SE animals). Similar results have been obtained for in vitro models of SE using 0 Mg^{2+}. We suspected that the reduction in amplitude is related to a decrease in the number of functional postsynaptic $GABA_A$ receptors. To analyze the results, mean-variance analysis (see the Methods section) of mIPSCs was performed with the basic assumptions that individual $GABA_A$ receptors open and close randomly, with an open probability depending on cleft [GABA]; mIPSCs represent the sum of the individual $GABA_A$ postsynaptic receptor current responses. The results of this analysis (in conjunction with receptor kinetic modeling) suggest that the number of postsynaptic $GABA_A$ receptors decreases 50%, from 36 $\pm$ 11 per synapse in controls to 18 $\pm$ 4 per synapse in slices obtained after 1 h of SE.

To confirm that the reduction in the number of functional $GABA_A$ receptors was from internal relocation of receptors towards the cell interior (away from synapses), immunocytochemical labeling of $GABA_A$ receptors was performed. In granule and pyramidal cells from control (left column) hippocampi, immunoreactivity against the $\beta2/\beta3$ (top row) or $\gamma2$ (bottom row) subunits of $GABA_A$ receptors outlines the cell membrane (red fluorescence), and frequently colocalizes with the presynaptic marker for synaptophysin (green); overlay of subunit and synaptophysin markers appears as yellow dots. After 1 h of SE in vivo, much of this $\beta2/\beta3$ and $\gamma2$ subunit immunoreactivity is in the interior of the cell (right column), surrounded by synaptophysin immunoreactivity outlining the cell membrane. The colocalization of $GABA_A$ receptor $\beta2/\beta3$ subunits with synaptophysin decreases from 43% (controls) to 13% (with SE in vivo). Similar results have been found in vitro, using 0 Mg^{2+} to produce an SE-like discharge pattern in hippocampal cultures. Recently, a $\gamma2$ subunit mutation that shifts trafficking of $GABA_A$ receptors away from the cell surface in a temperature-dependent manner has been associated with febrile seizures, suggesting a causal relation between the surface location of $\gamma2$ subunit-containing receptors and seizure susceptibility. Using inhibitors of dynein in recording electrodes, these in vitro studies showed that the internalization of $GABA_A$ receptors was clathrin-dependent.

Our results also indicate an increase in extracellular GABA during SE, likely from presynaptic release during seizure activity. To determine if an increase in extracellular GABA could mimic the effects of SE, hippocampal slices were perfused with aCSF containing 3 μM of GABA. A reduction of mIPSC amplitude similar to that seen with SE occurs under these conditions, suggesting that increased ligand exposure may contribute to desensitization and downregulation of $GABA_A$ receptors (**Figure 1**).

Unlike mIPSCs, the absolute amplitude of NMDA-mEPSCs increases 25% after SE (from 16 to 20 pA). Mean-variance analysis suggests that an increase from 8 to 11 NMDA receptors per synapse is responsible for the physiological changes. This shift is confirmed by immunocytochemistry, using antibodies to the NR1 subunit of NMDA receptors; these studies show a relocation

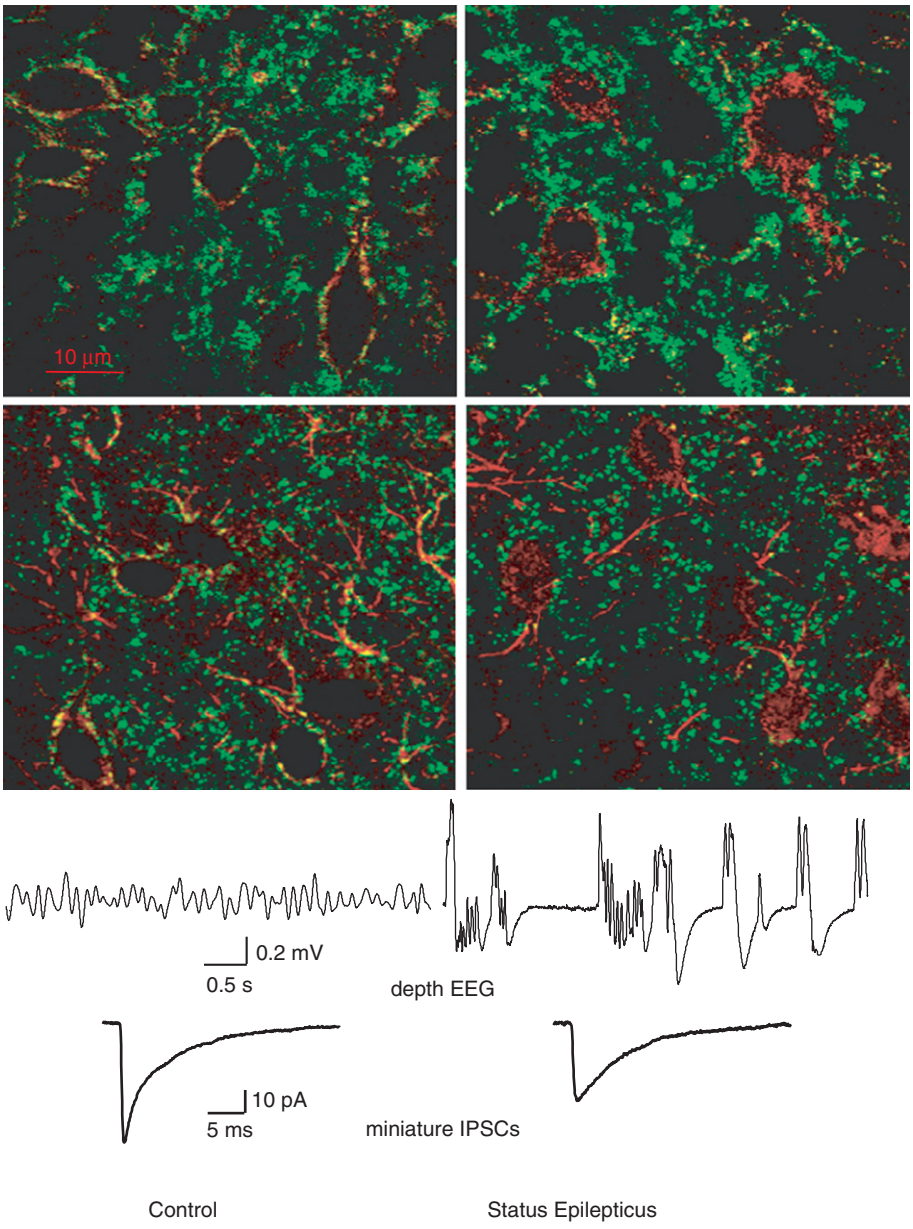


Figure 1 Subcellular redistribution of hippocampal $GABA_A$ receptor subunits reflects physiological changes in EEG and miniature inhibitory postsynaptic currents (mIPSCs) during status epilepticus (SE). $GABA_A$ β2/β3 (red, first row) and γ2 (red, second row) subunits relocate to the cell interior during SE, with decreased yellow colocalization of subunits with the presynaptic marker synaptophysin (green). Stereotaxic EEG recorded in the hippocampal dentate gyrus demonstrates polyspike and wave discharges during SE (third row), with a decrease in the amplitude of mIPSCs recorded from dentate gyrus granule cells (fourth row). The functional loss of synaptic inhibition associated with the internal aggregation of $GABA_A$ receptor subunits may help sustain SE. Adapted from Naylor DE, Liu H, and Wasterlain CG (2005) Trafficking of GABAA receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. *Journal of Neuroscience* 25(34): 7724–7733, with permission from Society of Neuroscience.

of subunits to the surface of granule and pyramidal cells in the hippocampus, with increased colocalization with synaptophysin at synapses. An increase in the amplitude of non-NMDA mEPSCs also occurs during SE, and is predicted to represent an increase (from 36 to 40) of non-NMDA glutamate receptors per synapse.

The direction of trafficking of receptors during SE appears to be specific to receptor type, with a downregulation in the number of $GABA_A$ receptors at inhibitory synapses and an increase in the number of NMDA and non-NMDA glutamate receptors at excitatory synapses. This shift should cause an imbalance between inhibition and excitation, and contribute to the initiation and maintenance of self-sustaining SE. The dramatic reduction (20-fold) in potency of benzodiazepines within minutes of the onset of SE can be explained

by the rapid loss of available $GABA_A$ receptors at postsynaptic sites. Similarly, the ability of NMDA antagonists such as ketamine or MK801 to abort SE, after more than an hour of seizure activity, can be attributed to the persistent availability and upregulation of NMDA receptors at postsynaptic sites. The rapid loss of inhibition is likely important in the initiation phase of SE (i.e., the transition from single seizures to self-sustaining SE), while NMDA receptor upregulation helps maintain SE.

Since seizure activity increases the level of extracellular GABA, and $GABA_A$ receptors at postsynaptic sites downregulate with GABA exposure, increases in firing/release should result in a loss of inhibition. This loss will then increase excitability/firing and lead to further GABA release and downregulation of synaptic inhibition mediated by $GABA_A$ receptors, establishing a pathophysiological cycle. At a critical threshold for the loss of synaptic inhibition, the resultant excitability will become self-sustaining. The increase in NMDA and non-NMDA receptors at excitatory synapses not only should accentuate excitation, but also can contribute to $GABA_A$ receptor downregulation/internalization, with chronic effects on $GABA_A$ receptor function.

Future Directions

Future research will explore the relation between NMDA activation and $GABA_A$ receptor trafficking and determine whether blockade of NMDA activity can reduce $GABA_A$ internalization. In addition, a time-course study of SE will help determine if $GABA_A$ receptor desensitization (reflected physiologically by a decrease in mIPSC amplitude) precedes internalization (as revealed by immunocytochemistry). Similarly, studies that prevent receptor internalization will show if mIPSC amplitude reduction can occur despite blockade of clathrin-dependent endocytosis; results from such an experiment also can elucidate whether receptor desensitization precedes internalization.

Many new potential therapies for SE may emerge from these studies. In particular, treatments that disrupt clathrin-dependent endocytosis, including treatment with osmotic agents, may prevent the internal relocation of postsynaptic $GABA_A$ receptors and help restore/maintain synaptic inhibition. Since extrasynaptic $GABA_A$ receptors are not down-regulated in the same way that synaptic $GABA_A$ receptors are down-regulated, potentiation of tonic (i.e., extrasynaptic) $GABA_A$ currents could provide a route towards restoration of inhibition. Therefore, pharmacological agents that enhance these currents, such as neurosteroids, may counterbalance losses of inhibition at synapses. Finally, since NMDA and non-NMDA glutamate receptors are upregulated at excitatory synapses and remain accessible as targets for pharmacological blockade, therapies that use antagonists directed at these receptors should improve the efficacy of pharmacotherapy, especially after the initiation of SE, over agents directed towards enhancing synaptic inhibition alone.

See also: **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor Plasticity in Epileptogenesis; Pathophysiology of Status Epilepticus; **Transporters**: GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability.

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NEUROTROPHIC FACTORS

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Activity-Regulated BDNF Expression: Contributions to Synaptic Plasticity and Neuroprotection

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Introduction

Some of the first evidence that neuronal gene expression in brain is influenced by physiological activity came from studies of the effects of seizures. Work in the early 1980s found that in rodents, seizures increase levels of the neuropeptide enkephalin throughout the limbic telencephalon. These results were quickly followed by reports that seizures increase mRNA levels for several transcription factors that, in turn, regulate genes encoding families of proteins; the breadth of this second order response was largely unknown. Findings of these types prompted efforts in many laboratories to test whether, *in the adult brain*, increases in neuronal activity during seizures initiate the expression of genes associated with growth and plasticity. That such was the case was first demonstrated for nerve growth factor (NGF), and soon thereafter for the structurally related neurotrophin, brain derived neurotrophic factor (BDNF). Both NGF and BDNF mRNA levels are increased over 10-fold within 3 h of seizure onset in rat hippocampus, and at somewhat greater latencies in the neocortex (**Figure 1(a)** and **1(b)**). It soon became clear that these alterations in neurotrophin gene expression were occurring within a context of myriad, seizure-induced changes in the expression of neuroactive peptides, transcription factors, adhesion proteins, structural proteins, and more. The trophic factors alone exhibited a staggering array of responses, including: increases in the expression of transforming growth factor (TGF) superfamily members such as the potent survival factor for basal ganglia neurons, glial cell-derived neurotrophic factor (GDNF), the fibroblast growth factors (FGF1 and FGF2), and vascular endothelial growth factor (VEGF); and decreases in neuronal expression of others mRNAs (e.g., neurotrophin-3). Surprisingly, several trophic factors that are predominantly expressed by glial cells and are induced during deafferentation (e.g., ciliary neurotrophic factor, insulin-like growth factor-1) respond little, if at all, to seizures (or to other increases in neuronal activity).

Background – BDNF Facilitates Synaptic Plasticity in Adult Brain

Among the trophic factors induced by seizures and considered likely to contribute to lasting changes in brain, BDNF stands out as being of particular interest. BDNF expression responds rapidly and with very low threshold to increases in neuronal activity and, along with its high affinity receptor TrkB, is expressed throughout the brain and spinal cord. As a consequence, even brief or localized increases in neuronal activity (e.g., epileptiform bursts in the absence of a frank seizure) increase BDNF production and, most probably, signaling. It is also the case that BDNF produces an impressive and diverse array of neuronal responses. Along with other members of the neurotrophin family (e.g., NGF, NT3, NT4/5), BDNF influences the differentiation, process outgrowth, gene expression, and survival of responsive neurons. Importantly, it is largely anterogradely transported, released from synapses in an activity-dependent fashion, and has acute effects on neuronal function that departs from classic actions on the trophic center of the cell. Through local signaling, including that occurring in the vicinity of the synapse, BDNF regulates properties of various ion channels, calcium influx, cytoskeletal remodeling, local protein translation, neurotransmitter release, and neurotransmitter receptor function. Moreover, and not

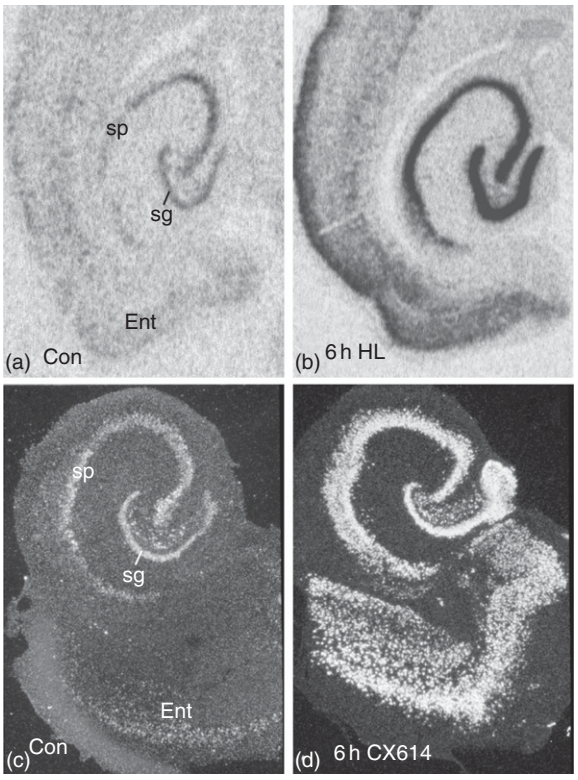


Figure 1 BDNF expression is positively regulated by neuronal activity. Photomicrographs show ^{35}S-cRNA in situ hybridization labeling of BDNF mRNA in sections through rat hippocampus (a, b) and cultured hippocampal slices (c, d). (a, b) Bright field photographs of film autoradiograms show labeling in hippocampus and adjacent entorhinal cortex (Ent) of a control rat (a) and an experimental seizure rat killed 6 h after limbic seizure onset (b): as shown, seizures increased BDNF mRNA in the granule and pyramidal layers of hippocampus (sg and sp, respectively) and in surrounding cortical fields. (c, d) Dark field photographs show labeling of BDNF mRNA in cultured hippocampal slices harvested without treatment (c) and after 6 h CX614 exposure (cRNA labeling appears white). Like seizure activity, ampakine treatment increased BDNF mRNA in granule and pyramidal layers of hippocampus and in adjacent cortical fields.

surprisingly considering this list of functions, BDNF also facilitates and enhances the magnitude of activity-dependent synaptic plasticity.

Long-term potentiation (LTP) is a principal form of synaptic plasticity in the mammalian brain, thought to underlie learning and memory and, more generally stated, enduring experience-dependent enhancement of synaptic transmission. In experimental settings LTP is typically induced by high-frequency (100–200 Hz) afferent stimulation, but studies have shown that it is more efficiently induced with patterns of afferent activity that simulate the natural, 5 to 8 Hz 'theta' rhythm of cell firing that can be recorded from hippocampus and limbic cortical regions during behavior. As neurons are known to discharge at high frequencies and in synchronous bursts during seizures, it has long been speculated that synaptic potentiation and, specifically LTP, contribute to the enhancement of circuit activity, reductions in seizure threshold with kindling in experimental models, and epileptogenesis in man.

The first evidence that BDNF may be involved in LTP came from the demonstration that infusion of this neurotrophin into rat hippocampus leads to an LTP-like enhancement of synaptic transmission. Although this response has proved to be variably obtained and is likely to be region-specific (i.e., it is reliably observed in the dentate gyrus but not hippocampal field CA1), the basic conclusion that BDNF facilitates potentiation when applied in combination with afferent stimulation has held. Studies in many laboratories have shown that infusion of exogenous BDNF both lowers the activity-dependent threshold and increases the magnitude of LTP induced by theta burst afferent stimulation, whereas reductions in BDNF availability or signaling (through genetic manipulation in mice or treatment of experimental tissue with reagents that impede BDNF delivery or receptor function) prevents LTP induction by theta stimulation.

While these findings show that BDNF is important for LTP, and therefore is likely to contribute to changes in circuit function with seizures, there are still questions as to the cellular mechanisms through which BDNF enhances synaptic transmission; it is also still unclear whether activity-regulated increases in the endogenous neurotrophin influence neuronal survival in response to insult in the developing and adult brain. The latter question is of particular importance in the instance of seizures and epilepsy, because experiments using in vitro preparations have shown that BDNF can reduce cell death in response to excitotoxic insult and ischemia, events thought to contribute to neuropathology as well as the likelihood of future seizure in man.

To address these issues, recent studies have made use of hippocampal slice preparations to elucidate mechanisms through which exogenous BDNF facilitates LTP, and to test if increases in the endogenous protein similarly facilitate LTP and reduce neuronal vulnerability to insult. Results of these studies indicate that activity-induced increases in BDNF expression in the adult brain both (i) enhance a form of synaptic plasticity that is likely to contribute to epileptogenesis (i.e., LTP) and (ii) give rise to secondary changes in gene expression that counteract epileptogenic influences and reduce vulnerability to ischemic insult.

Methods

Studies in our laboratory have made use of both acute and cultured hippocampal slices to test BDNF effects on synaptic transmission and neuronal survival. The acute hippocampal slice preparation has many advantages for studies of synaptic plasticity in adult brain. Hippocampal

slices, cut 400 μm thick perpendicular to the long axis of the structure, can be maintained for hours in a constantly perfusing, heated and oxygenated artificial cerebrospinal fluid (aCSF) bath, and drugs can be applied via the aCSF input line without disturbing the slice. A large proportion of the hippocampal circuitry lies within the cross sectional plane of the slice, and due to differences in lipid content, the cell layers and afferent lamina are clearly visible; thus, electrodes can be placed within specific hippocampal laminae to stimulate and record synaptic responses from known axonal systems. Our studies have focused on synaptic responses to the Schaffer collateral afferents to hippocampal field CA1. These axons arise from hippocampal CA3 pyramidal cells and innervate the apical and basal dendrites of CA1 pyramidal cells in a highly laminated fashion. Potentiation of CA3-to-CA1 synaptic transmission can be elicited by activation of the Schaffer collaterals with theta burst stimulation (TBS; i.e., four pulses at 100 Hz spaced by a 200 ms interburst interval). Hippocampal CA3 pyramidal cells fire in short trains of 3–5 bursts at theta frequency in vivo. Thus, TBS replicates the natural cellular firing pattern that occurs in hippocampus during behavior. Stimulation with 5 theta bursts is generally sufficient to induce stable LTP; a modestly suprathreshold train of 10 theta bursts is typically used.

The objective of testing the influence of activity-regulated changes in BDNF expression on synaptic function has been addressed using two experimental preparations. First, exogenous BDNF was added to the acute hippocampal slice aCSF bath during electrophysiological recordings; control preparations are bathed in aCSF alone or containing heat inactivated BDNF. Second, to evaluate the consequence of increases in endogenous BDNF levels, positive modulators of AMPA-class glutamate receptors (also known as ampakines) were employed. We have shown that spaced ampakine treatments increase BDNF gene expression and sustain increases in hippocampal BDNF protein content in vitro (**Figure 1(c)** and **1(d)**) and in vivo.

Finally, to test if increases in endogenous BDNF reduce neuronal vulnerability to insult, an in vitro model of oxygen–glucose deprivation (OGD) was used. Hippocampal slices were prepared from postnatal day 10 rat pups and explanted to interface culture 10 days before use. Over this time, the cultured slices exhibit maturational changes that parallel those in same-aged tissue in vivo. Slices were treated with the ampakine CX614 (3 h day^{-1} for 2 days), which causes a modest increase in neuronal firing during the CX614-on period, and both induces and sustains robust increases in endogenous BDNF protein content. One day after ampakine pretreatment, slices were exposed to 45 min OGD by submersion in oxygen- and glucose-depleted aCSF, and then returned to normal culture conditions until harvest 24 h later. Levels of cell death were assessed using FluoroJade staining and propidium iodide uptake.

Results

BDNF Facilitates Synaptic Responses through Effects on SK2 Channels

Although there is considerable evidence that BDNF facilitates activity-induced synaptic potentiation in hippocampus, the components of the BDNF-response profile contributing to this facilitation are not fully appreciated. In reexamining this issue, we evaluated effects of infusing BDNF on Schaffer collateral synaptic responses in field CA1. In agreement with results from many laboratories, BDNF infusion had negligible effect on field excitatory evoked potentials (fEPSPs), but did lower the stimulation threshold and increase the magnitude of LTP. In the presence of 2 nM BDNF, two theta bursts elicited modest LTP; this level of stimulation is not sufficient to potentiate transmission in control preparations. Moreover, the magnitude of maximal potentiation was elevated nearly twofold in the presence of added BDNF.

Examination of fEPSP responses to single theta bursts provided clues as to the locus of these BDNF effects. There is normally a modest facilitation of postsynaptic responses within a train of theta-bursts; in the presence of excess exogenous BDNF, this within-train facilitation is enhanced 60 to 100% – but effects are evident only after the first burst. This observation suggests that BDNF may attenuate the magnitude of the afterhyperpolarization (AHP) that follows a single burst response. Tests of this possibility proved positive: BDNF infusion reduced the postburst AHP as determined by whole cell recordings. The AHP reflects calcium-activated potassium currents and, at the latency of the BDNF-associated AHP change, the small-conductance calcium activated potassium (SK2) channels are thought to play a role. Indeed, treatment with SK2 inhibitors had the same effect as BDNF on within-train facilitation of burst responses and on the magnitude of maximal LTP. The effects of cotreatment with SK2 inhibitors and BDNF were not additive, suggesting that they act on the same pathway to influence LTP. These results indicate that BDNF facilitates LTP, at least in part, by reducing AHP potassium currents, thereby enhancing synaptic responses during a high-frequency train. Moreover, these findings suggest that endogenous BDNF will enhance synaptic responses to high-frequency burst activity such as seen in the epileptic brain.

BDNF Supports Cytoskeletal Changes that Stabilize Synaptic Potentiation

Recent studies indicate that LTP is associated with changes in synapse and spine morphology, and that both morphological changes and potentiation depend upon remodeling of the actin cytoskeleton. Knowing that BDNF influences actin polymerization in immature

systems, we tested the hypothesis that BDNF plays an instructive role in the activity-induced remodeling of the spine cytoskeleton involved in LTP. Using in situ infusion of rhodamine-phalloidin to label and quantify F-actin, we found that TBS induces large increases in spine F-actin that are restricted to laminae of afferent activation. This increase is blocked by latrunculin-A, which prevents the conversion of soluble monomeric- to filamentous-actin; this effect demonstrates that stimulation increases *new* actin filament assembly. With this result in hand, the effects of BDNF on stimulation-induced increases in spine F-actin were tested. BDNF decreased the stimulation threshold for eliciting both LTP and actin polymerization, although BDNF infusion alone (without stimulation) had no effect on spine F-actin content.

This study demonstrates that excess exogenous BDNF facilitates activity-induced increases in spine F-actin. To determine if endogenous neurotrophin is present and released at sufficient levels to have similar effects, slices were treated with BDNF antagonists and TBS was applied. TrkB-Fc is a chimeric protein containing the extracellular domain of the TrkB receptor that effectively scavenges released BDNF; K252a is a kinase antagonist that blocks TrkB autophosphorylation and signaling. TrkB-Fc and K252a block BDNF signaling through the high-affinity TrkB receptor. Both reagents, applied to hippocampal slices during or immediately after TBS, blocked LTP and increases in spine F-actin otherwise elicited by this level of stimulation.

These results demonstrate that BDNF facilitates the activity-induced increases in spine F-actin that are considered critical for the stabilization of synaptic potentiation, and that signaling by endogenous, synaptically released BDNF is needed for LTP stabilization and actin polymerization in the minutes immediately following theta pattern synaptic activity.

Ampakine Regulation of BDNF Expression in vitro and in vivo

Studies described above identified specific mechanisms through which (i) endogenous BDNF contributes to synaptic plasticity, and (ii) increases in BDNF availability might facilitate activity-dependent changes in circuit function in association with patterned neuronal activity and seizures. However, up to this point we had not shown that increases in endogenous BDNF protein content replicate the effects of adding excess BDNF on synaptic plasticity. This 'replication' cannot be assumed, since BDNF trafficking and release might be regulated to maintain, or set limits upon, trophic signaling. Further, BDNF arising from natural sources is not expected to be present throughout the tissue as is the case with added exogenous protein. BDNF is largely anterogradely transported, from perikarya to axon terminals, and released in an activity- and frequency-dependent fashion. As a consequence, secretion and signaling by the native neurotrophin is likely to occur preferentially in the region of the synapse and in association with high-frequency synaptic discharges. To better replicate this pattern of exposure and assess the functional consequences of fluctuations in endogenous BDNF content, we needed a method for manipulating the production of the neurotrophin in situ.

To this end, we have devised paradigms using positive allosteric modulators of AMPA class glutamate receptors (also known as ampakines) to increase BDNF expression. Early studies showed that BDNF gene expression and protein levels are positively regulated by AMPA receptor-mediated activity. The ampakines slow AMPA receptor desensitization and ligand-induced deactivation without direct agonist or antagonist activities, and without interfering with the native ligand binding site. As a consequence, they modestly enhance monosynaptic glutamatergic synaptic responses and more greatly enhance responses across the polysynaptic glutamatergic networks within hippocampus and neocortex. Ampakines cross the blood–brain barrier; hence, effects of ampakine treatment can be evaluated either in vitro or in vivo following systemic injection. Initial studies showed that in cultured hippocampal slices, prolonged ampakine exposure increased BDNF expression but also downregulated the AMPA receptors themselves. However, with shorter (<3 h), spaced ampakine treatments that simulate patterns of ampakine exposure likely to be obtained with intermittent dosing in vivo, BDNF protein levels are increased and can be sustained at elevated levels for days without effects on the AMPA receptor system.

BDNF is generated as a precursor protein (referred to as proBDNF) that is cleaved by intracellular and extracellular proteases to form the mature (m), 14–15 kDa BDNF. Both pro- and mature-forms are released and biologically active. There is evidence that pro- and mBDNF preferentially interact with the p75 low-affinity neurotrophin receptor ($p75^{NTR}$) and TrkB, respectively, and through these receptors facilitate potentiation (via TrkB) and depression (via $p75^{NTR}$) of synaptic responses. These findings raised concern as to whether the ampakines influence levels of proBDNF, mBDNF, or both. Using western blots to evaluate BDNF forms induced by ampakine treatment of cultured hippocampal slices, we found that 3 hr pulse ampakine treatments induced modest (20–50%) increases in 25–35 kDa proBDNF that dissipated within about 12 h, and both larger and more enduring increases in mBDNF (+100 to 200%) that lasted for days. Thus, the predominant effect of ampakine treatment is a lasting increase in the mature BDNF protein expected to facilitate LTP.

The next goal was to test if pulse ampakine treatments sustain increases in mBDNF protein content in vivo. Studies in rats and mice have examined effects of twice daily injections of a short acting ampakine (CX929, half

life of 15–20 min in rat). In one study, socially housed rats were handled daily for several days to reduce the stress of the injection procedure. Rats then received twice daily (morning, afternoon) intraperitoneal ampakine injections and were allowed to socially play for 20 min after each injection; within-group control rats received vehicle injections. The ampakine had no significant effect on locomotor activity, and experimental rats generally appeared indistinguishable from controls. Effects on BDNF protein levels were assessed by ELISA and western blot analyses of brain tissue samples from animals killed 1 day after the last injection. Both measures showed that ampakine treatment increased mBDNF protein concentrations by 50–100% in hippocampus and neocortex. Increases in BDNF content were dose-dependent, evident after the first day of ampakine treatment, and still in place after injection day 4. These and modest variations of these paradigms have been used in subsequent analyses of the consequence of activity-regulated increases in endogenous BDNF on properties of synaptic plasticity (i) in several animal models of cognitive dysfunction and (ii) in an in vitro model of ischemic insult, as described below.

Ampakine Treatment Increase BDNF and can Restore LTP in Models of Cognitive Impairment

As described above, BDNF facilitates LTP, and increases in BDNF availability from exogenous sources can compensate for deficiencies in induction or stabilization of potentiation, including otherwise subthreshold levels of synaptic stimulation. These findings have encouraged speculation that BDNF might be used to restore LTP, and cognitive processes deriving from it, in human syndromes involving cognitive impairment. Deficits in LTP have been described in association with normal aging and in animal models of Alzheimers disease, Fragile-X syndrome, and Huntingtons disease. In the latter two instances, electrophysiological studies of acute hippocampal and neocortical slices have shown that rhythmic or high-frequency activity induces short-lived potentiation that decays with time – i.e., the potentiation fails to stabilize. Nevertheless, infusion of exogenous BDNF fully restores stable LTP in both Fragile-X (**Figure 2(a)**) and Huntingtons disease mutant mice.

The most complete demonstration of the therapeutic potential of manipulating *endogenous* BDNF availability to offset deficits in synaptic plasticity has been obtained in studies targeting impairments in LTP with normal aging. In rats, learning and memory deficits first appear in 'middle age' (~12 months) and worsen with further aging. Prior studies of hippocampal LTP had not identified corresponding deficits in synaptic plasticity in middle-aged animals. However, these previous analyses focused on the dentate gyrus or the apical CA1 dendritic field. We reexamined the effects of aging on LTP of Schaffer collateral innervation of the CA1 basal dendritic field, known to exhibit some differences in the threshold and magnitude of potentiation. In the CA1 basal dendrites, TBS initially induced robust potentiation in slices from both young adult (2–3 months old) and middle-aged (8–12 months old) rats. In tissue from the young adults, potentiation stabilized at nearly twofold prestimulation values as measured 60 min post-TBS; in contrast, in the middle-aged hippocampus, basal dendritic potentiation failed to stabilize and decayed to baseline levels by this time point. Infusion of exogenous BDNF, initiated minutes before stimulation, rescued LTP in the basal dendrites of middle-aged rats.

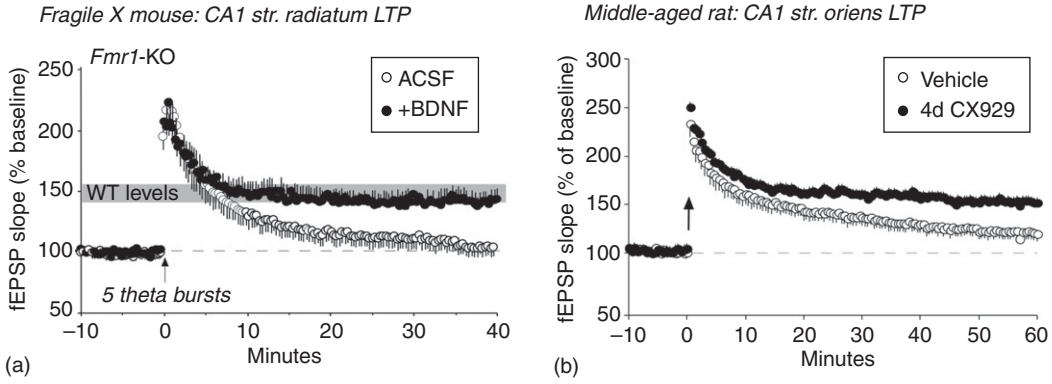


Figure 2 BDNF rescues LTP in rodent models of cognitive impairment. Plots of Schaffer collateral fEPSPs elicited by single test stimulation pulses applied before and after TBS (at arrow). (a) Plots of responses obtained in slices from Fmr1-KO mice (the murine model of Fragile X syndrome) show that in normal ACSF bath five bursts of TBS elicits an initial increase in the fEPSP that decays to prestimulation levels over about 30 min (i.e., potentiation fails to stabilize). In contrast, with 2 nM BDNF in the tissue bath the synaptic response is enhanced and potentiation stabilizes at about 150% baseline values. Grey bar shows the level of potentiation obtained in slices from wild-type mice. (b) Plots of Schaffer collateral fEPSP responses recorded from CA1 str. oriens in slices from vehicle-treated (open symbols) and CX929-treated (closed circles) middle-aged rats. As shown, TBS induced stable potentiation of the fEPSP (150% baseline values) in tissue from ampakine-but not vehicle-pretreated rats.

These results encouraged the thought that increases in endogenous BDNF levels might similarly rescue LTP in middle-aged animals. Using the spaced, systemic ampakine treatment strategy described above (i.e., twice daily injections of CX929 and sacrifice 1 day after the last injection) hippocampal and cortical BDNF protein levels were increased by about 60% in middle-aged rats. Electrophysiological analyses of hippocampi from ampakine-treated rats and age-matched vehicle-treated controls demonstrated that ampakine pretreatment fully rescued stable LTP to the basal dendritic field (**Figure 2(b)**) without changes to potentiation in the apical dendritic field (where age-related deficits were not observed). Moreover, in slices from ampakine pretreated rats, the restored LTP was eliminated by infusion of the BDNF scavenger TrkB-Fc, thereby demonstrating that endogenous BDNF was critical to the reestablishment of synaptic potentiation.

These findings demonstrate that increases in BDNF expression facilitate activity-dependent synaptic plasticity and can restore LTP in at least one condition (aging) involving deficits in both LTP and learning. Moreover, these results encourage the expectation that the more robust increases in BDNF expression and protein content induced by seizures will facilitate enduring synaptic plasticity.

Increases in Endogenous BDNF Reduce Vulnerability to Ischemia through Secondary Effects on NPY Gene Expression

BDNF and related neurotrophins are well known to reduce neuronal vulnerability to insult in a variety of model systems. These findings suggest that activity- and seizure-induced increases in endogenous BDNF protein might also be neuroprotective. However, other work has shown that in some instances (e.g., excitotoxic insults leading to necrotic cell death), applied BDNF can actually exacerbate toxicity. To test if increases in endogenous BDNF expression reduce vulnerability of hippocampal neurons to insult, we employed cultured hippocampal slices and a well-characterized oxygen–glucose deprivation (OGD) model of ischemia.

To increase endogenous BDNF content, the ampakine CX614 was applied to cultured slices for 3 h day^{-1} for 2 days. Such treatments increase mBDNF protein content over twofold. On day 3 (21 h after ampakine removal) the slices were exposed to OGD for 45 min and then returned to normal culture conditions until harvest 24 h later. As demonstrated using FluoroJade staining to label dying neurons, OGD alone caused robust neuronal cell death in hippocampal field CA1 pyramidal cells (**Figure 3(a)**). In contrast, there was little to no cell death in response to OGD in ampakine-pretreated slices. Thus, ampakine pretreatment markedly reduced neuronal vulnerability to ischemia. Cotreatment of cultures with the ampakine and a BDNF antagonist (either TrkB-Fc or K252a) during the 2 days prior to insult totally eliminated the protective effects of ampakine pretreatment, thereby demonstrating that protection was indeed BDNF-dependent.

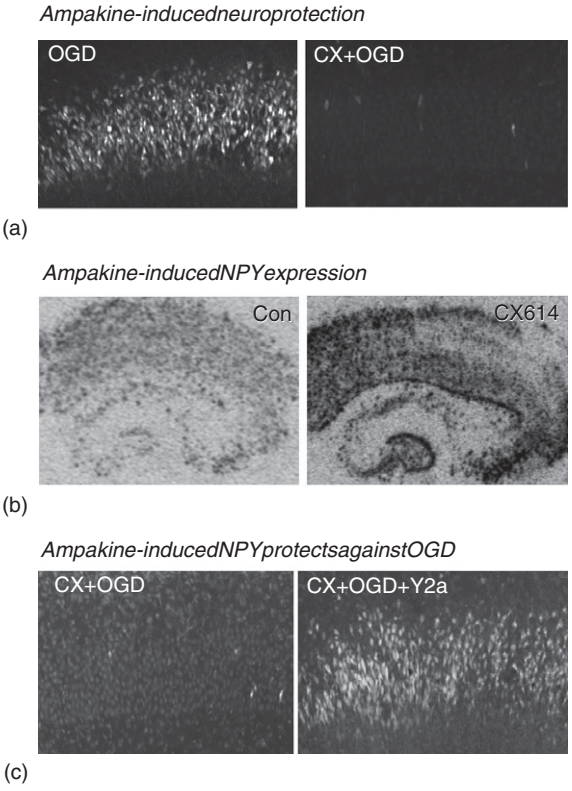


Figure 3 Neuroprotective effects of ampakine treatment are mediated by BDNF and NPY. Cultured hippocampal slices were pretreated with the ampakine CX614 (CX) (3 h day^{-1}, 2 days) then exposed to OGD. (a) Photographs of FluoroJade labeling in the CA1 pyramidal cell layer show that OGD elicits robust cell death in otherwise untreated slices (a, left; dying neurons appear white) but not in ampakine pretreated tissue (a, right). (b) In situ hybridization to NPY mRNA in an untreated cultured hippocampal slice (Con, left) and a slice treated with CX614 (3 h day^{-1}, 2 days) (right) shows that the ampakine increased NPY expression in hippocampal and cortical fields. (c) Photomicrographs of field CA1 showing FluoroJade labeling of dying neurons in slices exposed to OGD after CX614 pretreatment alone (left), or with Y2 receptor antagonist cotreatment (right): results show that ampakine-mediated neuroprotection depends upon the actions of NPY.

BDNF influences the expression of a battery of genes in target neurons, suggesting the possibility that secondary changes in gene expression might contribute to protective effects of ampakine pretreatment. In particular, BDNF stimulates the expression of neuropeptide Y (NPY), a neuroactive peptide that is reported to influence neuronal vulnerability to a variety of insults. To test if regulated changes in NPY expression contribute to changes in neuronal vulnerability with ampakine treatment, experiments were first conducted to determine

if the ampakines increase NPY expression in cultured hippocampal slices. Using in situ hybridization analysis, we found that spaced ampakine treatments elicited broadly distributed increases in NPY mRNA in hippocampus (pyramidal cells, granule cells, and interneurons) and by neurons distributed across all layers of neocortex (Figure 3(b)). NPY expression was elevated within 24 h of treatment onset and persisted through 4 days. Cotreatment with the ampakine ($3\,h\,day^{-1}$) and BDNF antagonists (continuous) completely blocked increases in NPY expression, demonstrating that this response is BDNF-mediated.

These results show that the ampakine treatments that reduced vulnerability to OGD, increased hippocampal NPY content well before the OGD insult. This result raises the possibility that BDNF-induced increases in NPY expression contribute to protection at the time of ischemia. To assess this hypothesis, and determine the relative contributions of BDNF and NPY to neuronal survival, we examined the effects of adding the BDNF scavenger TrkB-Fc or an NPY receptor antagonist to the culture media either throughout the 2 day period of ampakine pretreatment or just 2 h before and during the OGD insult. As shown in Figure 3c, ampakine (CX614) pretreatment alone eliminated OGD-induced cell death. This protection was reversed if either a BDNF signaling or an NPY Y2 receptor antagonist was present during the full pretreatment period, thereby indicating that, in the largest sense, protection was both BDNF- and NPY-mediated. In contrast, with the addition of the same antagonists 2 h before and during OGD, the NPY Y2 antagonist eliminated neuroprotection whereas TrkB-Fc had no significant effect.

These results support the somewhat surprising conclusion that, at the time of insult, protection is mediated by NPY acting through its Y2 receptor, and not by BDNF per se. NPY is localized to presynaptic elements forming axo-axonic contacts in hippocampus; through these contacts and Y2 receptor signaling, NPY is thought to attenuate presynaptic glutamate release. The reduction in glutamate release, in turn, mediates anticonvulsant and, presumably, neuroprotective effects of NPY. Our results indicate that activity-, and therefore seizure-, induced increases in BDNF expression likely go hand-in-hand with increases in the availability and action of NPY in hippocampal and cortical fields. Thus, although seizure-induced increases in BDNF might enhance regional excitability through potentiation of circuit relays, associated increases in NPY would be expected to offset some of these effects on circuit excitability through attenuation of transmitter release, and thus to protect cells from excitotoxic insult.

Future Goals

Evidence that BDNF is positively regulated by neuronal activity, and massively induced by epileptiform-like discharges, raised questions about its roles in plasticity and the network level changes that follow seizures. Studies from several groups established that BDNF lowers the threshold and increases the magnitude of LTP, but left unsettled the issue of how such effects might be achieved. The results described above provide a partial answer: BDNF (i) attenuates after-hyperpolarizing currents and thereby augments the repetitive EPSPs used to induce LTP, and (ii) via a quite different mechanism, potently facilitates activity-induced remodeling of the spine cytoskeleton and thus the stabilization of synaptic changes. The neurotrophin also affects other LTP-related events such as calcium influx and local protein synthesis. A critical objective for future research is to determine if these seemingly diverse effects are in fact interrelated and perhaps even sequential. There is good evidence, for example, that calcium contributes to cytoskeletal reorganization, which in turn has been linked to the initiation of dendritic protein synthesis. It is thus possible that released BDNF causes a relatively discrete set of early changes that, acting in concert with other signaling cascades set in motion by patterned afferent activity, modify the immensely complex apparatus that governs spine actin networks and local translation. Better understanding of these points is a likely prerequisite for formal hypotheses about BDNF's contributions to the long-lasting changes produced by seizure episodes.

The above observations have also prompted efforts to exploit the activity-dependent regulation of BDNF for the development of novel therapeutic strategies. Our work in this area showed that increasing endogenous BDNF, even by relatively modest amounts (+40 to 60%) rescues LTP in animal models of cognitive impairment. Increases in BDNF expression are far greater with seizures, and such changes could promote synaptic plasticity for days after a seizure. An effect of this kind might serve to partially offset the numerous postepileptiform events that depress LTP-like changes. Alternatively, there is the possibility that excessive levels of neurotrophin have effects on the spine cytoskeleton and dendritic protein synthesis that interfere with the production of long-lasting increases in synaptic strength. An important objective for future work is to test how partial blockade or sequestration of BDNF affects the threshold and magnitude of LTP in slices prepared from rats with recent limbic seizure activity.

Finally, studies showing that increased levels of endogenous BDNF reduce vulnerability to ischemic insult, via secondary increases in the expression of NPY, have important implications for epilepsy research. As suggested by a number of investigators, BDNF could potentially promote epileptogenesis because of its positive influence on LTP; the established anticonvulsant actions of NPY have the potential to offset such effects. A prominent goal for future research is to determine if pharmacologically induced increases in BDNF are reliably linked

to elevated levels of NPY, and then to test the significance of this link for seizure proneness. Given that we have been able to chronically increase BDNF levels using short half-life ampakines, it would be possible to carry out such tests under conditions in which the neurotrophin (and NPY) are present at high levels, but the inducing drug has long been cleared from the brain. Ampakine drugs are under development by several pharmaceutical companies and have been successfully used in clinical trials without evident side effects. Work on the above noted goals could thus have significant therapeutic implications with regard to the treatment of epilepsy.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Function in Temporal Lobe Epilepsy; **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; **Neurotrophic Factors**: Neuroprotective Strategies in Epilepsy; Role of BDNF in Animal Models of Epilepsy; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures.

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Fibroblast Growth Factor-2

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Introduction

Seizures can induce acute and delayed cellular alterations that may favor development and/or progression of epilepsy, including neuronal loss, neurogenesis, axonal and dentritic sprouting, gliosis and, ultimately, circuit remodeling. Neurotrophic factors are well-known physiological mediators in each of these cellular events, and thus they have been hypothesized to be involved in the pathogenesis of some types of epilepsy. Consistent with this idea, the expression of many neurotrophic factors has been reported to be altered in diverse, broadly distributed neuronal populations during epileptogenesis and following seizures.

Together with other neurotrophic factors, FGF-2 seems a good candidate for a role in the plastic changes associated with epilepsy. As described earlier, it regulates neurogenesis, cell survival, axonal sprouting, and synaptogenesis. Furthermore, as detailed in the following sections, (1) seizures increase FGF-2 mRNA and protein levels in specific brain areas and upregulate the expression of FGFR-1 receptors; (2) acute intrahippocampal injection of FGF-2 causes seizures, while chronic i.c.v. infusion of low dose FGF-2 does not affect kainate-induced seizures, but does promote behavioral recovery and reduces hippocampal damage; (3) seizure severity (in response to kainate) is not altered in FGF-2 knockout mice, but is increased in FGF-2 over-expressing mice; and (4) seizure-induced neuronal damage is attenuated in FGF-2 over-expressing mice.

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polypeptide composed of 146 amino acids. In humans and rodents, FGF-2 exists in multiple isoforms generated from a single mRNA by alternative translation. In the rodent brain, three high molecular weight (HMW; 22, 22.5, 24 kDa) and one low molecular weight (LMW; 18 kDa) form have been identified. The HMW forms contain a nuclear targeting signal and are predominantly localized in the nucleus, where they are thought to serve intracrine functions, to regulate gene transcription and activate programs for plasticity and proliferation. The LMW form is cytosolic, secreted, and capable of activating cell surface FGF receptors (FGFRs) – functioning in an autocrine and/or paracrine manner. Thus, different molecular weight proteins have different subcellular localizations and appear to serve different biological functions.

FGF-2 Receptors

FGFRs can be subdivided in four high- and one low-affinity receptor. The low-affinity receptor is a heparan sulfate proteoglycan. It seems to be critical for FGF signaling, because enzymatic removal of heparin/heparan sulfate from the cell surface or inhibition of sulfation abolishes the biological activity of FGFs. It has been proposed that heparan sulfate may facilitate activation of the high-affinity receptors.

The high-affinity FGFRs (FGFR-1 through FGFR-4) show substantial sequence homology and are similar in their general structure: they all are tyrosine kinase receptors, with one membrane spanning domain and two or three extracellular immunoglobulin-like domains. Alternative RNA splicing produces several different splice variants for each receptor mRNA. Binding of FGFs to these receptors leads to dimerization of receptor monomers, to activation of intracellular kinase domains, and to receptor transphosphorylation at multiple tyrosine sites. A number of Src-homology 2 (SH2) domain-containing signal transduction molecules can bind to these phosphotyrosine sites, potentially activating several signaling pathways, including the Ras/mitogen-activited protein kinase (MAPK) and the phospholipase Cγ (PLCγ). Depending on the activated receptor subtype and signaling pathway, different cellular responses may be produced. Apart from initiating tyrosine kinase signal transduction pathways, FGF-2 and FGFRs may internalize and translocate to the nucleus, influencing the cell cycle machinery. FGF-2 binds FGFR-1 with the highest affinity among high-affinity FGFRs.

Localization

FGF-2 has been found throughout the central nervous system, including the spinal cord, brainstem, midbrain, and forebrain (both subcortical and cortical regions). In the adult, FGF-2 mRNA and protein are present in the astrocytes of all these brain regions, and also in selected neuronal populations, most prominently in the pyramidal neurons of the CA2 hippocampal region and in neurons of the retrosplenial cortex.

Cellular Effects of FGF-2

FGF-2 is a pleiotropic factor that exerts its effects in different cells and organs. For example, it is a potent chemotactic factor for fibroblast and endothelial cells, and is a potent angiogenic molecule that stimulates wound healing and tissue repair via direct actions on endothelial and smooth muscle cells.

With reference to epilepsy, however, the most interesting effects of FGF-2 are neurotrophic. FGF-2 can regulate the proliferation and differentiation of neural stem cells and progenitors. Furthermore, it enhances the resistance to damage, survival, axonal branching, and synaptogenesis of mature neurons. FGF-2 acts as a neurotrophic factor both in the developing and in the adult nervous system. During development, it is thought to play a critical role in neurogenesis as well as in cell–cell signaling between neurons, astrocytes, and microglia. In the adult brain, it is involved in the regulation of plasticity and in the maintenance of structural integrity.

Proliferation and differentiation

FGF-2 is a potent mitogen, not only (as originally discovered) for fibroblasts, but also for a variety of cells of mesodermal and neuroectodermal origin. In vitro, exogenously administered FGF-2 exerts mitogenic and differentiative effects on embryonic hippocampal neurons in culture, stimulates proliferation of cortical precursor cells, and (with other growth factors) regulates the generation of neurons and astrocytes from progenitor cells. FGF-2 also favors the differentiation of cultured neonatal dentate granule cells. Different concentrations of FGF-2 may influence the fate of early cortical stem cells: low FGF-2 levels predominantly lead to development into neurons, whereas higher levels lead to generation into glia as well as neurons.

Similar effects have been observed in vivo. Injection of an antibody against FGF-2 in the neonatal rat brain inhibits neurogenesis; conversely, a single subcutaneous FGF-2 injection in neonatal rats elicits a significant increase in hippocampal cell number. The mitotic effects of exogenous FGF-2 persist in the adult brain in the regions (subgranular zone of the dentate gyrus and subventricolar zone) where neurogenesis persists during adulthood.

The mitogenic and differentiating effects of FGF-2 appear to vary depending on the specific developmental stage and on the cell type. It seems likely that different FGF-2 isoforms and/or receptor subtypes, as well as site- and time-specific synergies with other neurotrophic factors, account for this complexity of action.

Neuroprotection

A protective effect of FGF-2 has been documented in vitro. FGF-2 has been found to attenuate glutamate-induced death in cultures of neurons derived from many different brain regions. Since many of these preparations are essentially glia-free, these data suggest that at least part of the neuroprotective effect is exerted directly on neurons.

FGF-2 promotes neuronal survival and recovery against a remarkably wide variety of insults, reducing brain cellular damage and improving functional recovery in experimental models of stroke, of chemical, mechanical, and traumatic brain and spinal cord injury, and of epilepsy. In rats subjected to focal ischemia, for example, intravenous FGF-2 produces a significant reduction in cortical infarct volume and a marked improvement in rotarod performance. FGF-2 also increases long-term survival of spinal motor neurons and improves respiratory function after experimental spinal cord injury. More interestingly in the context of the present volume, chronic i.c.v. administration of FGF-2 protects against seizure-induced cell loss and reduces long-term behavioral deficits. Conversely, recovery from injury is impaired when neutralizing antibodies were used to prevent FGF-2 from activating its receptors during the postinjury period.

Neurite outgrowth and synaptogenesis

FGF-2 also produces morphogenic effects in mature neurons. It accelerates bifurcation and growth of axonal branches without affecting the elongation rate of primary axons in cultured rat hippocampal neurons, and it enhances neurite growth in hippocampal and cortical neurons. A greater number of axon branches is expected to produce a greater number of synaptic contacts, and indeed, local application of FGF-2 has been found to increase (in a concentration-dependent manner) the number of excitatory synapses between hippocampal neurons; these synapses are morphologically mature and functionally active.

Methods: Experimental Analyses

Gene Expression Studies

FGF-2 gene expression is induced with similar patterns in different acute seizure models: increased FGF-2 mRNA levels have been observed after application of bicuculline, lesion of the dentate gyrus hilus, kainate, and electroconvulsive shock. This phenomenon is marked and transient, peaking at 6–24 h in different hippocampal subfields and in the cortex. Increased levels of FGF-2 mRNA are observed in different regions (including neocortex, amygdala, and septum), but most prominently in the hippocampus. As described earlier, FGF-2 is expressed diffusely in astrocytes, and also in CA2 pyramidal neurons in the hippocampus of naive rodents. Following seizures induced by kainate or hilar lesion, increased FGF-2 mRNA levels have been observed in these cell populations, as well as new expression in CA1 pyramidal neurons and in dentate gyrus granule cells.

In the kindling model, induction of FGF-2 mRNA expression is observed in the hippocampus, neocortex, and hypothalamus; curiously, this expression is more pronounced after a single afterdischarge (not accompanied by behavioral seizures) than after a fully kindled, generalized tonic–clonic seizure lasting more than a minute. A higher responsiveness to low-level, compared with maximally-effective, stimulations has also been observed in the hippocampus after minimal, as compared with maximal electroshock, suggesting that the duration and intensity of seizures within a specific area does not necessarily correlate with the magnitude of FGF-2 mRNA level increase.

The induction of FGF-2 mRNA is followed by an increase in the protein levels. In the kainate model, FGF-2-like immunoreactivity is detectable 6 h after injection of the convulsant agent and peaks at 24 h, being mainly localized in the nuclei of astrocytes. After electroconvulsive shock, FGF-2 protein expression increases in the frontal and rhinal cortex, peaking at 20 h, and still elevated at 72 h. The HMW isoforms of FGF-2 appear to be produced more than the LMW isoform. In one study, FGF-2 protein levels were found to remain elevated in astrocytes up to 30 days following kainate-induced seizures, suggesting that the transient pattern of FGF-2 mRNA elevation has prolonged translational effects that may be capable of influencing long-term plasticity changes.

Seizure-induced changes in the expression of only two (FGFR-1 and FGFR-3) of the four high-affinity tyrosine kinase FGFRs have been studied in the kainate model. Seizure activity modulates FGFR-1 mRNA expression: in situ hybridization reveals increased FGFR-1 mRNA levels in the cell body of granular and pyramidal neurons, up to 24 h after kainate injection. At the protein level, FGFR-1 positive cells appear first in the molecular layer of the dentate gyrus after kainate seizures, then in other hippocampal subfields, and finally (by 24 h) very diffusely throughout the hippocampus and the cerebral cortex. Thirty days after kainate injection, strong FGFR-3 immunopositivity has been found in OX-42-positive reactive microglia in several brain areas, including hippocampus, amygdala, piriform, and entorhinal cortex.

In spite of some minor discrepancies between different models, these observations lead to the notion that seizures coordinately increase the expression of FGF-2 and its receptor(s), potentiating it signally potential in several brain regions. Interestingly, the ability of experimental seizures to produce damage is not essential for induction of FGF-2 expression; activation of the FGF-2 system can be also observed after relatively mild, noninjurious seizures. In other words, not only the actual production

of injury, but even the simple threat or anticipation of injury, may be sufficient for potentiating the endogenous FGF-2 system.

The conditions that have been found to trigger FGF-2 expression encompass a wide spectrum, ranging from physiological to pathological. At one end of this spectrum is sustained neuronal activity and at the other end is injury and cell death; in between, there are a number of other conditions, like stress and noninjurious seizures, that induce FGF-2 expression. Accordingly, the roles that newly-produced FGF-2 may exert appear to span in a wide range, from activity-dependent plasticity to resistance to damage to rescue, repair, and regeneration.

In the frame of epileptic conditions, potentiation of the FGF-2 system is likely to occur as part of an adaptive response to excessive activation of specific neuronal circuitries as well as a response to seizure-induced damage. The hypothesis that FGF-2 takes part in the plastic changes associated with epilepsy has been investigated using pharmacological and genetic tools.

Pharmacological Studies

Injection of FGF-2 into the dentate region of the ventral hippocampus causes an excitatory effect culminating in electrographic and behavioral seizures. This acute effect appears to be dose-dependent, with the percentage of seizing animals and the duration of seizures greater after injections of 50 than of 25 ng FGF-2. These acute electrical alterations of hippocampal activity cannot be ascribed to trophic effects, and have been proposed to result from a fast signal transduction mechanism leading to changes in excitability.

In contrast with the effects of acute administration, the chronic infusion of relatively low FGF-2 doses ($2.5\,\mathrm{ng\,h^{-1}}$) into the cerebral ventricle does not modify latency and duration of kainate seizures; however, it effectively prevents seizure-induced hippocampal cell loss and improves long-term behavioral recovery. This neuroprotective action of FGF-2 may depend on potentiation of Ca^{2+}-dependent inactivation of *N*-methyl-D-aspartate (NMDA) currents in hippocampal neurons, an effect produced by chronic (hours), but not by acute, treatment with FGF-2. Another possible mechanism of this effect may be the induction of activin A (ActA), a cytokine belonging to the transforming growth factor-beta superfamily. When coinjected with kainate in the hippocampus, FGF-2 prevents the loss of CA3 neurons in mice. In mice treated with kainate and FGF-2, but not in those treated with kainate alone, ActA-immunoreactivity was found to be high in CA3 as well as in CA1 and CA2 neurons. Critically, FGF-2 fails to protect CA3 neurons against kainate-induced death in presence of the ActA-neutralizing protein, follistatin.

A neuroprotective effect of FGF-2 has also been demonstrated in a quite different experimental situation – the grafting of fetal cells in the sclerotic hippocampus. Cell transplantation may offer a new therapeutic approach to reduce hippocampal cell loss, to prevent acute or delayed plastic brain alterations, and to restore the disrupted circuitries in the seizure-injured or epileptic hippocampus. Pretreatment of CA3 fetal hippocampal cells with FGF-2, alone or together with other trophic factors, is associated with a robust increase in long-term cell survival and enhanced graft neuronal integration in the injured hippocampal region. The efficacy of this cell grafting strategy appears to depend on the trophic action of FGF-2.

Genetic Studies

The generation of mice with homozygous deletion of the FGF-2 gene (FGF-2 knockout mice), and with a species-specific transgene inserted in the genome (human FGF-2 transgenic mice), has provided new tools to study the molecular, physiological, and pathological roles of FGF-2 in vivo. The FGF-2 knockout mice are viable, fertile, and without any obvious phenotypical difference from their wild-type (WT) littermates. However, these mice exhibit a significant reduction in the number of neurons in the neocortex, in particular in the motor areas. Furthermore, they display delayed wound healing. The susceptibility to seizures of FGF-2 knockout mice has been studied in the kainate model. The severity of kainate seizures is not different between knockout and WT mice. Under resting conditions, neurogenesis in the dentate gyrus is not significantly different between FGF-2 knockout and WT mice. In response to kainate seizures, however, an increase in FGF-2 protein levels and neuroproliferation were observed in the hippocampus of WT mice, while very low levels of neurogenesis were observed in the knockouts. Using a viral gene delivery system, high FGF-2 levels can be restored in the mutant mice, also restoring levels of neurogenesis comparable to those of WT littermates. These data suggest that seizure-induced FGF-2 expression is necessary to prime proliferation and differentiation of progenitor cells in the adult hippocampus.

Recent Results

In an attempt to further elucidate the effect of FGF-2 in epilepsy, we studied transgenic mice expressing the human FGF-2 (TgFGF2 mice). These mice have been previously characterized molecularly and phenotypically. Western blot analysis has shown that the murine and human FGF-2 isoforms are tissue-specifically expressed and translationally regulated. In gross examination, these transgenic mice are often affected by skeletal malformations (such as shortening and flattening of long bones) and moderate macrocephaly. In addition, without having any spontaneous or inherent vascular defect, they exhibit a

predisposition to angiogenic reactions with subsequent amplified angiogenesis.

The human FGF-2 transgene is highly expressed in the brain of these mice. Based on in situ hybridization and immunohystochemistry, we found that TgFGF2 mice express FGF-2 mRNA and protein in all hippocampal principal neurons (pyramidal and granule cells), not only in CA2 pyramidal neurons as in WT animals. This pattern of overexpression is remarkably similar to the one caused by seizures in WT animals (above).

These mice that 'overexpress' FGF-2 at baseline have a latent epileptic phenotype (**Figure 1(a)**), in that latency to generalized seizure onset is reduced, and behavioral seizure scores and lethality are increased. Some of the pathological sequelae of seizures (namely increased neurogenesis, cell damage, and sprouting of the mossy fibers) were also examined. First, in spite of the fact that FGF-2 is one of the most potent mitogenic factors for hippocampal neural stem cells, its over-expression in FGF-2 transgenic mice did not lead to increased neurogenesis under basal conditions or after kainate-evoked seizures. Under basal conditions, the number of BrdU immunoreactive cells in the dentate gyrus (an index of neuroregeneration) did not differ between FGF-2 transgenic and WT mice. Seizures increased cellular proliferation in a seizure severity-dependent manner (that is, the number of BrdU-positive cells increases as a function of seizure severity). However, seizures of similar grade increased the number of BrdU-positive cells in a similar manner in the two experimental groups. Together with the earlier-described data showing that seizure-induced neurogenesis is abolished in FGF-2 knockout mice, the data suggest that FGF-2 is necessary, but not alone sufficient, for seizure-induced neurogenesis.

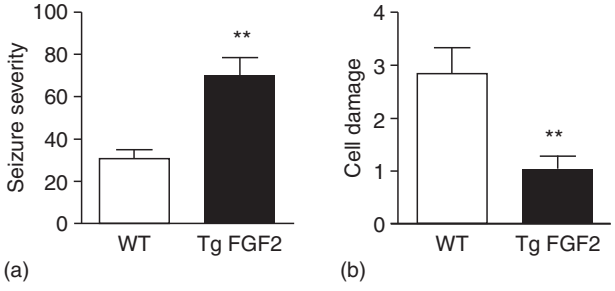


Figure 1 (a) Kainate seizure severity in wild-type (WT) and FGF-2 transgenic mice (TgFGF2). Seizure severity was scored according to a published scale (cumulative seizure score) and refers to the 2 h of behavioral observation following kainate injection. Data are the mean ± SE of 20 animals per group. $^{**}P < 0.01$ versus WT; Mann–Whitney *U* test. (b) Quantification of kainate-induced neurodegeneration in the hippocampus of WT and FGF-2 transgenic mice (TgFGF2) following seizures of similar grade (cumulative seizure score of 50). The degree of neurodegeneration was scored according to a published scale (degeneration index). Data are the mean ± SE of six animals per group. $^{**}P < 0.01$ versus WT; Mann–Whitney *U* test.

Second, neuroprotection was explored using several markers of cell death and apoptosis: Fluoro-Jade B labeling, silver impregnation, active caspase 3 immunohistochemistry. Following kainate-induced seizures of similar severity, we observed greater cell damage in WT than in FGF-2 transgenic mice (**Figure 1(b)**). In WT animals, Fluoro-Jade B labeling displayed the typical pattern of neurodegeneration associated with kainate seizures, involving different hippocampal subfields (CA1, CA3, CA4) and extrahippocampal regions like thalamus and cortex. In contrast, in transgenic mice that underwent severe kainate seizures, Fluoro-jade B-positive neurons were observed only in the hilar dentate gyrus region. These data are in good agreement with the neuroprotective action of FGF-2 in injury models. We hypothesize (see above) that seizures prompt the overproduction of FGF-2 in astrocytes and neurons; in turn, this newly produced FGF-2 enhances the production of ActA in selected neurons. In the hippocampus, ActA may reach high levels in CA1 and CA3 neurons, protecting these cell types from injury. Accordingly, we observed in TgFGF-2 mice the preservation from degeneration of CA1 and CA3 neurons, but not of neurons in the hilus of the dentate gyrus. Further experiments are in progress to challenge this hypothesis.

Third, we examined a long-term seizure-induced synaptic rearrangement, the sprouting of mossy fiber axons. Under control condition, neither control nor FGF-2 overexpressing mice display sprouting of the mossy fibers (as detected using the Timm staining). Thirty days after kainate seizures of similar grade, a similar degree of sprouting was observed in transgenic and in control mice. Thus, the overexpression of human FGF-2 does not seem to be sufficient to influence seizure-induced morphological reorganization.

Therefore, the latent epileptic phenotype of FGF-2 overexpressing mice cannot be explained on the basis of effects on neurogenesis (not altered in the mutant mice), cell death (not altered under basal conditions, and actually reduced after kainate seizures), or sprouting of the mossy fibers (not altered). Another hypothesis to explain the latent epileptic phenotype focuses on the ability of FGF-2 to increase the number of excitatory synapses. To explore this possibility, we have investigated the effect of FGF-2 overexpression on excitatory synaptic transmission. To estimate the density of excitatory terminals, we evaluated the expression of the glutamatergic vesicular transporter VGLUT1. A clear enhancement of VGLUT1 immunoreactivity was observed in the hippocampus of FGF-2 transgenic compared with WT mice. In contrast, no differences were observed in the density and distribution of VGAT, the vesicular transporter of GABA and a marker of GABAergic terminals.

To explore the functional correlates of these observations, we compared the basal properties of the Schaffer collateral-CA1 synapse in hippocampal slices. Extracellular field potential recordings in the area CA1 have been

performed to generate input/output (I/O) curves for each mouse strain. We found that the I/O curve was shifted leftwards in FGF-2 transgenic compared with WT mice, indicating increased excitability in the former.

Furthermore, in an attempt to trigger release from only one axon connected to the neuron under recording, and to measure changes in neurotransmitter release due to alterations in the quantal content, we used paired-pulse minimal stimulation of Shaffer collaterals to evoke EPSCs in CA1 pyramidal neurons. When pairs of EPSCs were elicited every 60 s, at intervals of 50 ms, the mean amplitude of the second EPSC (EPSC2) was larger than EPSC1, an effect known as paired-pulse facilitation (PPF). If the fluctuation in the amplitude of EPSC1 results from changes in its quantal content, then the amplitude of the second response is inversely related to the quantal content of the first response; that is, increased probability of glutamate release results in a decrease of PPF. In slices prepared from FGF-2 transgenic mice, the mean amplitude of EPSC1 was larger and EPSC2 was smaller than in WT. These results suggest an increase in the amount of neurotransmitter released in TgFGF-2 mice due to an increase of the quantal content in the Schaffer collaterals. Thus, FGF-2 transgenesis either increases the number of vesicles per glutamatergic terminal or the number of terminals per axon. Although our data do not allow distinction between these two hypotheses, the evidence (see above) that FGF-2 has synaptogenetic effects strongly supports the latter. Together, these data suggest that an anomalously increased excitatory synaptic input may cause an increased propensity to seizures in TgFGF2 mice.

Future Goals

In conclusion, the experimental evidence presented in this article suggests the following: (1) seizures (most notably epileptogenic seizures) induce the synthesis of FGF-2 in astrocytes and neurons constitutively expressing its gene, and ectopically in neurons that do not normally produce this neurotrophic factor; (2) pharmacological and genetic data suggest that increased availability of FGF-2 increases seizure susceptibility, but reduces seizure-induced cell death.

The multiple effects of FGF-2 (increased hippocampal excitability and reduced cell death) have apparently contrasting physiopathological implications, like increased susceptibility to seizures but decreased seizure-induced cell death and, thus, may depend on different mechanisms (for example, on different isoforms or different receptor subtypes). Identifying these mechanisms will be critical in pursuing therapeutic goals (controlling seizures and their deleterious consequences) through modulation of the FGF-2 system.

See also: **Neurotrophic Factors**: GDNF: A Neurotrophic Factor Against Epilepsy; The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy; **Peptides**: Dynorphin and Nociceptin/Orphanin FQ; **Plasticity**: Inositol Lipid Signaling in Synaptic Activity, Neuronal Plasticity and Epileptogenesis; Seizure-Related Changes in Dendritic Spine Structure.

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GDNF: A Neurotrophic Factor Against Epilepsy

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Introduction

The glial cell line-derived neurotrophic factor (GDNF) is a member of the transforming growth factor-beta superfamily. Initially GDNF was discovered as a survival factor for dopaminergic neurons. Subsequently, motoneurons and noradrenergic neurons were found to be sensitive to GDNF for their survival. Parkinson's disease is a primary candidate for GDNF-based treatment, but stroke and motoneuron injury are also being considered. Members of the GDNF family include neurturin, artemin, and persephin. All these proteins have their selective receptors (i.e., GFRa1, 2, and 3), which as a complex bind to a common transmembrane receptor tyrosine kinase (RET) to induce signaling.

Wide distribution of GDNF and its receptors in the rat and human central nervous system and their regulation by seizures suggested a role for GDNF in seizure activity. It has also been demonstrated that intraventricular infusion, as well as adenoviral vector-mediated overexpression of GDNF in the hippocampus, suppresses generalized seizures and kindling epileptogenesis. Most recently, we have shown that hippocampal GDNF overexpression, mediated by an adeno-associated viral vector, could suppress seizure activity in various electrically induced animal models of epilepsy.

Background

Gene transfer has developed into one of the main experimental tools in neuroscience to explore and to understand the functions of various proteins. This technology is designed to manipulate gene expression in large populations of neural cells, in dissociated and organotypic slice cultures, and in vivo. Moreover, gene transfer is beginning to be used for therapeutic purposes. Recombinant viral vectors provide one of the most efficient methods of gene transfer in the CNS.

The two main routes to transfer genes to the CNS, the indirect (ex vivo) and direct (in vivo), have their advantages and disadvantages. The direct in vivo gene transfer, by recombinant viral vectors such as adenoviral vectors, adeno-associated viral vectors, and lentiviral vectors, entails transduction of nondividing resident host cells and modifying them genetically, with long-lasting and nonreversible expression of the transgene. In ex vivo gene transfer, the vector is first delivered to a cell line in vitro, and these cells are then transplanted into the brain. In this case, there is no integration of foreign DNA causing a genome dysregulation of the host cells. Further, the special case of encapsulated cell biodelivery (ECB) provides an additional safety aspect to the procedure, as it is possible to remove the cell-containing capsules in case of unwanted side effects. Both direct (in vivo) and indirect (ex vivo) gene transfers have been used to control epileptic seizures in animal models with variable success, depending on the targeted genes and cells.

Methods

In our laboratory, we use recombinant adeno-associated viral (rAAV) vectors to transfer GNDF gene into the rat brain, particularly into the hippocampus. rAAV is produced according to the standard procedures, and is obtained through a collaboration with Dr. Deniz Kirik from Wallenberg Neuroscience Center, Lund. In brief, a chicken β-actin promoter, a hybrid between a cytomegalovirus enhancer element and a chicken β-action promoter, was driving the expression of the GDNF or the GFP gene. Vectors further contained a woodchuck hepatitis virus post-transcriptional regularity element downstream of the transgene. The injection procedure is as follows: The animals are anaesthetized and placed in a stereotactic frame; a hole is drilled in the skull according to the preselected coordinates, and the glass pipette filled with viral vector solution is inserted into the brain through the skull hole. Injection of 1 μl solution is performed over several minutes, and the pipette is left in position after the injection for another few minutes to prevent back-flow of the injected viral vector into the pipette tract.

To test the seizure-suppressant effect of such gene transfer, we use various models of epilepsy, such as electrical kindling and electrical status epilepticus. In kindling, initially focal seizures, induced by daily stimulations, gradually generalize and can be scored by behavioral seizure manifestations. Once animals reach a generalized seizure stage, stimulations that initially induced only focal seizures induce generalized convulsion, and animals are said to be kindled. This process of gradual generalization of seizures is considered as a model of epileptogenesis, which can be studied experimentally. In our status epilepticus model, we use 10 s trains of 1-ms duration square-wave pulses (at 50 Hz) over the course of 1 h, interrupted every 10 min by 1-min EEG recording. Such

stimulation leads to self-sustained status epilepticus-like activity, and after a few weeks, results in the development of spontaneous generalized seizures in these animals.

Characterization of the tissue is performed at the end of these experiments using conventional immunocytochemistry and general tissue staining methods. The role of neurogenesis in the effects of GDNF is evaluated by intraperitoneal injections of BrdU and other conventional immunostaining methods (e.g., immunostaining for DCX).

Another approach for ex vivo gene therapy is to implant an encapsulated GDNF-producing cell line. For this purpose, we use Encapsulated Cell Biodelivery (ECB) devices from NsGene A/S, Denmark. These devices consist of a semipermeable membrane, which allows exchange of extracellular fluids across the mebrane, and delivery of the cell secretion products into the surrounding host tissue. The insertion of the ECB devices into the brain is performed via preimplanted canulas.

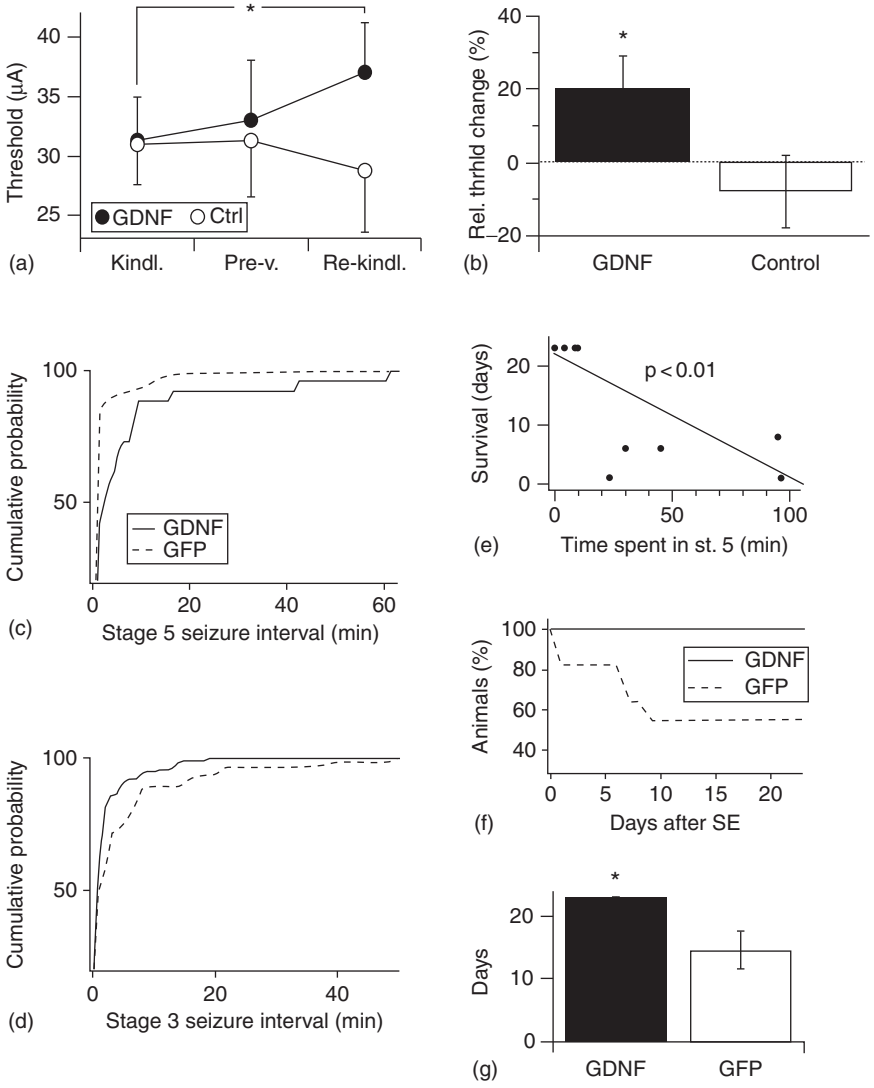


Figure 1 Transgene GDNF increases the seizure-induction threshold in kindled animals, and decreases severity of SE seizures and survival rate of animals. (a) Relative change of the threshold from kindling to 3 weeks post-virus (Post-v.), i.e., injection of rAAV–GDNF (* indicates $p < 0.05$ for the absolute threshold change, Student's paired *t*-test). (b) Similar to (a) in rAAV–GFP and vehicle treated controls. Dashed lines represent values of individual animals, thick lines show group averages. rAAV–GDNF, $n = 10$ and control, $n = 8$ (rAAV–GFP, $n = 5$ and vehicle $n = 3$). (c) The cumulative probabilities of generalized stage 5 and (d) focal stage 3 seizures during the self-sustained phase of SE. (e) The number of animals (as percentage of the initial group size) surviving at the days following SE. (f) The correlation between the number of stage 5 seizures during SE and the survival time of GFP expressing animals (corr. coefficient $r = -0.7$, $p < 0.01$). (j) The survival time (in days) following SE (*$p < 0.05$, Student's unpaired *t*-test). rAAV–GDNF, $n = 10$ and rAAV–GFP, $n = 11$. Error bar is mean ± SEM. Reproduced from Kanter-Schlifke I, Georgievska B, Kirik D, and Kokaia M (2007) Seizure suppression by GDNF gene therapy in animal models of epilepsy. *Molecular Therapy* 15: 1106–1113.

Recent Results

The main findings of our studies demonstrate that the rAAV–GDNF vector does transduce hippocampal cells, GDNF is produced and released in the whole hippocampus, and has inhibitory effect primarily on generalized seizures. Moreover, seizure induction threshold and mortality after status epilepticus are decreased in treated animals. These results are summarized in Figure 1.

To apply these findings in more clinically safe and relevant delivery systems, we have used GNDF-producing encapsulated cells transplanted into the hippocampus

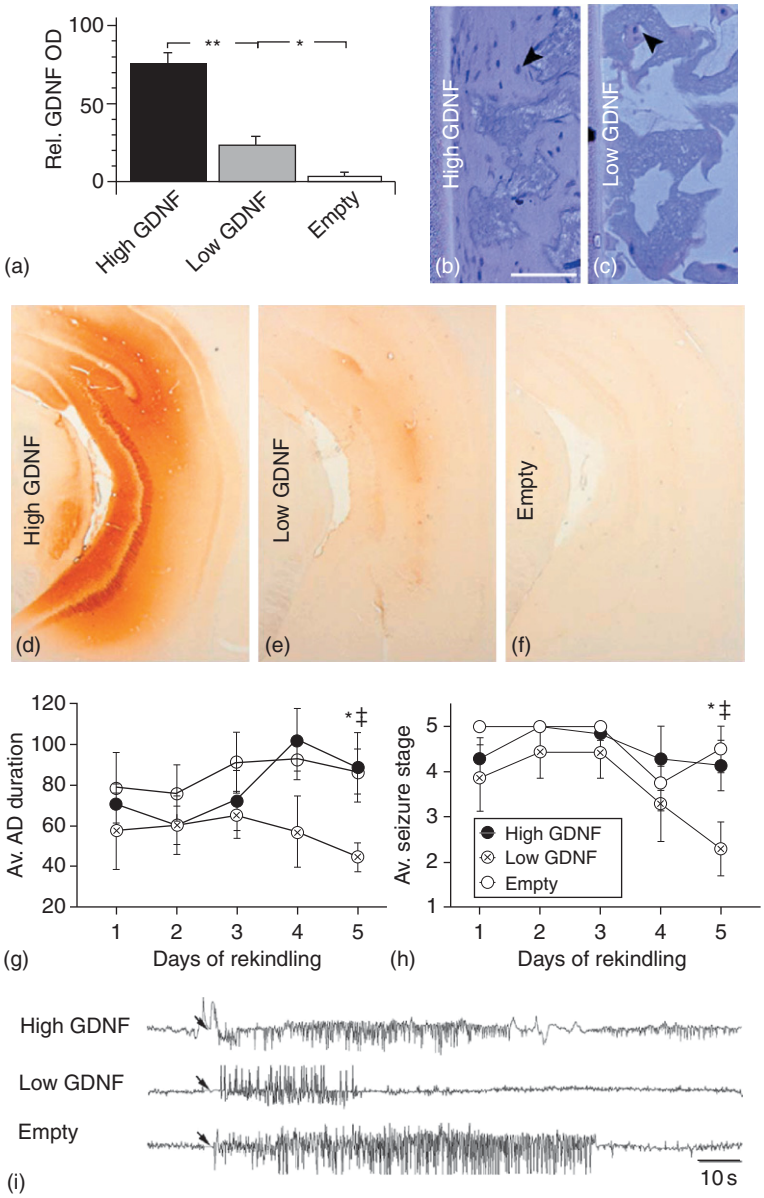


Figure 2 GDNF release from ECB devices in vitro and in vivo, and its seizure-suppresses activity in rekindling. (a) Relative optical density (OD) of GDNF-IR in the hippocampus of animals assigned to the three experimental groups. OD was measured adjacent to the device implantation site, and values are averaged for left and right portion of the hippocampus ($n = 7$ for high and low GDNF, and 4 for empty, respectively, $^{**}p < 0.01$, $^{*}p < 0.05$). (b) Example of an ECB device of the high GDNF group containing some living cells. (c) Example of an ECB device of the low GDNF group containing hardly any living cells. Arrowheads in (d) and (e) indicate viable cells. GDNF-IR in the hippocampus of an animal with high (d) or low (e) GDNF release from ECB devices, and in the hippocampus of an animal implanted an empty ECB device (f). Scale bar are 100 μm in (b, c) and 1 mm in (d, e). (g) The average duration of afterdischarges (ADs) on the five days of rekindling. (h) The average seizure stage on the 5 days of rekindling. Stars indicate $p < 0.05$ for low GDNF compared to high GDNF, and ‡indicates $p < 0.05$ for low GDNF compared to empty, respectively. Representative EEG traces of ADs are shown in (i). Arrows indicate the electrical stimulation and the beginning of epileptiform EEG activity. Reproduced from Kanter-Schlifke I, Fjard-Larsen L, Kusk P, Angehagen M, Wahlberg L and Kokaia M (2009) GDNF released from encapsulated cells suppresses seizure activity in the epileptic hippocampus. *Experimental Neurology* 216: 413–419.

(ECB devices provided by NsGene A/S, Denmark). These experiments showed that such an approach is also effective in decreasing seizure severity and duration in kindled animals, and that the effect is dependent on the relative amount of GDNF released from these capsules. Unexpectedly, lower levels of released transgene GDNF seemed to be more effective rather than higher levels, as assessed by the relative intensity of GNDF immunoreactivity in the host hippocampal tissue surrounding the transplants (**Figure 2**).

We then explored some possible mechanisms that could underlie the seizure-suppressant effect of transgene GDNF. Among others, increased expression of neuropeptide Y (which has been shown to be induced, e.g., by BDNF exposure), decreased GFRalpha-1 receptor, and altered neurogenesis have been explored. However, none of these mechanisms seems to be critically responsible for transgene GDNF effects.

Future Goals

What remains to be explored is whether transgene GDNF can alter synaptic transmission in the hippocampus (directly or indirectly). It has been shown to upregulate glutamate transporters, in particular GLAST and GLT-1, which would result in more effective removal of elevated extracellular glutamate in chronic models of epilepsy. More directly, GDNF could modulate excitatory or inhibitory synaptic transmission in the brain, as shown in studies of GDNF effects on myocytes or cultured midbrain neurons. To achieve these goals, specific immonstainings and whole-cell patch clamp techniques will be used. Such experiments will reveal the mechanisms behind GDNF antiepileptic activity and will open possible novel research perspectives in using GDNF as a potential target molecule in gene therapy for epilepsy.

See also: **Antiepileptic Drugs**: Local Delivery of Antiepileptic Drugs; **Neurogenesis**: Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; **Neurotrophic Factors**: Fibroblast Growth Factor-2; **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides.

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The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy

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Introduction

The idea that the hippocampus is related to epilepsy, specifically temporal lobe epilepsy, has a long history of support from basic and clinical studies. One of the seminal questions is why the hippocampus appears to be 'seizure-susceptible' – in other words, why epileptiform discharges are readily evoked, and seizures often begin, within this structure. One hypothesis is that the hippocampus is designed to support diverse types of structural and functional plasticity under normal conditions, which is important to hippocampal-dependent functions, such as learning and memory. However, the mechanisms that support plasticity, especially mechanisms that increase glutamatergic transmission and synchronization, may lead to a greater risk of seizures.

Here we examine one of the neurotrophins that is expressed in hippocampus, BDNF. BDNF is one example of a modulator of hippocampal function that needs to 'balanced,' because it can induce plasticity that enhances normal hippocampal function, and it can induce seizures. It may contribute to plastic changes in the brain that are

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Neuroprotective Strategies in Epilepsy

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Introduction

From the earliest attempts to develop treatments for epilepsy, the primary goal of epilepsy therapeutics has been seizure control. Since seizures are not only pathognomonic for epilepsy, but also represent a significant problem for patients, this strategy has been both rational and fruitful. However, in spite of the development of a wide array of anticonvulsant drugs, some patients have poorly controlled seizures while many more report breakthrough seizures. Efforts to seek better anticonvulsant treatments are ongoing, and these efforts have been accompanied by a concurrent effort to protect the brain from the damaging effects of seizures.

Using animal models, epilepsy researchers have discovered that seizures lead to permanent changes in the brain circuitry. Some of these changes, such as mossy fiber sprouting, could potentially be associated with seizure progression. In addition, uncontrolled seizures may cause a loss of brain cells. Humans with chronic temporal lobe epilepsy (TLE) show neuronal loss, sclerosis, and atrophy, especially in the hippocampus. In animal models, the occurrence of severe seizures leads to cell loss in various regions of the brain, especially the limbic system. As illustrated in **Figure 1**, the hippocampus of rodents has proved to be exquisitely sensitive to the excitotoxic cell death induced by seizures, and this cell loss in the animal model reflects the damage profile seen in human TLE. Behavioral studies in animals have also shown that seizure-related cell loss is associated with functional impairments, such as memory decline and emotional deficits, similar to findings in humans where epilepsy has been associated with functional impairments ranging from memory loss to alterations in mood. Protecting neurons in the human epileptic brain may protect against functional impairments.

As a consequence of the realization that seizures beget neuronal loss and behavioral impairment, epilepsy researchers are increasingly interested in pursuing neuroprotective strategies in epilepsy. Protecting neurons from death or functional compromise after seizures would, in the short term, prevent some of the far-reaching consequences of breakthrough seizures. In the longer term, the optimal treatment for epilepsy would simultaneously prevent seizures and protect neurons from the damaging consequences of seizure activity.

Background

Neurotrophic Factors and Neuroprotection

The past two decades have seen a surge in interest in the potential of protein growth factors to protect cells throughout the body. From the perspective of an epilepsy researcher, those protein factors capable of protecting cells in the nervous system, termed neurotrophic factors,

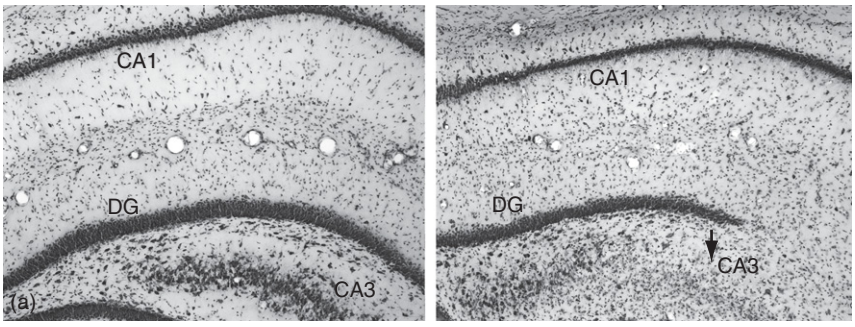


Figure 1 Neuronal loss induced by status epilepticus in rat hippocampus. (a) Photomicrograph of a normal cresyl violet-stained rat hippocampus showing CA1, CA3, and the dentate gyrus granule cell layer (DG). (b) A similar photomicrograph from a hippocampal section taken 24 h after the animal experienced pilocarpine-induced status epilepticus showing severe neuronal loss in CA3 (arrow) and thinning of the cell layer in CA1.

represent promising therapeutic candidates. These proteins – which protect neurons from insults ranging from oxygen deprivation and serum starvation to excitotoxic damage – have moved from the Petri dish into animal models. A number of these factors have been tested in animal models of seizure disorders, with varying degrees of success.

A Tale of Two Factors

Our laboratory has a long-standing interest in the potential of growth factors to protect neurons, and has focused on the potential of two growth factors – brain-derived neurotrophic factor (BDNF) and vascular endothelial growth factor (VEGF) – as treatments for epilepsy.

BDNF

BDNF is a member of the neurotrophin family of growth factors. Receptors for the neurotrophins include the p75 neurotrophin receptor, which binds to neurotrophins with low affinity, and the Trk (tropomyosin-related kinase) family of receptors, which bind preferentially to certain neurotrophins with high affinity. Of the three Trk receptors, only TrkB binds BDNF. While both BDNF and its relative, nerve growth factor (NGF), are upregulated after seizures, BDNF and TrkB are more widely distributed in limbic structures, making BDNF the more promising candidate for seizure neuroprotection. Before being tested as a protective factor in the context of epilepsy, BDNF proved to be protective in a variety of other situations, ranging from developing cells in vitro through in vivo models of various disorders. BDNF was first tested for neuroprotective potential after seizures using the kainic acid model of status epilepticus. In this model, animals are administered kainic acid intraperitoneally and develop status epilepticus (SE) within about an hour. Starting 24 h after SE, significant neuronal loss can be detected in the CA1 and CA3 pyramidal neurons as well as in the hilar neurons of the hippocampus. When exogenous recombinant BDNF was delivered in this model, it failed to significantly protect against neuronal death in CA1, and actually significantly worsened damage in CA3. Since BDNF had previously been considered a well-established neurotrophic factor, this result was unanticipated.

VEGF

In contrast to BDNF, VEGF was first described as a vascular factor. It was only a decade later that its potential as a neurotrophic factor began to be appreciated. Perhaps most relevant for seizure researchers, VEGF was shown to protect against excitotoxic damage in hippocampal cultures. However, because VEGF's primary signal-transducing receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1, KDR), were not constitutively expressed on adult neurons, it was unclear whether VEGF would work as a protective factor in adult epilepsy. The discovery that VEGF receptors were upregulated after brain insult – e.g., after stroke or after status epilepticus – made this possibility more likely. Using pilocarpine-induced status epilepticus in rats (a chemoconvulsant model resembling the kainic acid model), we discovered that VEGF significantly protected hippocampal neurons from damage (see **Figure 2**).

Physiological Effects of Neurotrophic Factors

The interpretation of the protective effects of various neurotrophic factors has been complicated by the initially unexpected finding that many of these factors have acute physiological effects on neurons. Traditionally, these protein factors were thought to mediate only longer-term effects on cell survival, function, and morphology. As physiologists became more interested in studying growth factors, the initially heretical idea that these protein modulators could cause relatively rapid alterations in cell firing became a well-established fact. Because excitability is an important contributor to excitotoxicity, enhancement or suppression of neuron excitability has the potential to complicate both the interpretation of and the efficacy of neuroprotective effects of growth factors, including BDNF and VEGF.

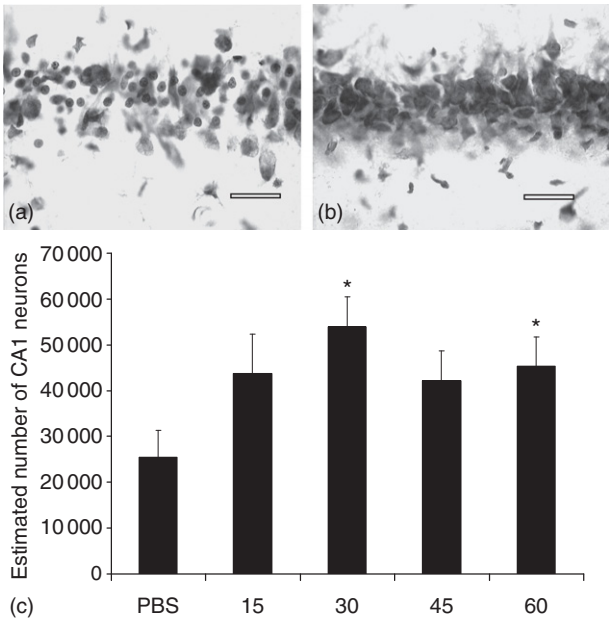


Figure 2 Rescue of hippocampal CA1 neurons by treatment with VEGF. (a) Rats treated only with vehicle (phosphate-buffered saline, PBS) show a normal loss of CA1 neurons 24 h after pilocarpine-induced status epilepticus. (b) CA1 neurons appear to be protected from status-epilepticus-induced cell death in the presence of VEGF. (c) This protection is statistically significant, as determined by stereological counting of CA1 neuronal profiles, *significantly more cells than PBS vehicle, $p < .05$, scale bars in (a, d) = 50 μm. Reproduced from Nicoletti JN, Shah SK, Goodman JH *et al.* (2008) Vascular endothelial growth factor (VEGF) is upregulated after status epilepticus and serves a neuroprotective role. *Neuroscience* 151(1): 232–241, with permission.

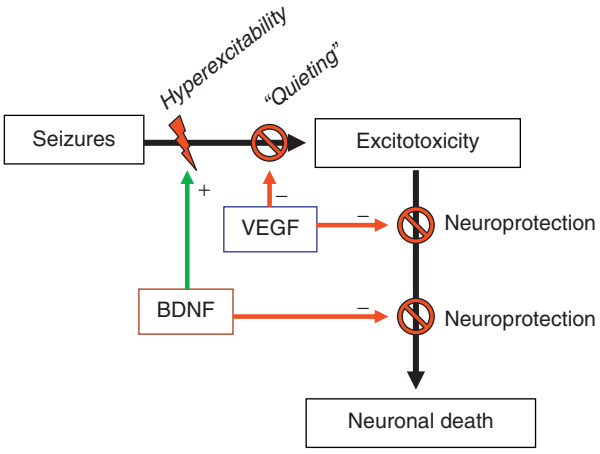


Figure 3 Dual actions of neurotrophic factors. Protein growth factors can prevent neuronal death either by signaling through cell survival pathways in neurons, thereby inducing direct neuroprotection, or by reducing neuronal excitability, thereby reducing excitotoxicity. Examples of two neurotrophic factors which can induce direct neuroprotection are BDNF and VEGF. However, one of these factors, VEGF, also has the potential to decrease excitotoxicity by reducing cell excitability while the other, BDNF, has the opposite effect.

Based on its established protective effects, BDNF was predicted to protect neurons from seizure-induced cell death. It was therefore surprising when BDNF increased neuronal loss in CA3, as indicated earlier. However, BDNF had also been shown to enhance synaptic transmission in hippocampal slices and to induce hyperexcitability – resembling epileptiform activity – in entorhinal/hippocampal slices. In vivo, mice transgenically overexpressing BDNF seized more readily while those deficient in BDNF were more resistant to seizure activation. Further, administration of BDNF protein to normal rats induced seizures in the absence of other convulsant stimuli. Some laboratories, however, have reported beneficial effects of BDNF against epileptogenesis, perhaps as a consequence of TrkB downregulation during certain administration paradigms. Given these results, BDNF's ability to increase excitability, potentially leading to increased excitotoxicity, could outweigh its ability to directly protect neurons (see **Figure 3**). It is for this reason that it is crucial for all potential neuroprotective agents to be evaluated for effects on neuronal excitability.

In contrast to the proexcitability effects of BDNF, the neurotrophic factor VEGF appears to 'quiet' neurons, making them less excitable. Like BDNF, this factor showed promising neuroprotective effects in other situations before being tested for efficacy against status epilepticus-induced neuronal loss. But unlike BDNF, VEGF showed significant efficacy in protecting hippocampal neurons from death after status epilepticus. While it is tempting to attribute this effect solely to VEGF's demonstrated protective effects, the interpretation of the mechanism of its efficacy is complicated by its physiological effects (see **Figure 3**). Specifically, since VEGF decreases the excitability of neurons in hippocampal slices, its protective effects could be mediated through reduced excitability rather than direct neuroprotection. The frustrations involved in interpreting the nature of VEGF's protective effects are, however, more than compensated for by the exciting possibility that VEGF may represent a therapeutic approach which both protects neurons from excitotoxic damage and reduces the likelihood that the patient will have seizures.

Methods

While there are many animal models of epilepsy and epilepsy-induced neuronal loss available, our laboratory has chosen to use the pilocarpine and kainic acid models of acute SE. Both models induce severe SE in the majority of animals injected, and both produce replicable damage to the brain, most striking in the pyramidal cells of the hippocampal CA1 and CA3 subfields, as well as hilar neurons. Damage continues to progress for several days

to a week after SE, which allows us the flexibility to study the brain either several days after SE to determine the full extent of damage, or 24 h after SE to study the process of cell death.

In either model, we introduce growth factors directly into the brain via implanted cannulae, because these large growth factors do not cross the blood–brain barrier. Growth factors can be administered acutely via injection through the cannula, chronically via infusion into the cannula by means of a pump, or genetically by introducing a virus delivering DNA or RNA for the growth factor into brain cells. This latter approach results in longer-term delivery of physiologically relevant levels of growth factors since the brain cells will then make increased levels of growth factor protein using the extra genetic material. In our laboratory, we deliver growth factors into the hippocampus, both because the cell loss after SE is striking in that structure, and also because hippocampal damage has been associated with well-defined behavioral impairments.

In our laboratory we use two approaches to quantifying the neuroprotective effect of growth factors – measuring neuronal density and evaluating behavioral outcome. Neuronal density is determined using stereological software which allows for unbiased estimates of cell density. Behavioral outcome is determined using the Morris water maze for learning and memory ability and the light-dark exploration task for anxiety levels, a measure of emotional tone. It has been well-established that hippocampal damage leads to impairment in both of these behavioral tests.

Results

Functional Preservation

While some work has been done looking at the potential of growth factors to protect against the neuronal loss caused by severe seizures, less work has been carried out to determine if this protection translates into behavioral rescue. Should neuroprotective factors rescue neurons from death, but fail to preserve their ability to function normally, these treatments would ultimately be of little clinical value.

Memory impairment stands out as the most carefully validated impairment in epileptic patients, especially those showing hippocampal sclerosis. While memory and emotional disturbances have been reported repeatedly in animal models of epilepsy, we have found that neuronal rescue and functional rescue after status epilepticus are not always linearly related. With VEGF, for example, we have documented approximately 50% rescue of neurons after status epilepticus. Two weeks after SE, VEGF-treated animals show full rescue from impairments in anxiety, but only partial rescue from learning and memory impairments in the Morris water maze. This 'discrepancy' points to the likelihood that the relation between neuronal and behavioral rescue will be complex, and that the ultimate functional outcome of neuronal rescue may depend on the type and location of rescued neurons, on the nature of the behavior measured, and on the functional status of the hippocampal circuitry that remains intact. Behavioral impairments can be induced in animals, for instance, simply by causing abnormal electrical spiking in the intact hippocampal circuit. In addition, abnormal brain chemistry has been found to be correlated with cognitive deficits in human epileptic patients, independent of gross anatomical lesions. Given the importance of functional preservation, it seems appropriate to eventually expand our definition of 'neuroprotection' to cover not just prevention of neuronal loss, but also protection of normal neuronal functioning. Indeed, researchers increasingly include preservation of normal neuronal physiology and circuitry in their definition of neuroprotection.

Directions for Future Research

While work in the area of neuroprotection in epilepsy is progressing, challenges remain. In addition to our work with BDNF and VEGF, promising data have been obtained by others with protein factors such as glial-derived neurotrophic factor (GDNF) and fibroblast growth factor-alpha (FGFα). Perhaps the biggest impediment to using any of these growth factors in human epilepsy is that the factors are too large to cross the blood–brain barrier. Therefore, chronic administration of these factors requires the introduction of an indwelling cannula into the brain, an approach which would not only be invasive, but would expose human epileptics to the risk of infection.

In an attempt to minimize the invasive nature of growth factor treatment, more research is currently being conducted using acute administration of viral vectors containing DNA or RNA (i.e., gene therapy) for growth factors. While this approach is invasive, it requires only one surgery, after which the brain can be left to heal. Preliminary data from our laboratory has suggested that gene therapy introducing extra copies of the VEGF gene is at least as effective as protein infusions in preventing neuronal death after status epilepticus. Gene therapy using other protective agents (e.g., GDNF) has already shown therapeutic potential in animal models of epilepsy. Once gene therapy is optimized in animal models, it may become possible to move this treatment strategy into humans. However, this approach is only likely to be undertaken in severe cases because it requires brain surgery. Treatment with growth factors for less severe cases may need to await the development of small molecules targeting growth factor receptors and signaling pathways.

In order to minimize the need for invasive therapies, our work is increasingly focusing on identifying the receptors and signaling pathways involved in growth factor-induced neuroprotection. Once these pathways are established, small molecule reagents could be sought which would emulate the beneficial effects of large protein factors without the need for brain surgery. The development of a neuroprotective small molecule would pave the way for a systemically administered drug (such as a pill) which could combat the brain damage and resultant behavioral impairments suffered by some epileptics.

Another advantage of identifying the particular receptors involved in the protective effects of factors such as VEGF is that most growth factors bind at multiple receptors which mediate multiple effects within the brain (e.g., VEGF signals through both VEGFR1 and VEGFR2 which have been proposed to modulate inflammation and vascular plasticity as well as protection). Selective targeting of the receptor which mediates neuroprotection would reduce side effects that could result from binding at those receptors not involved in the protective effects. Our laboratory is currently using reagents which preferentially block one VEGF receptor at a time in an attempt to discover which receptor mediates VEGF's protective effects in epilepsy.

While the search for therapeutic reagents emulating protein growth factors is ongoing, other laboratories are targeting the pathways which lead to neuronal death. All cells, for instance, share common biochemical cascades which mediate apoptosis, or programmed cell death. Protein growth factors are known to act via these pathways, with phosphorylation of their receptors leading to activation of the mitogen-activated protein kinase (MEK), extracellular signal-regulated protein kinase (Erk1/2), and/or phosphatidylinositol-3-kinase (PI3K)-Akt survival pathways (see **Figure 4**). Indeed, much of VEGF's neuroprotective effect in other systems appears to be mediated via the Akt pathway, but it also has the potential to mediate survival via the Erk pathway. Both of these pathways, as well as other trophic signaling pathways, lead to effects on components of cell death and survival pathways such as caspases, BAD, Bcl-2, Bax, Bim, and Bcl-w – all of which could impact on neuronal survival during seizures (**Figure 4**). For instance, animals deficient in Bcl-w and animals treated with a caspase-3 inhibitor both show enhanced cell protection after status epilepticus, validating the idea that neurons could be protected from SE-induced neuronal death by blocking the biochemical pathways leading to cell death.

While it is challenging to identify small molecule reagents that cross the blood–brain barrier, are soluble in water-based solutions, and selectively activate prosurvival signaling pathways, aggressive research in this area should lead us ever-closer to viable therapeutics. Inhibitors of programmed cell death would have to be used with care in young children with epilepsy, as programmed cell death is an important component of normal development. In older children and adults, however, this 'downstream' strategy for neuroprotection may one day be possible.

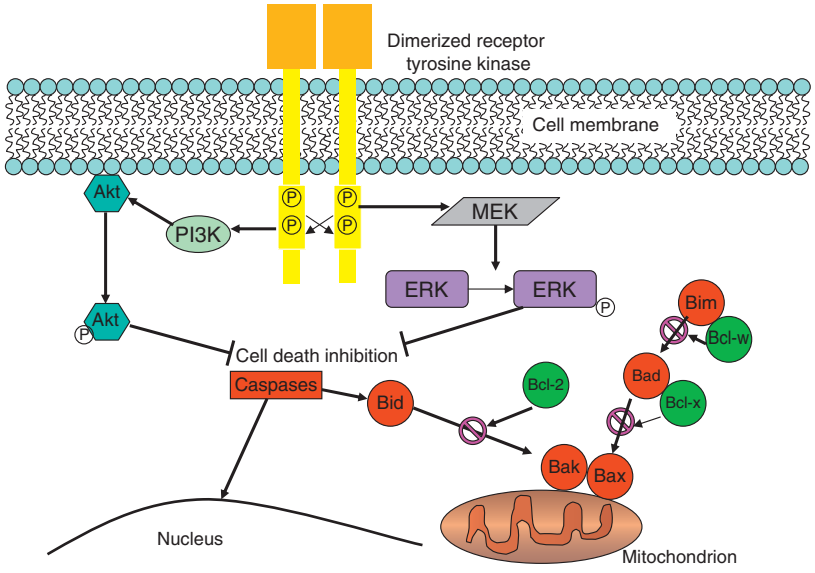


Figure 4 Signaling cell survival. Most protein growth factors transduce signals through biochemical pathways which can inhibit cell death. Two common prosurvival pathways are the PI3K/Akt pathway and the MEK/Erk pathway, both of which can be induced by transphosphorylation of dimerized receptor tyrosine kinases. This simplified cartoon illustrates some of the key players in these pathways, including the procell death mediators Bim, Bad, Bax, Bid, Bak, and cleaved caspases in red and the prosurvival mediators Bcl-2, Bcl-w, Bcl-x, in green. These substances could, themselves, become potential targets for neuroprotective therapeutics.

Whatever the eventual neuroprotective approach taken in humans, be it growth factor-related or not, preserving the brain of the epileptic patient is a promising strategy for maintaining normal functioning. The success of neuroprotective approaches could lead to a substantial enhancement in the quality of life of epileptic patients.

Acknowledgements

I thank my many collaborators and students for thought-provoking discussions which have shaped my thinking about seizure neuroprotection. I am especially indebted to my colleagues Drs. Helen Scharfman, Daniel McCloskey, Pat Rockwell, Jeffrey Goodman, Stanley Wiegand, and John Rudge, and my students Jamee Nicoletti, Elisa Salerni, and Janice Lenzer.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Neurotrophic Factors**: Activity-Regulated BDNF Expression: Contributions to Synaptic Plasticity and Neuroprotection; Role of BDNF in Animal Models of Epilepsy; The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy.

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Role of BDNF in Animal Models of Epilepsy

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Introduction

Elucidating the cellular and molecular mechanisms of epileptogenesis could lead to novel therapeutic approaches aimed at the prevention or management of the disease. The discovery that limbic seizures increase nerve growth factor (NGF) mRNA levels led to the idea that seizure-induced expression of neurotrophic factors might contribute to the lasting structural and functional changes underlying epileptogenesis. Similarly, increases in neurotrophin expression that follow other insults (e.g., ischemia and traumatic brain injury) could also contribute to epileptogenesis. In the past decade, there has been an exciting confluence of in vitro and in vivo findings that strongly implicate the neurotrophin, brain-derived neurotrophic factor (BDNF), in particular, limbic circuits involved in the cascade of electrophysiological and behavioral changes underlying the development of the epileptic state.

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In particular, seizure activity increases the expression of BDNF mRNA and protein, and interfering with BDNF signal transduction inhibits the development of the epileptic state in vivo. The purpose of this article is to summarize the evidence regarding a role of BDNF in epileptogenesis, and to examine the implications of BDNF's involvement in epileptogenesis for new therapeutic targets.

Background

Historical Background

In 1982, BDNF, the second member of the 'neurotrophin' family of neurotrophic factors, was shown to promote survival of a subpopulation of dorsal root ganglion neurons, and subsequently purified from pig brain. The amino acid sequence of BDNF has a strong homology with NGF, the neurotrophin first described because of its trophic (survival and growth-promoting) effects on sensory and sympathetic neurons. Since the discovery of NGF in the early 1950s by Rita Levi-Montalcini and Viktor Hamburger, and the discovery of BDNF by Yves Barde and colleagues in 1982, other members of the 'neurotrophin' family such as neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5) have been described. Each appears to have a distinct profile of trophic effects on distinct subpopulations of peripheral nervous system (PNS) and central nervous system (CNS) neurons.

BDNF Structure

The gene for the mature form of human BDNF has been mapped to chromosome 11p. The protein shares about 50% amino acid identity with human NGF, NT-3, and NT-4/5. The structure of each neurotrophin protein molecule contains: (1) a signal peptide important for intracellular sorting of the protein; (2) a pro-region containing an N-linked glycosylation site and a proteolytic cleavage site for furin-like pro-protein convertases; (3) the mature BDNF protein sequence; and (4) a distinctive three-dimensional structure containing two pairs of antiparallel β-strands and cysteine residues in a cystine knot motif. Neurotrophins are initially produced as proneurotrophins; prohormone convertases, such as furin, cleave the proneurotrophins (MW ~30 kDa) to the mature neurotrophin protein. Proneurotrophins have altered binding characteristics and distinct biologic activity in comparison with mature neurotrophins. Mature neurotrophin molecules are noncovalently-linked homodimers, with molecular weight about 28 kDa. The BDNF gene has four 5′ exons (exons I–IV) that are associated with distinct promoters, and one 3′ exon (exon V) that encodes the mature BDNF protein. Eight distinct mRNAs are transcribed, with transcripts containing exons I–III expressed predominantly in brain and exon IV found in lung and heart.

BDNF Signal Transduction

Each neurotrophin binds one or more of the trk (tropomyosin-related kinase) receptors, members of the family of receptor tyrosine kinases (RTKs). Trk proteins are transmembrane RTKs homologous to other RTKs, such as the epidermal growth factor (EGF) receptor and insulin receptor family. Ligand-induced receptor dimerization results in kinase activation; subsequent receptor autophosphorylation on multiple tyrosine residues creates specific binding sites for intracellular target proteins, which bind to the activated receptor via SH2 domains. The intracellular proteins that bind to the activated receptor include PLCγ1 (phospholipase C), p85 (the noncatalytic subunit of PI-3 kinase), and Shc (SH2-containing sequence); activation of these target proteins can then lead to a variety of intracellular signaling cascades, such as the Ras-MAP (mitogen-activated protein) kinase cascade and phosphorylation of CREB (cyclic AMP-response element binding protein).

Binding specificity is conferred via the juxtamembrane Ig-like domain of the extracellular portion of the receptor in the following pattern: trkA binds NGF (with low-affinity binding by NT-3 in some systems); trkB binds BDNF and NT-4/5 (with lower-affinity binding by NT-3); and trkC binds NT-3. Trk receptors exist in both a full-length (trkB.FL) form as well as truncated (trkB.T1, trkB.T2) forms lacking the kinase domain. Although most functions attributed to BDNF are associated with trkB.FL, several roles have been suggested for truncated receptors, including growth and development and negative modulation of trkB receptor expression and function. Expression of truncated trk receptors on astrocytes is upregulated following injury, and may modulate neuronal vulnerability and sequestration of BDNF in astrocytes. Recent studies have shown that BDNF activates glial calcium signaling by truncated trk receptors.

In addition, all of the neurotrophins bind to the p75 receptor, designated $p75^{NTR}$. $p75^{NTR}$, related to proteins of the tumor necrosis factor (TNFR) superfamily, has a glycosylated extracellular region involved in ligand binding, a transmembrane region, and a short cytoplasmic sequence lacking intrinsic catalytic activity. Neurotrophin binding to $p75^{NTR}$ is linked to several intracellular signal transduction pathways, including nuclear factor-κB (NF-κB), Jun kinase, and sphingomyelin hydrolysis. $p75^{NTR}$ signaling mediates biologic actions distinct from those of the trk receptors, notably the initiation of programmed cell death (apoptosis). It has also been suggested that p75 may serve to determine neurotrophin binding specificity.

Localization, Transport, and Release of BDNF

BDNF mRNA has a widespread distribution in the central nervous system, including limbic forebrain and

neocortex, and is abundant in all principal neurons of the hippocampus. Like BDNF mRNA, constitutive BDNF protein expression is widespread, localized on neuronal cell bodies, axons, and dendrites. The mossy fiber axons of hippocampal dentate granule cells display especially intense BDNF immunoreactivity. TrkB mRNA and protein are widespread in hippocampus. Truncated trkB is also found in the ependymal cells lining the ventricular cavities, effectively limiting diffusion of intraventricularly administered BDNF.

Unlike the classical target-derived trophic factor model in which neurotrophins (such as NGF) are retrogradely transported, there is now abundant evidence that BDNF is also anterogradely transported in brain. First, BDNF protein is localized to nerve terminals, and pathway transection or axonal transport inhibition eliminates this terminal expression. Second, high-resolution microscopic studies have shown that BDNF is associated with dense-core vesicles, which are the primary site for neuropeptide storage and release from nerve terminals. Third, functional studies have supported the anterograde transport hypothesis. Fourth, pro-BDNF is shuttled from the trans-Golgi network into secretory granules, where it is cleaved by prohormone convertase 1 (PC1).

In addition, emerging evidence suggests that both BDNF and trk receptors may undergo regulated intracellular transport. For example, seizures lead to redistribution of BDNF mRNA from hippocampal CA3 cell bodies to their apical dendrites. Trk signaling is now thought to include retrograde transport of intact neurotrophin–trk complexes to the neuronal cell body.

Evidence also indicates that neurotrophins are released acutely following neuronal depolarization. In fact, direct activity-dependent pre- to postsynaptic transneuronal transfer of BDNF has been demonstrated using fluorescently-labeled BDNF. The released form of BDNF is thought to be pro-BDNF, raising the possibility of postsecretory proteolytic processing by membrane-associated or extracellular proteases in the modulation of BDNF action.

BDNF Gene Regulation

A multitude of stimuli have been described that alter BDNF gene expression in both physiologic and pathologic states. Physiologic stimuli are known to increase BDNF mRNA content. For example, light stimulation increases BDNF mRNA in visual cortex, osmotic stimulation increases BDNF mRNA in the hypothalamus, and whisker stimulation increases BDNF mRNA expression in somatosensory barrel cortex. Electrical stimuli that induce long-term potentiation (LTP) in the hippocampus (a cellular model of learning and memory) increase BDNF and NGF expression. Even physical exercise has been shown to increase NGF and BDNF expression in hippocampus. Interestingly, BDNF levels vary across the estrus cycle, with BDNF levels correlated with changes in neural excitability.

Distinct BDNF 5′ exons are differentially regulated by different stimuli (such as neural activity). For example, exons I–III, but not exon IV, increase after kainic acid-induced seizures or other stimuli that increase activity. Protein synthesis is required for the effects of activity on exon I and II, but not on III and IV, raising the possibility that the latter act as immediate-early genes. The transcription factor CaRF activates transcription of exon III under the control of a calcium-response element, CaRE1. CREB, which can be stimulated by diverse stimuli ranging from activity to chronic antidepressant treatment, also modulates exon III transcription. Recent evidence also indicates that neural activity triggers calcium-dependent phosphorylation and release of MeCP2 (methyl-CpG binding protein 2) from BDNF promoter III to de-repress transcription.

Effects of BDNF on Synaptic Transmission

BDNF has an enormous range of physiologic actions at both developing and mature synapses, overall enhancing synaptic transmission by both pre- and postsynaptic mechanisms. The first studies of BDNF effects on synaptic transmission showed that BDNF increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) at neuromuscular synapses in *Xenopus*. Since then, numerous studies have examined the actions of BDNF. Overall, BDNF appears to strengthen excitatory (glutamatergic) synapses and weaken inhibitory (GABAergic) synapses. Exposure of adult rat hippocampal slices to BDNF leads to a long-lasting potentiation of synaptic strength at Schaffer collateral-CA1 synapses. Subsequent studies have supported a role of BDNF in long-term potentiation (LTP). For example, incubation of hippocampal or visual cortical slices with trkB inhibitors inhibits LTP, and hippocampal slices from $BDNF^{-/-}$ mice exhibit impaired LTP induction – which is restored by reintroduction of BDNF.

Whether BDNF-induced synaptic potentiation occurs primarily by a presynaptic action (e.g., through enhancement of glutamate release) or postsynaptically (e.g., via phosphorylation of neurotransmitter receptors) is intensely debated. A number of studies have provided evidence for a presynaptic locus, but evidence for postsynaptic actions has also been obtained. Both pre- and postsynaptic trkB receptors in the hippocampus may be important.

A role for BDNF in GABAergic synapses was first raised by studies showing that BDNF influences GABAergic neuronal phenotype. Subsequently, BDNF was shown to decrease inhibitory (GABAergic) synaptic transmission. Recent evidence shows that BDNF can modulate the

function of $GABA_A$ receptors via modulation of phosphorylation state. Interestingly, BDNF may also regulate the efficacy of GABAergic synapses by direct downregulation of the neuronal K^+–Cl^- cotransporter, which would impair neuronal Cl^- extrusion and weaken GABAergic inhibition. Indeed, BDNF selectively decreases the efficacy of inhibitory neurotransmission by downregulation of Cl^- transport.

Methods

BDNF gene regulation by seizure activity has been studied at both the mRNA and protein levels. Changes in mRNA induced by seizure activity have been studied by Northern blot and in situ hybridization analysis. In Northern blot analysis, total RNA is extracted from a tissue sample, run on an RNA gel, and a specific labeled antisense oligonucleotide probe is used to detect the RNA of interest. Similarly, in situ hybridization utilizes a specific radiolabeled or chemically labeled antisense RNA strand to detect specific genes (in this case BDNF) in ex vivo tissue sections. While it is less quantitative than Northern blot analysis, the advantage of in situ hybridization is that not only the level but the anatomic site(s) of BDNF mRNA regulation can be determined. Immunohistochemistry, Western blot analysis, and ELISA (enzyme-linked immunosorbent assay) have been used to examine BDNF protein regulation following seizures. Immunohistochemistry, using a specific antibody to BDNF, has shown levels and anatomic sites of BDNF protein upregulation by seizure activity. ELISA and Western blot can be used to evaluate, in a more quantitative manner, BDNF protein levels in tissue extracts.

Studies of the function of BDNF on synaptic transmission and epilepsy have used both in vitro and in vivo techniques. Potentiation of synaptic strength by BDNF has been most commonly studied in ex vivo slices of hippocampus. In these studies, specific hippocampal synaptic pathways (e.g., Schaffer collateral-CA1 synapses) are stimulated electrically and the responses recorded in the presence and absence of bath-applied BDNF. Likewise, inhibitors of BDNF transmission (such as trkB inhibitors) or studies of hippocampal slices from $BDNF^{-/-}$ mice have shown impaired induction of long-term potentiation, a common model of synaptic plasticity and learning and memory.

In vivo studies of the role of BDNF in epilepsy have used animal models of epilepsy, such as the pentylenetetrazol (PTZ) model, kainic acid (KA) model, and kindling model. In these studies, BDNF or BDNF inhibitors are given in the attempt to affect epileptogenesis. For example, trkB receptor bodies (which sequester endogenous BDNF) have been injected chronically via an intracerebroventricular route and have been shown to inhibit epileptogenesis in the kindling model.

Epilepsy-Relevant Results

Role of BDNF in Animal Models of Epilepsy

The discovery that limbic seizures increase NGF mRNA levels led to the idea that seizure-induced expression of neurotrophic factors may contribute to the lasting structural and functional changes underlying epileptogenesis. It is now clear that BDNF and trkB are markedly upregulated by seizures. A wide variety of seizure paradigms (kindling, kainic acid, pilocarpine, PTZ, electroconvulsive shock (ECS)) rapidly and markedly increase expression of BDNF mRNA in dentate gyrus as well as in other areas of the hippocampus and cortex. This mRNA increase is associated with a transient upregulation of BDNF protein. TrkB mRNA and protein in the dentate gyrus are also upregulated following various seizure protocols. TrkB mRNA expression is increased in dentate granule cells 2–6 hours after rapid electrical kindling, hippocampal afterdischarge, PTZ kindling, ECS, or pilocarpine status epilepticus. Subcellular studies have demonstrated targeting of BDNF and trkB mRNAs to dendrites in CA3 neurons following kindled seizures.

Studies using the kindling model of epilepsy have functionally implicated BDNF in epileptogenesis. In the kindling model, repeated, focal application of initially subconvulsive electrical stimuli eventually results in intense focal and tonic–clonic seizures. Once established, this enhanced sensitivity to electrical stimulation persists for the life of the animal. The kindling model has been an important tool, since it allows experimental control over seizures and precise quantitation of effects of experimental manipulation on epileptogenesis in vivo.

The development of kindling is partially inhibited in heterozygote knockout BDNF mice or mice with conditional BDNF gene deletion. Intraventricular infusion of trkB-Fc, a chimera of human IgG-Fc domain and the extracellular domain of the trkB receptor that would sequester and limit the activity of endogenous BDNF, inhibits kindling development. Mice overexpressing a truncated trkB display reduced seizure susceptibility. Interestingly, conditional homozygous deletion of trkB appears to prevent kindling. These results are consistent with studies demonstrating that increasing BDNF promotes excitability. For example, direct application of BDNF to hippocampal slices induces hyperexcitability in vitro; overexpression of BDNF in transgenic mice leads to spontaneous seizures; and intrahippocampal infusion of BDNF is sufficient to induce seizure activity in vivo.

A separate group of experiments has demonstrated that chronic BDNF infusion can inhibit kindling. These inhibitory effects appear to be due to trkB receptor downregulation following chronic BDNF administration, and hence are still consistent with the 'proepileptogenic BDNF' hypothesis. This interpretation is supported by

the observation that chronic exposure to BDNF in vitro leads to downregulation of trkB mRNA and protein. Similarly, continuous in vivo intrahippocampal BDNF infusion results in downregulation of trkB protein by as much as 80%. Thus, whereas chronic BDNF infusion inhibits kindling progression, acute microinjections of BDNF enhance epileptogenesis in the absence of effect on trkB expression. Furthermore, chronic infusions of BDNF may upregulate the inhibitory neuropeptide Y.

Whether BDNF has a significant effect on seizure-associated mossy fiber sprouting is not clear. Although mossy fiber sprouting has been reported in $BDNF^{+/-}$ mice and following BDNF infusion, there is no effect on mossy fiber sprouting in BDNF-overexpressing mice or following chronic infusion or bolus injection of BDNF in other studies. However, BDNF overexpression does increase dendritic length and complexity in the hippocampus. The relative role of BDNF in synaptic changes vs. larger-scale morphological changes during epileptogenesis remains to be clarified.

Trk Receptor Activation Following Seizure Activity

The ability to monitor trk receptor activation following seizures using phospho-specific trk antibodies has enabled investigators to identify the anatomical, time course, and threshold characteristics of trk receptor activation in the hippocampus following seizure activity. Following seizure activity, phospho-trk immunoreactivity is selectively increased in dentate hilus and CA3 stratum lucidum of hippocampus. This distribution precisely coincides with the 'mossy fiber' pathway of dentate granule cell axon terminals. In addition, this anatomic pattern coincides with the distribution of both basal and seizure-induced BDNF protein. Basal BDNF protein is also localized in hilus and CA3 stratum lucidum, and seizures increase levels of BDNF protein in dentate gyrus and CA3 (and BDNF immunoreactivity in hilus and CA3 stratum lucidum). This precise anatomic colocalization of increased phospho-trk immunoreactivity and increases in BDNF protein suggests that the phospho-trk immunoreactivity is caused by seizure-induced increases in BDNF. However, interesting recent evidence also demonstrates transactivation of trkB in the mossy fiber pathway by the divalent cation zinc, in a neurotrophin-independent manner.

Perspective and Goals for Future Research

Since the discovery of BDNF in the 1980s, a great deal of evidence has mounted for roles of BDNF in development, physiology, and pathology. BDNF appears essential to molecular mechanisms of synaptic plasticity and larger-scale structural rearrangements of axons and dendrites. Basic activity-related changes in the central nervous system are thought to depend on BDNF modulation of synaptic transmission. However, pathologic levels of BDNF-dependent synaptic plasticity appear to contribute to epileptogenesis in a variety of animal models. On the basis of distribution, regulation, and localization studies, the hippocampus and closely associated limbic structures are thought to be particularly important in the proepileptogenic effects of BDNF. Specimens from patients with temporal lobe epilepsy have also demonstrated BDNF upregulation. It is hoped that understanding of the hyperexcitability associated with BDNF in epilepsy animal models may lead to novel anticonvulsant or antiepileptic therapies.

New methods of regulation of neurotrophins have been developed to achieve therapeutic efficacy in diseases of neurotrophin deficiency. For example, 'ampakines' represent a new class of compounds that have been shown to upregulate BDNF over a long period of time. Determination of proper dosing protocols, so as not to trigger downregulation of important neurotrophin signaling pathways, will be critical to avoiding deleterious side effects of these potential new therapies. Nevertheless, these and similar compounds are under active clinical investigation as cognitive and memory-enhancing drugs.

Manipulating neurotrophin action by simple up- or downregulation may lead to many nonspecific effects. For ultimate clinical application in specific conditions such as epilepsy, it will be very helpful to elucidate downstream neurotrophin signaling pathways responsible for specific phenotypic effects. For example, BDNF activation of trkB downregulates hippocampal KCC2, a K^+–Cl^- cotransporter; this suppresses chloride-dependent fast GABAergic inhibition and may partially account for BDNF modulation of GABAergic synapses. In addition, BDNF phosphorylates specific subunits of both the NMDA receptor and the $GABA_A$ receptor, altering their function. Longer-term effects of BDNF must take into account the fact that it upregulates many other plasticity-related genes, such as neuropeptide Y. NPY, for example, may not only modulate excitability but also other phenomena such as neurogenesis. Targeting the downstream pathways specifically associated with epileptic hyperexcitability, in an anatomically and temporally restricted manner, will be necessary for optimal therapeutic effect.

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Glia/Astrocytes**: Role of Astrocyte Dysfunction in Epilepsy; **Neurotrophic Factors**: Activity-Regulated BDNF Expression: Contributions to Synaptic Plasticity and Neuroprotection; Neuroprotective Strategies in Epilepsy; The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy;

Plasticity: Dendritic Abnormalities in Epilepsy; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy.

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NON-SYNAPTIC MECHANISMS

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Changes in Extracellular Ionic Composition

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Introduction

All neurons and glia maintain an electrical potential across the cell membrane, which is negative relative to the extracellular environment. This negative potential provides a driving force for charged particles to move across the membrane. Every nerve impulse is dependent on this movement of ions. Most of these ions move through ion channels, along concentration gradients generated by electrogenic pumps, but some ions move across membranes on transporters. Abnormalities in the control of ionic movement across membranes can result in abnormalities in neuronal excitability.

With neuronal activity, action potential firing results in movement of potassium out of the cell and sodium into it. A single action potential will result in little change in the ion concentrations in the extracellular space. During seizures (i.e., intense neuronal activity), however, the amount of potassium moving out of neurons exceeds the ability of the brain to regulate precisely the ion concentrations, and the extracellular potassium will increase from a resting level of around 3 to 10–12 mM.

(ECB devices provided by NsGene A/S, Denmark). These experiments showed that such an approach is also effective in decreasing seizure severity and duration in kindled animals, and that the effect is dependent on the relative amount of GDNF released from these capsules. Unexpectedly, lower levels of released transgene GDNF seemed to be more effective rather than higher levels, as assessed by the relative intensity of GNDF immunoreactivity in the host hippocampal tissue surrounding the transplants (**Figure 2**).

We then explored some possible mechanisms that could underlie the seizure-suppressant effect of transgene GDNF. Among others, increased expression of neuropeptide Y (which has been shown to be induced, e.g., by BDNF exposure), decreased GFRalpha-1 receptor, and altered neurogenesis have been explored. However, none of these mechanisms seems to be critically responsible for transgene GDNF effects.

Future Goals

What remains to be explored is whether transgene GDNF can alter synaptic transmission in the hippocampus (directly or indirectly). It has been shown to upregulate glutamate transporters, in particular GLAST and GLT-1, which would result in more effective removal of elevated extracellular glutamate in chronic models of epilepsy. More directly, GDNF could modulate excitatory or inhibitory synaptic transmission in the brain, as shown in studies of GDNF effects on myocytes or cultured midbrain neurons. To achieve these goals, specific immonstainings and whole-cell patch clamp techniques will be used. Such experiments will reveal the mechanisms behind GDNF antiepileptic activity and will open possible novel research perspectives in using GDNF as a potential target molecule in gene therapy for epilepsy.

See also: **Antiepileptic Drugs**: Local Delivery of Antiepileptic Drugs; **Neurogenesis**: Newborn Neurons Mitigate Synaptic Excitability in Epileptic Brain; **Neurotrophic Factors**: Fibroblast Growth Factor-2; **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides.

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The Influence of Neurotrophins on Excitability in Hippocampus and its Potential Relevance to Epilepsy

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Introduction

The idea that the hippocampus is related to epilepsy, specifically temporal lobe epilepsy, has a long history of support from basic and clinical studies. One of the seminal questions is why the hippocampus appears to be 'seizure-susceptible' – in other words, why epileptiform discharges are readily evoked, and seizures often begin, within this structure. One hypothesis is that the hippocampus is designed to support diverse types of structural and functional plasticity under normal conditions, which is important to hippocampal-dependent functions, such as learning and memory. However, the mechanisms that support plasticity, especially mechanisms that increase glutamatergic transmission and synchronization, may lead to a greater risk of seizures.

Here we examine one of the neurotrophins that is expressed in hippocampus, BDNF. BDNF is one example of a modulator of hippocampal function that needs to 'balanced,' because it can induce plasticity that enhances normal hippocampal function, and it can induce seizures. It may contribute to plastic changes in the brain that are

associated with epileptogenesis, and hence 'pathological plasticity.' The paragraphs below provide an overview of the evidence that hippocampal BDNF may be one of reasons why the hippocampus is predisposed to seizures.

Background

Neurotrophins, their Receptors, and Distribution within Hippocampus

The neurotrophin family is composed of nerve growth factor (NGF), BDNF, neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). There are two receptors for neurotrophins: p75 and trk. All neurotrophins bind p75, which mediates important actions in development to prune excess neurons. In adult brain, and hippocampus specifically, the role of p75 is not yet clear. Effects of neurotrophins at trk receptors are better understood. Neurotrophins bind to selected trk receptors, as follows: NGF binds trkA, BDNF and NT-4/5 bind to trkB, and NT-3 binds to trkC. Compared to BDNF expression, the expression of NT-4/5 in hippocampus is weak, and the hippocampal effects of NT-4/5 are poorly characterized. Therefore, most consider BDNF the primary ligand for trkB in hippocampus.

Receptor dimerization after ligand binding is followed by receptor autophosphorylation and its participation in a variety of signaling pathways, such as the MAP kinase pathway, and many others. Trk activation ultimately leads to synthesis of transcription factors such as cyclic-AMP response element binding (CREB) proteins, which exert effects such as stabilization of LTP. Truncated trk receptors, which lack the internal kinase domain, appear to be expressed primarily on glia, and it has been suggested that the function of truncated receptors is to limit the effects of neurotrophins. However, truncated receptors exert specific effects besides simply binding the ligand even though they lack the kinase domain. However, the effects of truncate trkB in hippocampus are not clear at present time.

BDNF is synthesized from a precursor, proBDNF, which is thought to act primarily at p75 rather than at trkB receptors. Under normal conditions, proBDNF has actions that are limited by extracellular proteases such as furin, plasmin, or matrix metalloproteases that normally cleave proBDNF. Nonetheless, it is thought to play an important role in long-term depression (LTD) since proBDNF increases LTD in CA1.

BDNF mRNA is present in all principal cells of hippocampus, but normally protein expression appears to be primarily in granule cells, where BDNF is anterogradely transported to the mossy fiber axons. Interestingly, some antibodies detect BDNF preferentially in somatic locations, possibly because BDNF has different conformations in the soma versus axon.

After seizures, there are many changes in the expression of BDNF and its associated receptors. To date, evidence for the upregulation of proBDNF by seizures is weak, but there is robust evidence for a rapid increase in BDNF and trkB in granule cells. These changes appear to occur after a variety of types of seizures. The increase in BDNF and trkB may initiate changes associated with seizures, such as increased neuropeptide Y; BDNF induces NPY by trkB activation. p75 also increases after seizures, and may contribute to seizure-induced apoptosis.

BDNF may have widespread actions after seizures, beyond those that occur normally, because its expression increases in cells that normally translate message only at a low level, such as hippocampal pyramidal cells. BDNF may also change its subcellular location (somatic to dendritic). Therefore, after seizures, BDNF may have a more widespread role than its normal expression pattern would suggest.

The changes in BDNF and its receptors after status epilepticus (SE) in the adult rodent is especially interesting because chronic seizures develop in the subsequent weeks. It has been proposed that the upregulation of BDNF by the prolonged seizures that characterize status epilepticus may initiate epileptogenesis. It is possible that upregulation of BDNF after intermittent seizures also contributes to epileptogenesis, because deleting trkB in forebrain impairs epileptogenesis in the kindling model.

BDNF and Synaptic Plasticity in Hippocampus

Evidence that BDNF influences hippocampal synaptic transmission began with studies of Kang and Schuman, who showed that Schaffer collateral-evoked transmission was potentiated by perfusion of the slice ('bath-application') with recombinant BDNF. This effect was long-lasting, and it was suggested that BDNF induced a unique form of LTP. Subsequent studies extended these findings by showing that LTP was impaired in mice that lacked BDNF, as was learning and memory. Interestingly, many laboratories had difficulty replicating the BDNF-induced LTP in slices, and the reasons have never been completely clarified. Current ideas to explain discrepancies across laboratories include differences in the source of BDNF, its concentration, the flow rate, slice procedure, constituents of the buffer, and animal age. Age is a particularly interesting variable in the context of epilepsy, because there appears to be a decrease in epileptogenesis in aged animals; this decrease could be due to the decline in BDNF that occurs with aging.

Although there are discrepancies concerning BDNF-induced potentiation in slices, there currently is agreement that BDNF plays a critical role in the late phase of tetanus-evoked LTP in area CA1 and the dentate gyrus. It has been suggested that BDNF exerts its effect by influencing the factors that stabilize LTP. These are likely to involve ERK1/2 and the MAP kinase pathway in the dentate gyrus. The phospholipase Cγ (PLCγ) pathway could be

critical in CA1; disrupting trkB activation of the PLCγ pathway appears to block CA1 LTP. These, and other studies, suggest that both pre- and postsynaptic mechanisms contribute to the effects of BDNF on LTP, and mechanisms in dentate gyrus may be different from CA1.

BDNF has many other actions besides the potentiation of glutamatergic transmission on principal cells of hippocampus. Dentate gyrus neurogenesis is increased by BDNF by actions at trkB receptors on progenitors in the subgranular zone. The density and complexity of dendritic spines are increased by BDNF. Interneuron expression of neuropeptides is induced by BDNF, and BDNF downregulates KCC2, a potassium-chloride cotransporter that controls the transmembrane chloride gradient. $GABA_A$ receptor distribution is altered by BDNF. BDNF also influences specific sodium channels (NaV1.9), leading to depolarization. These actions, and others, make it highly likely that BDNF could influence multiple mechanisms involved in seizures, epileptogenesis, and epilepsy (Figure 1).

Methods

To address an issue such as the role of hippocampal BDNF in seizures and epilepsy, it is important to use methods that can examine the hippocampus at relatively high resolution, typically in vitro. It also is important to validate in vitro findings using an intact system, i.e., an in vivo approach. In vitro methods discussed below include electrophysiology in hippocampal slices, or morphological/structural studies in sections from perfusion-fixed brain. In vivo methods include recordings from animals that have chronic electrodes in hippocampus. Pharmacological analysis is challenging when neurotrophins are used, because there are few antagonists, and agonists are large molecules that do not diffuse well. Therefore, peripheral systemic injection – or even i.c.v. infusion – is not ideal. Direct application of drugs into hippocampus is one resolution to this problem, but controls are essential, because insertion of a cannula or needle into hippocampus can upregulate BDNF locally, and subsequently induce neuromodulators such as NPY. Obtaining proper controls is not trivial, however, because saline or buffer injection is inadequate to control for the effects of a large protein such as BDNF. Using cytochrome C as a control for BDNF is useful, because it has similar physical chemistry to BDNF but lacks activity at trkB. Another important consideration in these experiments is stress to the animal prior to use; stress has profound and complex time-dependent effects on BDNF levels, and

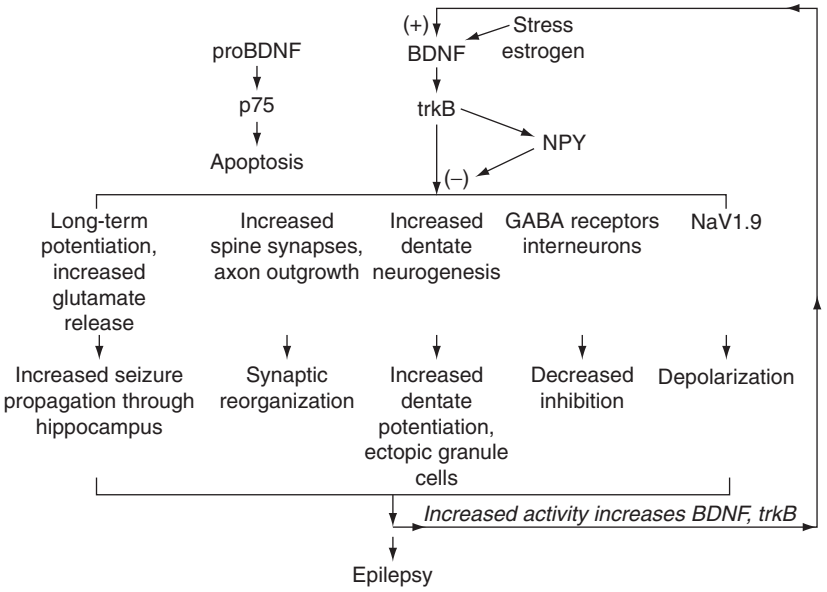


Figure 1 BDNF and epileptogenesis. A schematic illustrates the actions of proBDNF and BDNF that may contribute to epileptogenesis. ProBDNF via p75 could lead to seizure-induced apoptosis. BDNF via trkB has many potential actions, and most would be likely to facilitate seizures. For example, BDNF stabilizes LTP, and increases glutamate release in hippocampus, which could facilitate seizure propagation through hippocampus. Also, BDNF may contribute to synaptic reorganization because it promotes spine synapse formation and axonal outgrowth, although evidence for a role in mossy fiber sprouting, a striking example of synaptic reorganization that is thought to be relevant to epileptogenesis by many investigators, is unclear. BDNF increases neurogenesis of granule cells and ectopic granule cells in the hilar region, which could increase excitability of the dentate gyrus by increasing perforant path – evoked LTP, or by increased synchronization between granule cells and pyramidal cells. BDNF has many actions on GABA receptors and interneurons, and some of these effects would lead to disinhibition. Actions at NaV1.9 may depolarize hippocampal neurons. An important consideration is the fact that activity increases BDNF and trkB, which would potentially create a positive feedback loop in this schematic, and ultimately could underlie the development of chronic seizures (epilepsy).

glucocorticoids have effects on the target neurons of BDNF-expressing axons (e.g., CA3 pyramidal cells). Social housing and regular handling are important methods for reducing stress in experimental subjects.

Results

Initial Studies

Our initial experiments addressed the role of BDNF in mossy fibers. Mossy fibers appear to contain the most BDNF protein of all neurons in hippocampus, but most studies focused on area CA1 rather than mossy fibers. The high BDNF protein content of mossy fibers suggested retrograde effects on granule cells or anterograde effects on mossy fiber targets. To address potential effects on granule cells and mossy fiber transmission, the slice preparation was used with recording electrodes in multiple sites to detect potential effects. We found that application of recombinant BDNF potentiated mossy fiber transmission, with little detectable effect on granule cell physiology. The effect on mossy fiber transmission appeared to be selective for the excitatory input to pyramidal cells; inhibition was unaffected when IPSPs were recorded in pyramidal cells. In addition, even low doses of convulsants and GABA receptor antagonists could not reproduce the BDNF effect. Inputs to CA3 pyramidal cells from the fimbria and recurrent collaterals did not appear to be influenced by exposure to BDNF. Subsequent studies have shown high levels of BDNF in the large boutons of mossy fibers that innervate pyramidal cells, but not in the smaller boutons that innervate interneurons, explaining the preferential increase in glutamatergic mossy fiber transmission to CA3 pyramidal cells.

Mossy fiber stimuli (at 1–2 Hz) led to repetitive population spikes in area CA3 in the presence of BDNF; after three pairs of stimuli at 1 Hz, each stimulus evoked more than one population spike rather than a single population spike. This stimulation frequency is very low compared to that traditionally used to induce LTP, and stimulation was much shorter in duration compared to the stimuli required to induce LTD. The trains of population spikes occurred approximately in the range of gamma frequency, suggesting a novel mechanism to induce gamma oscillations in CA3. In some cases, the repetitive discharge was followed by spreading depression episodes, suggesting hyperexcitability. Indeed, gamma oscillations have been observed to precede epileptiform discharges. These data suggested a potential role of BDNF in seizure susceptibility of hippocampus.

Recombinant BDNF was perfused onto the slice at concentrations (50–250 ng ml^{-1}) that were in the range used by others, and thought to be physiologically relevant. However, the physiological in situ concentration of BDNF remains unclear. It is also unclear how much BDNF is retained in tissue slices (i.e., when tissue is prepared for in vitro studies), and some have suggested it actually is increased by the slice procedure. Therefore, alternate methods are necessary to confirm that the results seen in slices were not a pharmacological phenomenon. One approach to this problem involved using slices from a BDNF-overexpressing mouse line that had elevated BDNF in mossy fibers. BDNF was not added to the slice at all, but the same type of responses to mossy fiber stimuli were observed as those that were recorded from normal male rat slices after exposure to BDNF. Pairs of mossy fiber stimuli at 1 Hz led to repetitive population spikes and spreading depression. An in vivo approach was then used to examine the question of in vivo versus in vitro effects. BDNF was infused into hippocampus directly, and results were compared to the effects of cytochrome C or another large protein, albumin. Infusion of BDNF into the adult rat brain led to seizures in many of the rats, but no seizures were detected in controls.

Surprisingly, after BDNF was infused in vivo and seizures were observed, seizures did not continue, despite the fact that BDNF continued to be perfused for 2 weeks or more. In addition, some rats that were infused with BDNF did not exhibit seizures (although seizures were not monitored continuously, so some seizures could have been missed). One possible explanation for this loss of effect is that membrane trkB receptors are internalized when high doses of BDNF are infused. BDNF also induced NPY, which depresses mossy fiber transmission and has been shown to be anticonvulsant. It is intriguing to consider that BDNF potentiates the mossy fiber pathway, and also induces a potential 'brake' by increasing the synthesis of NPY. One would predict that an imbalance in BDNF and NPY might lead to seizures. Indeed, NPY knockout mice have increased seizure susceptibility, possibly by leaving BDNF–mediated excitation 'unchecked.'

Additional Studies from Other Laboratories

Studies from other laboratories have provided substantial evidence that BDNF plays a role in epilepsy. Seminal studies of Gall and colleagues first showed that BDNF message and protein were elevated after seizures, and the effect was extremely robust and rapid in granule cells – possibly more rapid here than in any area in the brain. In light of these data, as well as results from more recent studies, some have suggested that BDNF may be functionally similar to an immediate early gene, initiating a cascade of events that leads to changes in gene expression, which in turn alters the structure of synapses, axons, and excitability. These data support the concept discussed earlier that seizure-induced elevation in BDNF could initiate epileptogenesis.

Other studies led to more direct lines of evidence that BDNF was important to epileptogenesis. Using the

kindling model, and a scavenger of BDNF, Binder and colleagues demonstrated that scavenging BDNF impaired kindling. Binder and colleagues also showed the trk was phosphorylated in mossy fibers after seizures. Taken together, the results suggest that seizure-induced trk activation in mossy fibers could contribute to epileptogenesis in the kindling model. Other investigators infused BDNF into the hilus of the dentate gyrus and examined kindling. Given the results summarized earlier, one would predict that kindling might occur more rapidly. Instead, kindling was delayed, a result that caused some confusion in the field at the time. However, the issue was resolved after it was demonstrated that high doses of BDNF can down-regulate its receptor, trkB.

Transgenic approaches also provided evidence that BDNF influenced epileptogenesis. A number of transgenic lines have been developed, and the majority of the data from these mice indicate that BDNF is either proconvulsant or facilitates the process of epileptogenesis in animal models of temporal lobe epilepsy. For example, BDNF-overexpressing mice had a more severe seizures in response to the convulsant kainic acid, and gene mutations leading to elevated BDNF or trkB had similar actions. Interestingly, a conditional forebrain knockout of BDNF did not alter kindling epileptogenesis, but the knockout that lacked trkB had a robust effect: after-discharges were difficult to elicit by stimulation at the kindling site, thus retarding kindling. More recently it was shown that the ketogenic diet may act by blocking BDNF and trkB upregulation after seizures. Use of 2-deoxyglucose to inhibit glycolysis, an approach that simulates the effects of the ketogenic diet, led to a sequence of events that ultimately inhibited BDNF and trkB promoters.

The Role of BDNF in Epileptogenesis

As discussed earlier, the results of many studies implicate BDNF and trkB in seizures and epilepsy. How exactly do BDNF and trkB contribute? It seems likely that the normal actions of BDNF would facilitate the processes currently thought to play a role in epileptogenesis, as shown in Figure 1. These actions are discussed later in the context of the status epilepticus models for temporal lobe epilepsy. In these studies, status epilepticus is initiated in the adult rodent, and then is followed weeks later by recurrent limbic seizures.

One of the first questions one might ask is whether BDNF plays a role in the initial stage of the status models – i.e., in status epilepticus itself. On the basis of studies of a forebrain knockout mouse without trkB, it appears that trkB may indeed play a role in the induction of status epilepticus. In this mouse, it was difficult to elicit after-discharges using implanted electrodes, suggesting seizure activity was 'inhibited' relative to controls containing trkB.

Another question is whether BDNF plays a role in neuronal damage, one of the consequences of status epilepticus that is thought to be important in epileptogenesis. It seems likely that BDNF could increase neuronal damage by increasing glutamate release, which one would predict would contribute to excitotoxicity. Indeed, BDNF exposure does make excitotoxicity worse in CA3. In addition, proBDNF is likely to act on p75 to initiate apoptosis, since this is a normal function of proBDNF. This effect might be increased after status epilepticus, because p75 levels increase at this time. However, BDNF has also been reported to have neuroprotective actions. Therefore, the role of BDNF in seizure-induced neuronal damage is not clear at the present time.

There are several ways that BDNF could contribute to epileptogenesis; status epilepticus increases BDNF levels, and BDNF promotes synaptic plasticity. Therefore, after status epilepticus, elevated BDNF might induce long-term potentiation of glutamatergic circuits. Potentiation of glutamatergic circuits could increase the facility with which seizures propagate through brain areas such as hippocampus, thus enhancing the ability of focal seizures activity to generalize. Independent of its ability to potentiate synapses, BDNF could mediate some of the structural and circuit modifications that are thought to play a role in limbic epileptogenesis, such as increasing recurrent excitatory circuits. However, the evidence that BDNF mediates phenomena like mossy fiber sprouting, a classic example of synaptic reorganization, is unclear – despite numerous attempts to provide a causal link.

Another way that BDNF could facilitate epileptogenesis is related to its ability to increase the rate of dentate gyrus neurogenesis. In the normal brain, BDNF appears to increase survival of newly born granule cells, and the same may be true for granule cells that are born after seizures. However, whether an increase in survival of new granule cells increases or decreases seizure activity is hard to say, because those granule cells that migrate into the granule cell layer after status epilepticus appear to have normal excitability, whereas those that migrate to ectopic locations seem to develop abnormal excitability. At present, the influence of increased neurogenesis in the dentate gyrus on epileptogenesis is unclear.

Figure 1 illustrates these points, and also makes an additional point that is important to the argument that BDNF may contribute to epileptogenesis: even an increase in neuronal activity that is subthreshold for seizure generation can increase BDNF. That fact leads to the prediction that periods of brief, subthreshold seizure activity during early stages of epileptogenesis may increase BDNF repeatedly. Periodic elevations in BDNF may be important for the continued activation of plastic changes during epileptogenesis. After the first spontaneous seizure, a rise in BDNF would also be predicted, and

could explain the progression of epilepsy that many suggest continues long after the first spontaneous seizure.

Relevance of BDNF to Women with Epilepsy

Most studies of BDNF and trkB in rodents have been conducted in males, and clinical studies have not described gender-specific effects. The lack of investigation in females is surprising given that estrogen has a response element on the BDNF gene, and BDNF appears to fluctuate as a function of estrogen, particularly in the mossy fiber pathway. Therefore, the female brain may inherently change BDNF protein content as estrogen varies; that in turn could change seizure susceptibility. In the epileptic brain, when BDNF levels are chronically high, a normal fluctuation in estrogen may elevate BDNF well above the range that is normal and lead to seizures.

This hypothesis is relevant to women with epilepsy where seizures fluctuate across the menstrual cycle – e.g., when estrogen surges just before ovulation (the 'periovulatory period'). It is also potentially relevant to the subset of women who have seizures which increase in severity at the end of the cycle, the 'perimenstrual' period, because estrogen rises and falls just before this time of the cycle also. Therefore, elevated BDNF could occur in the 'perimenstrual period,' when seizures have been reported to increase in a subset of women with epilepsy. Hormonal regulation of BDNF could also play a role in the changes in seizures that occur during pregnancy, when estrogen rises.

We hypothesized that estrogen would induce BDNF in the mossy fibers during the normal preovulatory estrogen surge, and this BDNF increase would lead to a similar increase in CA3 evoked responses as observed in the BDNF-overexpressing male mouse (or normal male rat that was exposed to exogenously-applied BDNF). Using intact female rats, and then ovariectomized and estrogen-replaced rats, we found that BDNF was elevated in mossy fibers after estrogen was increased. In the intact rat, the elevation in mossy fiber BDNF was present on the morning of proestrous, when estrogen reaches a peak. It was also detected for the next 24 h, presumably because BDNF stores had not been depleted. Similar results were obtained from ovariectomized rats when proestrous morning was simulated by timed injections of estradiol to mimic the proestrous surge in estradiol. In slices from these ovariectomized, estrogen-replaced rats, mossy fiber stimuli evoked repetitive population spikes and spreading depression, similar to slices from BDNF-overexpressing male mice, or slices from the normal rat that were exposed to BDNF. K252a, an inhibitor of trk receptors, blocked the repetitive spiking, and the spreading depression.

These data lead to the hypothesis that BDNF plays a role in the hormone-sensitive seizures of women with epilepsy. Is there evidence for this hypothesis from clinical studies? So far there have been no studies of BDNF levels in women with epilepsy. Serum or CSF values of BDNF would be potentially useful, but they are unlikely to be as informative as mossy fiber BDNF levels. To examine mossy fiber BDNF, an animal model would be useful, but there are few animal models that simulate women with epilepsy. Indeed, most animals with recurrent seizures lose reproductive function. However, the data from female rodents that have been administered estradiol suggest that seizure threshold declines in response to estradiol administration (but not always). A mechanism that is dependent on BDNF/trkB has not been explored.

Future Goals

It has been widely hypothesized that BDNF and trkB are proconvulsants, and contribute to epileptogenesis. Exactly how they do so, and how critical they are, remain to be seen. If one could downregulate BDNF in the epileptic brain, would that manipulation stop seizures? These questions become a priority for future studies. Unfortunately, specific antagonists to BDNF and trkB are critical to address these questions, but limited in availability.

Another priority is to establish how important mossy fiber BDNF is to epileptogenesis. Silencing the mossy fiber pathway selectively could address the issue, but compensatory changes could confound such an experimental approach.

A third question is whether women with epilepsy have seizures at particular times when estrogen rises because of BDNF induction. An animal model of epilepsy that replicates the female condition would help address the issue. So also would be a way to block – selectively – the response element on the BDNF gene. Unfortunately, this approach is difficult because most animal models of epilepsy in female rodents lead to a loss of reproductive function.

Finally, if BDNF and trkB are critical, what about the other neurotrophins? Mossy fibers also express NT-3, and seizures appear to downregulate NT-3 when they upregulate BDNF. NT-3 also appears to regulate other neurotrophins. It may be necessary to consider not only BDNF but also other neurotrophins in order to gain a complete perspective and address therapeutic strategies with the best chances of success. There are many hypotheses still to be tested, exploring the potential for new therapeutic approaches to epilepsy based on BDNF mechanisms.

See also: **Neurogenesis**: Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; **Neurotrophic Factors**: Activity-Regulated BDNF Expression: Contributions to Synaptic Plasticity and Neuroprotection; Neuroprotective Strategies in Epilepsy; Role of BDNF in Animal Models of Epilepsy.

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Neuroprotective Strategies in Epilepsy

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Introduction

From the earliest attempts to develop treatments for epilepsy, the primary goal of epilepsy therapeutics has been seizure control. Since seizures are not only pathognomonic for epilepsy, but also represent a significant problem for patients, this strategy has been both rational and fruitful. However, in spite of the development of a wide array of anticonvulsant drugs, some patients have poorly controlled seizures while many more report breakthrough seizures. Efforts to seek better anticonvulsant treatments are ongoing, and these efforts have been accompanied by a concurrent effort to protect the brain from the damaging effects of seizures.

Using animal models, epilepsy researchers have discovered that seizures lead to permanent changes in the brain circuitry. Some of these changes, such as mossy fiber sprouting, could potentially be associated with seizure progression. In addition, uncontrolled seizures may cause a loss of brain cells. Humans with chronic temporal lobe epilepsy (TLE) show neuronal loss, sclerosis, and atrophy, especially in the hippocampus. In animal models, the occurrence of severe seizures leads to cell loss in various regions of the brain, especially the limbic system. As illustrated in **Figure 1**, the hippocampus of rodents has proved to be exquisitely sensitive to the excitotoxic cell death induced by seizures, and this cell loss in the animal model reflects the damage profile seen in human TLE. Behavioral studies in animals have also shown that seizure-related cell loss is associated with functional impairments, such as memory decline and emotional deficits, similar to findings in humans where epilepsy has been associated with functional impairments ranging from memory loss to alterations in mood. Protecting neurons in the human epileptic brain may protect against functional impairments.

As a consequence of the realization that seizures beget neuronal loss and behavioral impairment, epilepsy researchers are increasingly interested in pursuing neuroprotective strategies in epilepsy. Protecting neurons from death or functional compromise after seizures would, in the short term, prevent some of the far-reaching consequences of breakthrough seizures. In the longer term, the optimal treatment for epilepsy would simultaneously prevent seizures and protect neurons from the damaging consequences of seizure activity.

Background

Neurotrophic Factors and Neuroprotection

The past two decades have seen a surge in interest in the potential of protein growth factors to protect cells throughout the body. From the perspective of an epilepsy researcher, those protein factors capable of protecting cells in the nervous system, termed neurotrophic factors,

Plasticity: Dendritic Abnormalities in Epilepsy; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy.

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NON-SYNAPTIC MECHANISMS

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Changes in Extracellular Ionic Composition

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Introduction

All neurons and glia maintain an electrical potential across the cell membrane, which is negative relative to the extracellular environment. This negative potential provides a driving force for charged particles to move across the membrane. Every nerve impulse is dependent on this movement of ions. Most of these ions move through ion channels, along concentration gradients generated by electrogenic pumps, but some ions move across membranes on transporters. Abnormalities in the control of ionic movement across membranes can result in abnormalities in neuronal excitability.

With neuronal activity, action potential firing results in movement of potassium out of the cell and sodium into it. A single action potential will result in little change in the ion concentrations in the extracellular space. During seizures (i.e., intense neuronal activity), however, the amount of potassium moving out of neurons exceeds the ability of the brain to regulate precisely the ion concentrations, and the extracellular potassium will increase from a resting level of around 3 to 10–12 mM.

At the same time calcium, magnesium, sodium, and chloride enter cells, and the extracellular concentrations of these ions will decrease. After any activity that alters the ionic gradients, there needs to be 'correction' of the concentrations back to the baseline, to 'resting' state. Potassium, which is elevated, can diffuse away, can be taken up into neurons or glia by active pumps, or can be taken up by passive processes into astrocytes. Ions that are low in the extracellular space (Na^+, Ca^{2+}, Mg^{2+}, Cl^-) can be adjusted by diffusion back into the area of low concentration, or ions can be pumped out of cells back into the extracellular space.

Astrocytes are the most numerous type of glial cell. They are involved in the maintenance of the blood–brain barrier, in the regulation of water and ion homeostasis, and in metabolism of amino acid transmitters. Astrocytes are proposed to play a major role in the maintenance of the extracellular space, including the concentrations of the various ions. In particular, astrocytes are thought to contribute a significantly to the removal of excess potassium through glial 'spatial buffering,' through activation of the glial Na–K pump, and via activation of inwardly rectifying potassium channels. Astrocytes also have sodium, chloride, and bicarbonate transporters and exchangers that contribute to the regulation of these ions in the extracellular space. And astrocytes express multiple calcium channels, suggesting that they also play a role in regulation of calcium levels.

Background

Since ion-selective electrodes have been available, a number of investigators have measured changes in extracellular potassium in a several brain regions during neuronal activity. During seizures or intense neuronal activity, the extracellular potassium increases to 10–12 mM. In the hippocampus, the peak potassium levels measured during seizures are slightly higher in the dentate gyrus than in CA1, but overall the changes are similar. At the same time as the potassium is increasing, calcium, magnesium, sodium, and chloride enter cells, thus lowering the extracellular concentrations of these ions. As a result of these ionic shifts, the extracellular space shrinks and becomes acidic.

Increases in extracellular potassium ($[K^+]_o$) are known to increase neuronal excitability. The small increases in $[K^+]_o$ measured with interictal bursts have been postulated to contribute to the transition between interictal and ictal activity. The increases in $[K^+]_o$ have also been shown to facilitate spread of seizure activity to nearby and synaptically connected regions. Interestingly, during seizure activity, the $[K^+]_o$ increases to a ceiling of about 10 mM – it does not continue to rise with ongoing neuronal activity. This observation suggests that the mechanisms that are clearing the $[K^+]_o$ are keeping pace with the amount of potassium moving out of the neurons with each action potential. Understanding the mechanism(s) that clear the potassium from the extracellular space would lead to an understanding of how the brain can regulate neuronal excitability.

Intracellular and extracellular pH changes may have fundamental importance in regulating neuronal excitability. Small changes in intracellular and extracellular pH have been shown to alter the function of ligand- and voltage-gated channels. Since transient pH changes may be confined to localized regions of the extracellular space, these pH transients may play a role in signaling by altering neuronal excitability locally. Movement of hydrogen ions across membranes in the central nervous system is controlled largely by two families of acid–base transporters. The Na/H (NHE) and the bicarbonate-dependent transport families are a diverse group of transport proteins. The sodium/HCO_3 cotransporter (NBC) is found in glia throughout the hippocampus and in the granule cells of the dentate gyrus, supporting a role of glia in the regulation of pH in the hippocampus and providing a possible explanation for the differential pH regulation in the dentate gyrus compared to CA1. In one model of pH regulation, neuronal activity causes an increase in extracellular potassium and depolarization of astrocytes, which stimulates the movement of H^+ out of the astrocytes; as a result, the intracellular space within the glia becomes more alkaline while the extracellular space becomes more acidic. The combination of the regional diversity in pH regulation and the sensitivity of neuronal excitability to small changes in extracellular pH suggest that pH regulation may play a part in spread of epileptiform activity.

Intracellular and extracellular calcium also contribute to neuronal excitability. Normally intracellular calcium is maintained at very low levels, but measurable increases occur in neurons with action potential firing. Changes in extracellular calcium are somewhat more complex, in that neuronal activity alone does not necessarily result in significant and sustained decreases in calcium. However, any decrease in extracellular calcium would increase neuronal excitability, in part due to a loss of surface charge screen. Decreasing calcium in the perfusion solution results in multiple population spikes in CA1 in response to a single stimulation, indicating multiple action potential firing instead of the normal single action potential. If the extracellular calcium decreases below 0.5 mM, spontaneous epileptiform activity has been reported. In the presence of small elevations in extracellular potassium, neurons in the hippocampus are more sensitive to small decreases in calcium.

Changes in extracellular magnesium, sodium, and chloride have not been studied to the same extent as potassium, calcium, and pH. However, during epileptiform activity, decreases in extracellular sodium and biphasic changes in extracellular chloride have been measured.

Lowering of the extracellular magnesium would be predicted to increase transmitter release from presynaptic terminals, thus increasing neuronal excitability. Lowering of magnesium would also decrease the surface charge screen, and if the decrease is sufficient, NMDA receptors may be affected. Since chloride concentrations affect function of GABA receptors, alterations in the extracellular chloride could produce changes in inhibitory function.

Methods

There have been two major approaches to studying changes in extracellular ion concentrations and their roles in epileptogenesis. One is to monitor ion concentrations (in either anesthetized rats or in in vitro brain slice preparations) with ion-sensitive electrodes – before, during, and after seizure activity induced by any of a number of methods. The second approach is to alter the extracellular ion concentration to determine the effect this manipulation has on epileptiform activity. The latter procedure is most commonly done in in vitro systems of brain slices or organotypic cultures.

Recording of extracellular ion concentrations is carried out using double-barrel ion-sensitive electrodes. Double-barrel electrodes are prepared by silanizing one barrel of the electrode to make it hydrophobic and then filling just the tip of that side with an ion-exchange resin for the ion of interest. The remainder of the electrode is then filled with a solution containing the ion to be measured. The other side of the electrode is filled with a saline solution for recording of the extracellular field potentials. The electrode is calibrated in a series of standard solutions around the expected concentrations of that ion in the brain. The electrode is placed into the brain region of interest, and the ion signal is determined by a differential amplifier. Both barrels of the electrode measure the field potential changes, and the side with the ion-exchange resin also measures the concentration of that particular ion. The differential amplifier then subtracts the field potential from the additive signal, providing an output that reflects the changes in ion concentration. These double-barrel electrodes have been used in both in vivo and in vitro preparations. A newer method involves insertion of one barrel (of a 'double electrode') inside a second barrel. This modification shortens the effective column length of the ion-exchange resin, which improves the response time of the electrode. However, because of the physical properties of this electrode, it works well only in in vitro preparations. Using either method, one can record changes in extracellular ions before, during, and after epileptiform activity.

The effects of changes in extracellular ions can be determined in in vitro systems by altering the perfusion solution to levels of ions recorded in vivo. For example, when recording extracellular calcium in vivo, it was determined that the calcium falls to various levels during different types of epileptiform activity. These levels of extracellular calcium can then be reproduced (in isolation) in vitro to determine the significance of these changes. One can ask, for example, if this lower level of calcium is sufficiently low to block synaptic transmission, or only low enough to increase excitability. Such information is, in turn, used to address the question of whether/how the changes in physiological function due to the change in ionic concentration contribute to epileptiform activity. Of course, changing levels of only one ion does not mimic the situation in vivo, where many ions will change simultaneously during epileptiform activity. However, combinations of ion manipulations can be reproduced in in vitro preparations.

Recent Results

It is well known that extracellular potassium concentrations ($[K^+]_o$) increase during intense neuronal activity, including seizures (Figure 1). Glial cells are believed to play a major role in the regulation of the $[K^+]_o$ increase. Using ion-selective electrodes, we examined the $[K^+]_o$ changes in the dentate gyrus of urethane-anesthetized adult rats, comparing the changes in the presence of reactive astrocytes and after reduction of glial function. The regulation of $[K^+]_o$ in the dentate gyrus was determined by measuring the ceiling level of $[K^+]_o$ and the half-time of recovery of $[K^+]_o$, during and after seizures produced by 20-Hz trains of stimulation to the angular bundle. Reactive astrocytes were induced by repeated seizures and their presence was confirmed by a qualitative increase in glial fibrillary acidic protein (GFAP) and appearance of vimentin immunoreactivity. In the presence of reactive astrocytes, there was no significant change in the peak $[K^+]_o$ during seizures, but there did appear to be

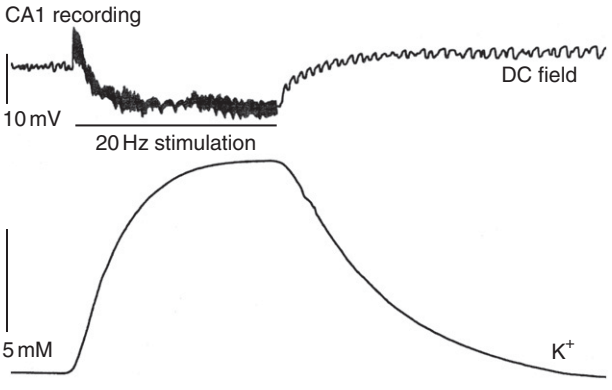


Figure 1 Field recording in CA1 of the extracellular field potential (top) and the extracellular potassium concentration (bottom) before, during and after a 20 Hz stimulation to the contralateral CA3 region in vivo.

a trend toward a faster recovery of the $[K^+]_o$ toward baseline as compared with control animals. To inhibit glial function, fluorocitrate (FC), a reversible metabolic inhibitor, was injected into the dentate gyrus region and the regulation of $[K^+]_o$ was monitored for 8 h. Fluorocitrate significantly slowed the rate of recovery of $[K^+]_o$. Overall, the results suggested that normal glial function is required for the recovery of elevated $[K^+]_o$ after seizures in vivo.

It was somewhat surprising that the ceiling level of $[K^+]_o$ was not elevated after loss of astrocytic function. At this ceiling level, it is presumed that the potassium is in a state of active equilibrium (or steady-state). Active neurons release potassium, which is then taken up by neurons or glia at the same rate. Based on this hypothesis, it would be predicted that changing glial cell function (either increase or decrease) would result in a change in the ceiling level of $[K^+]_o$ during seizures. The results of these experiments did not support this hypothesis. In addition, although there was a statistically significant change in the rate of recovery of $[K^+]_o$ after seizures when glia function was altered, the change was relatively small. One must wonder what physiological significance this small change would have on neuronal function or excitability.

In order to investigate further the mechanisms that might contribute to regulation of activity-dependent variations in $[K^+]_o$, we examined spontaneous recurrent epileptiform activity induced in the dentate gyrus of hippocampal slices from adult rats. Seizures were induced by perfusion with 8 mM potassium and 0-added calcium medium. Local application of TTX blocked local $[K^+]_o$ changes, suggesting that potassium is released and taken up locally. Perfusion with barium (0.2–0.3 mM) or cesium (1–3 mM), blockers of the inward rectifying potassium channel that is postulated to mediate glial uptake of potassium, did not alter any of the measures of $[K^+]_o$ regulation. If gap junctions between glial cells are critical for the spatial buffering of the $[K^+]_o$, then reduction of gap junction conductance should alter $[K^+]_o$ regulation. Decreasing gap junctional conductance only decreased the ceiling level of $[K^+]_o$. Perfusion with furosemide (which blocks cation/chloride cotransporters) or perfusion with low chloride increased the ceiling level of $[K^+]_o$. Bath or local application of ouabain, a Na^+/K^+-ATPase inhibitor, increased the baseline $[K^+]_o$, slowed the rate of $[K^+]_o$ recovery, and induced spreading depression. Together, these findings suggest that potassium redistribution by glia plays only a minor role in the regulation of $[K^+]_o$ in this model. The major regulator of $[K^+]_o$ in this model appears to be uptake via a Na^+/K^+-ATPase, most likely neuronal.

Studies of changes in hydrogen ion concentration (pH) have proved to be quite interesting, in part because the changes during seizure activity are not the same in all brain regions. It is clear that neuronal activity results in alterations in both intracellular and extracellular pH. The measured changes in extracellular pH are within the range of pH values that have been shown to alter neuronal activity. In vivo and in vitro, in the CA1 region, recurrent epileptiform activity induced by stimulus trains, bicuculline, and kainic acid results in biphasic pH shifts, consisting of an initial extracellular alkalinization followed by a slower acidification (**Figure 2**). In CA1, the acidification consistently peaks after termination of the after-discharge, and the peak level of acidification correlates with the duration of the after-discharge, suggesting a contribution of neuronal activity to the level of acidification. Both GABAergic and glutamatergic neurotransmission have been reported to cause alkalinization. In the CA1 region, this alkalinization was decreased by inhibition of carbonic anhydrase and increased by addition of GABA agonists (diazepam and phenobarbital), which is consistent with a key contribution to pH changes by activation of $GABA_A$ receptors (rather than through activation of glutamatergic receptors). The observation that pH fluctuation is linked to synaptic transmission is supported by the observation (in slices) that, in nonsynaptic conditions, there is no initial alkalinization. In the dentate gyrus in vivo and in vitro, seizure activity induced by stimulus trains to the angular bundle, by injection of either bicuculline or kainic acid, or in in vitro nonsynaptic conditions was only associated with extracellular acidification.

The role of astrocytes in these extracellular pH changes during neuronal activity was examined using local injection of fluorocitrate and fluoroacetate into the CA1 cell layer, where there is both alkalinization and acidification. Both glial toxins reduced the peak level of acidification, without changing the lengthening of the after-discharge. The peak level of acidification was still correlated with the total discharge duration, but the levels of acidification were consistently lower than in control animals. Administration of either glial toxin had no effect on the peak alkalinization during the stimulus train, on the rate of recovery from peak level of acidification, or on the amplitude or frequency of the neuronal discharge during the after-discharge. These results suggest that,

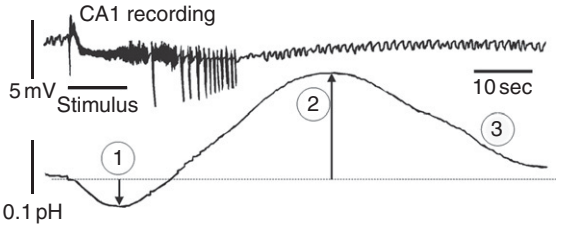


Figure 2 Field recording (top) and extracellular pH (bottom) are shown before, during and after a stimulus train to the contralateral CA3 region in the hippocampus in vivo. Early on (1) synaptic activity results in alkalinization. The peak acidification (2) is dependent on the after-discharge duration. The recovery phase (3) is the least well understood.

in normal conditions, astrocytes contribute to the acidification of the extracellular space that occurs in response to intense neuronal activity. Such acidification may contribute to feedback regulation of neuronal excitability.

Changes in extracellular calcium ($[Ca^{2+}]_o$) have also been studied in the hippocampus in vivo. In urethane-anesthetized Sprague-Dawley rats, changes in $[Ca^{2+}]_o$ were measured in response to stimulus frequencies of 10 and 20 Hz. Maximal changes in $[Ca^{2+}]_o$ were recorded in the cell body layers of CA1 and the dentate gyrus. In the dentate gyrus there was a secondary drop in $[Ca^{2+}]_o$ with the appearance of maximal dentate activation (appearance of the large amplitude population spikes, Figure 3); that is, the largest changes in $[Ca^{2+}]_o$ in the dentate gyrus were recorded during maximal dentate activation. To investigate the role of astrocytes in regulation of in $[Ca^{2+}]_o$, local injections of fluorocitrate, a metabolic toxin selectively taken up by astrocytes, were used. FC, but not vehicle, caused a small but significant decrease in the maximal changes in $[Ca^{2+}]_o$ in CA1, but an increase in the dentate gyrus. The results suggest that astrocytes play a minimal role in the regulation of $[Ca^{2+}]_o$ during epileptiform activity.

Future Goals

Because concentrations of the various ions in the extracellular space clearly contribute to neuronal excitability, understanding the mechanisms by which these ions are regulated will further our understanding of the manner in which neuronal excitability can be controlled. This insight is especially important for understanding the conditions in which seizures begin and how seizures are terminated. Action potentials open channels, allowing ions to flow down concentration gradients, resulting in changes in the extracellular ion concentrations. Much of the work in the past has focused on the significance of these changes in ionic concentrations on neuronal function. But it is also important to determine the mechanisms by which the ion concentrations are returned to the baseline levels, the 'recovery' of the ion concentrations, especially after intense neuronal activity such as a seizure.

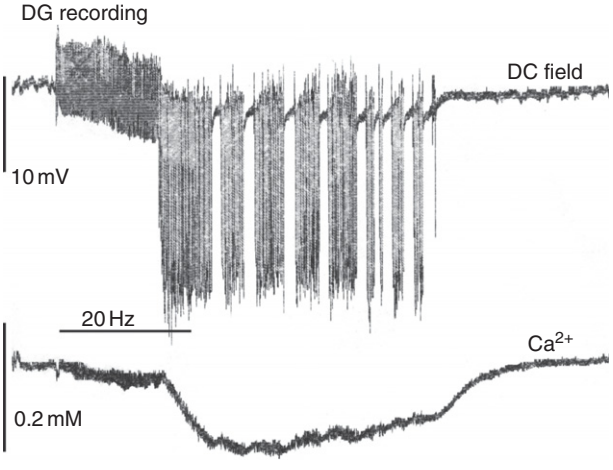


Figure 3 Extracellular DC field recording (top) and extracellular calcium concentration (bottom) in the dentate gyrus (DG) before, during and after a 20-Hz stimulus train to the contralateral CA3 region in vivo.

Largely because they do not fire action potentials, the study of glial cells and their role in brain function is an area of investigation that has lagged behind the study of neurons. However, it is now clear that glia contribute to neuronal function. One way in which they can control neuronal excitability is in the maintenance of the extracellular environment. There are many unanswered questions about the relationship between glial function in controlling extracellular ion concentrations and neuronal activity. For example: Does the glial spatial buffer for potassium slow the recovery of the potassium gradient that is necessary for normal neuronal function? Can the astrocytes regulate extracellular calcium concentrations – and if so, how does that affect neurotransmitter release?

Are there changes in the mechanisms that regulate the extracellular environment in a chronically epileptic brain (compared with 'normal' brain), and do these changes contribute to the epileptic state? It is known that a number of ion channel mutations are present in families with epilepsies. Do these channel mutations alter the ionic composition in the extracellular space? Channel biophysics would suggest that a mutation that results in a channel staying open longer would allow more ions to flow through that channel. But would this difference in ion flow contribute to the seizures in this instance, or are the seizures arising from another mechanism? It is known that a number of pumps, transporters or cotransporters are responsible for the maintenance of ion gradients. One could postulate that a single nucleotide change in a transporter protein could result in a shift in the efficiency of the transport, resulting in an increased (or decreased) seizure threshold.

Finally, are there methods by which one could intervene to influence the regulation of these ion gradients, and thus to reduce the likelihood of a seizure starting – or to augment the ability of the brain to stop a seizure once it has started? For example, more rapid or efficient transport activity could shorten the post-ictal state and improve recovery after a seizure.

See also: **Glia/Astrocytes**: Role of Astrocyte Dysfunction in Epilepsy; **Hormones and Gender**: Hormones and Absence Epilepsy; **Imaging**: Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In vitro; **Metabolic Changes**: Role of Metabolic Substrates and Acid-Base Balance in Seizures; **Non-Synaptic**

Mechanisms: Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; **Seizures**: Seizure Initiation and Termination; **Treatments (non-Antiepileptic Drugs)**: Ginseng and Other Herbal Treatments for Epilepsy.

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http://www.brain-map.org/welcome.do – Allen Institute for Brain Science includes The Allen Brain Atlas, which is an interactive, genome-wide image database of gene expression in the mouse brain. A combination of RNA in situ hybridization data, detailed reference atlases and informatics analysis tools are integrated to provide a searchable digital atlas of gene expression.

Changes in the Extracellular Space as a Modulator of Excitability and Epileptogenicity

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Introduction

The global behaviour of a neuronal population is largely determined by its connectivity patterns, established through chemical synapses and gap junctions. The excitability of individual neurons within a network is usually assumed to be fixed once their synaptic inputs and distribution of ion channels are specified. In view of this assumption, it is not surprising that the vast majority of the research on basic mechanisms of, and therapeutic treatments for, epilepsy focuses on synaptic mechanisms and ion channel properties.

Another means through which neurons can communicate is the extracellular space (ECS). Although the role of the ECS in epilepsy has been much less studied than synaptic mechanisms, it has long been hypothesized that alterations of the ECS can modulate neuronal synchrony and excitability – through nonsynaptic mechanisms. ECS-dependent mechanisms that may play important roles in epilepsy include changes in the electrical resistivity of brain tissue, local ephaptic interactions, long-range electric field effects, changes in extracellular ion concentrations, and the diffusion pathways of neuromodulating molecules.

The size of the ECS is a dynamic entity that can undergo both long-term and short-term changes. Two important measurable variables used to quantify changes of the ECS are (i) extracellular volume fraction (EVF), a measure of the relative volume of the tissue that is available for the diffusion (between cells) of extracellular substances and (ii) tortuosity, a measure of the increase in the diffusion path length between two points in the tissue, beyond what it would be without any pathway barriers. The baseline values of EVF and tortuosity, as well as their susceptibility to change, can vary significantly between

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different anatomical regions. These region-specific differences are due, in part, to differences in cell packing and geometry, the relative proportion of glial cells to neurons, and metabolic rates. For example, Nicholson and colleagues have reported that the resting EVFs of the CA1 and CA3 regions in hippocampal slices are approximately 0.14 and 0.20, respectively – a difference that reflects the denser cell packing in the CA1 region. Pathological conditions, such as those observed during ischemia, have been noted to cause rapid and distinctly different effects on the EVFs and tortuosities of the different hippocampal regions. In particular, ischemia causes a dramatic reduction of the EVF in area CA1 with little effect on its tortuosity, whereas the tortuosity of area CA3 significantly increases with much smaller effects on its EVF. It might be expected that conditions which favor a significant reduction in the EVF in a region may enhance electric field interactions between neurons, by increasing the electrical resistivity of the tissue and by bringing neuronal cell bodies closer together. In contrast, conditions that favor an increase in tortuosity of a region may enhance the duration and magnitude of transient increases in extracellular potassium, since the K^+ is less capable of dissipating through passive diffusive forces.

Background – Overview of Mechanisms and Evidence of ECS Modulation of Epileptogenicity

Specific ECS-related mechanisms are often described as if they are independently acting mechanisms. However, it is important to note that ECS-dependent mechanisms are coupled, interdependent, and work in parallel with each other and with synaptic mechanisms. For example, changes in extracellular potassium concentrations following large increases in neuronal activity may cause a reduction of the size of the ECS that can further concentrate extracellular ions and increase ephaptic interactions, thus leading to an additional enhancement of neuronal firing and synaptic activity.

Modulation of Epileptogenicity Through Osmolarity Effects on the ECS

It has long been noted in the clinical literature that decreases in plasma osmolality, which presumably lead to cell swelling and a decrease of the ECS, increase electrographic brain activity and the likelihood of developing seizures. Examples of this phenomenon are seen in patients suffering from hyponatremic seizures resulting from water intoxication. The acute hyponatremia, induced by the dilution of extracellular fluids, leads to significant cerebral cell swelling. In contrast, increased plasma osmolality has been observed to suppress seizure susceptibility. In support of this notion, it has been reported that intravenous administration of large quantities of osmolytes such as urea can stop status epilepticus in human patients.

The effects of changes in osmolality can be reproduced and studied in a variety of experimental settings. Osmolytes such as mannitol, when administered intravenously in sufficient quantities, can block kainic acid induced status epilepticus in rats and suppress interictal spiking in human subjects. Epileptiform activity in various in vitro slice models can also be up- or down-regulated by decreasing or increasing the osmolarity of the perfusion medium. Observations that osmolarity alters both the size of the ECS and epileptogencity in parallel have been interpreted as supporting the view that the size of the ECS itself may directly modulate epileptogenicity.

Extracellular Ion Concentrations

Changes in the extracellular concentrations of all ion species are associated with increases in neuronal activity. For example, in vivo rat studies with ion-selective microelectrodes have shown that normal increases in neuronal activity in the hippocampus and neocortex can result in the increase of extracellular potassium concentrations ($[K+]_0$) from 3 to 5–6 mM, and intense epileptic activity can further increase this concentration to 12 mM. At the same time, extracellular calcium ($[Ca^{2+}]_0$) can drop from its resting levels of 1.0–1.4 mM to as low as 0.6 mM with intense neuronal activity. In vitro slice studies by Stringer and Lothman have shown that simultaneous alterations of $[K^+]_0$ and $[Ca^{2+}]_0$ can work synergistically to modulate epileptogenicity. Even moderate alterations of the concentrations of these ion species together can dramatically influence the propensity of tissue to develop spontaneous discharges. Observations such as these suggest a number of possibilities through which the ECS and ion concentrations can interact to modulate excitability and epileptogenicity. For example, an impairment of the ion homeostatic mechanism in the ECS, as observed in some experimental seizure and trauma models, might increase seizure susceptibility. Additionally, conditions that lead to a significant alteration of the ECS, such as changes in neuronal firing activity or in osmolarity, in effect, change the concentration of extracellular ions by shifting water between intra- and extracellular compartments.

Cation-Dependent Chloride Cotransport

In vitro hippocampal slice studies (using tissue from adult rats) have shown that the loop diuretic, furosemide, a cation chloride cotransporter antagonist, blocks epileptiform activity in a wide variety of seizure models, regardless of the underlying synaptic mechanisms. For example, furosemide blocks epileptiform activity in the 'zero-calcium' model in which all chemical synaptic activity is

absent, and in which the epileptiform discharges must be mediated entirely through mechanisms other than chemical synapses. Other in vitro studies have shown that furosemide diminishes glial cell swelling during conditions of increased $[K^+]_0$, and blocks decreases in the ECS of hippocampal slices during stimulation-evoked and epileptiform discharges. Some evidence suggests that the furosemide blockade of epileptiform activity occurs concomitantly with the blockade of activity-evoked cell volume and ECS changes.

Taken together, these results suggest that cation chloride antagonists affect some mechanism common to all the tested seizure models (even those that are not dependent on chemical synaptic interactions) that is necessary for the maintenance of epileptiform activity. The antiepileptic effects of furosemide may be related to its action on an ECS-dependent mechanism, such as its blockade of activity-evoked cell swelling.

Ephaptic and Other Electrical Interactions

There are several ways in which neurons that are not physically connected through gap-junction or chemical synapses can interact electrically through the ECS. One such type of electrical interaction, called ephaptic coupling, is usually meant to describe electrical interactions between neuronal processes that are in close apposition. This interaction occurs when the extracellular potential generated by a neuron causes depolarization of other nearby neurons. Ephaptic interactions are increased with increasing electrical resistivity of the tissue. Ephaptic interactions may be an influential neuronal synchronizer under conditions where the resistivity of the tissue is significantly increased or when neuronal processes are brought closer together. Such changes would occur when water moves from the ECS into intracellular compartments, causing cell swelling and the reduction of the EVF. Intense neuronal activity and increases in plasma osmolality are two examples of conditions that can lead to enhanced electrical coupling through this mechanism.

Other types of longer-range electrical effects are also present in neuronal tissue, and are usually referred to as electric field interactions. Electric fields generated by the activity of a subpopulation of neurons have been shown to influence the excitability of inactive neurons, nonsynaptically, by decreasing their membrane potentials and thus bringing them closer to threshold for action potential generation. For example, intracellular recordings of transmembrane potentials of CA1 hippocampal neurons have demonstrated that population spikes can excite inactive neurons within the population. Evoked field potentials in laminar structures such as hippocampus and neocortex can be relatively large, reaching over $50\,\mathrm{mV\,mm^{-1}}$ in hippocampus. Electric field oscillations, such as 1 Hz slow waves observed in neocortex, can reach several millivolts per millimeter. Under normal conditions, the amplitudes of these types of electric field effects are not of sufficient magnitude to elicit action potential firing from surrounding neurons. However, they may contribute importantly to action potential firing and the spread of epileptic activity under conditions in which the excitability of neurons has already been increased through other means. Shrinkage of the ECS following intense neuronal activity has been shown to enhance these electric field effects, enabling them to elicit action potentials from neurons that would otherwise be below their firing threshold.

Effects of Osmolarity and Cell Volume Changes on Neuronal Ion Channels

The transition of an ion channel between its open and closed states can be thought of as a change between two conformational states of a protein. A channel spanning a cell membrane has an aqueous core with an internal osmolarity that is different from the osmolarities of the solutions comprising the intra- and extracellular spaces. For the channel to open in the presence of solutes inaccessible to its pore, it must do work to extract water from the solutions outside of its opening. The amount of work that a channel must do to transition between these states is determined by an osmotic pressure gradient (i.e., by the differences in the concentrations of solutes between the inside of the channel and its external environments). It is a theoretically intractable problem to calculate how changes in the osmolarity of the ECS might bias the state-preference of a channel, since osmolarity is a concept defined within the framework of equilibrium thermodynamics, whereas the physiological situation is one of nonequilibruim due the different concentrations of solutes within the fluids comprising the intracellular and extracellular compartments. Therefore, empirical measurements are required to determine whether the kinetics of a given type of channel is affected by changes in the osmolarity of the ECS in a way that can influence the excitability of a neuron. In view of this requirement, it is important to test the effects of osmolarity on channel properties in preparations in which in the various subpopulations of neurons and their synaptic contacts are present, and under conditions that mimic the types of osmolarity changes that might be observed in vivo.

Studies performed on hippocampal slices demonstrate that osmolarity can affect properties of specific channels, on subtypes of neurons, in important ways that could modulate network epileptogenicity. Yaari and colleagues have shown that changing the osmolarity of the ECS by 13% can significantly affect the persistent Na^+ current in hippocampal CA1 pyramidal neurons, a current that plays an important role in determining neuronal excitability and synaptic integration. In their studies, a reduction of

the osmolarity of the ECS converted nonbursting pyramidal cells into bursting neurons. Baraban and colleagues demonstrated that decreasing the osmolarity of the ECS by 10% in hippocampal slices resulted in a significant enhancement of a voltage-activated potassium current of inhibitory CA1 interneurons, while leaving the corresponding current in excitatory CA1 pyramidal cells unaffected. This effect would likely reduce the firing activity of inhibitory cells and thus contribute to an overall increase in network excitability.

A number of studies on cultured neurons have demonstrated the presence of volume-sensitive, stretch-activated ion channels. Many cell types undergo a regulatory volume decrease (RVD) in response to cell swelling following a decrease in extracellular osmolarity. A number of mechanisms are known to be involved in mediating the RVD, including changes in the activities of various ion cotransporters and the activation of volume-sensitive K^+ and Cl^- channels. Okada and colleagues have reported that volume-sensitive outwardly rectifying Cl^- channels are activated in cortical neurons during cell-swelling. These channels could contribute to altering neuronal excitability, and have been linked to apoptotic cell death; blockade of these channels prevents swelling-evoked cell death in various types of cultured neurons. It has not yet been determined whether these volume-sensitive anion channels are present and play a role in modulating epileptogenicity and apoptosis in vivo or in acute slice preparations.

Methods – Measuring Ion Concentrations and Properties of the ECS

Quantitative measurements of activity-evoked changes in extacellular ion concentrations, EVF, and tortuosity have most commonly been accomplished with ion-selective microelectrodes (ISMs). A typical ISM consists of a double-barreled glass microelectrode, with the tip of one barrel containing a liquid membrane that allows for selective transport of a specific ion (i.e., the ion exchanger), and a second barrel that is a potential-sensing electrode, used as a reference. The barrel with the liquid membrane tip is filled with an electrolyte solution containing the ion of interest, so that a Nernst potential is generated across the ion-exchanger when the tip of the ISM is immersed in solutions containing different concentrations of the ion. The signal recorded by the potential-sensing reference barrel is subtracted from the one recorded by the ion-selective barrel as a means to compensate for changing background potentials. Ion exchangers are available that make it possible to measure the concentrations of various ions of physiological interest, including K^+, Na^+, Ca^{2+}, Cl^-, and H^+. In practice, ISMs have poor sensitivity for measuring extracellular changes in Na^+ and Cl^- because activity-evoked changes of these ions are relatively small with respect to their ambient concentrations.

ISMs can also be used to measure changes in tortuosity and volume fraction of the ECS. This measurement process involves iontophoresing the membrane-impermeant treamethlyammonium ion (TMA^+) into the tissue at a fixed location, and measuring the concentration-time profiles of this ion with an ISM at a known distance from the TMA^+ source. Software can then be used to fit these data to a solution of the diffusion equation, from which EVF and tortuosity can be numerically estimated. References providing the details involved in making these types of measurements are provided in Further Reading.

Recent Results

Molecular Mechanisms Associated with the ECS

Aquaporin-4 and epileptogenicity

The aquaporins are membrane-bound proteins that function as water channels and are found in a variety of cell types. The presence of these water channels facilitates the transport of water between the intra- and extracellular spaces, and they may play an important homeostatic role under conditions of osmolarity changes. The predominant aquaporin found in the brain is aquaporin-4 (AQP4), which has been found to be expressed in glial cells, lining surfaces that are in contact with cerebrospinal fluid in the ventricular system and subarachnoid space. AQP4 is also expressed in the process of astrocytes that are in contact with blood vessels.

The putative role of AQP4 in epileptogenesis has recently been investigated in knockout mice (AQP4−/− mice). AQP4−/− mice have been found to have higher seizure thresholds than normal mice in response to PTZ and electrical stimulation-evoked seizures. The expression of AQP4 has also been studied in sclerotic hippocampal tissue acquired from patients suffering from medically intractable temporal lobe epilepsy. A significant increase in AQP4 expression has been observed in sclerotic, but not in nonsclerotic hippocampal tissue. These data suggest that alterations in the expression of AQP4 – which likely plays a role in the redistribution of water between cellular, ECS, and ventricular/subarachnoid/vascular compartments during increased neuronal activity – may modulate epileptogenicity.

Volume changes regulating phosphorylation and activity of ion cotransporters

Transient volume changes in the ECS involve the redistribution of water between intra- and extracellular spaces, resulting in either cell swelling or shrinking. There are several cell volume-regulating mechanisms in neurons and glia that may play a role in modulating the epileptogenicity of neuronal tissue. Critical for maintaining

transmembrane chloride gradients in neurons are the Na^+–K^+–$2Cl^-$ (NKCC1) and K^+–Cl^- (KCC2) cotransporters. NKCC2 typically transports solutes, and in particular, chloride, from extracellular to intracellular spaces, using the abundance of extracellular sodium to drive potassium and chloride into cells. Conversely, KCC2 moves solutes from intracellular compartments into the ECS by using the high intracellular potassium concentration to drive chloride against its concentration gradient.

In addition to being important regulators of intracellular chloride (and therefore of $GABA_A$-mediated synaptic events), these cotransporters are thought also to be important regulators of cell volume. Evidence suggests that during cell-swelling, KCC2 becomes upregulated and increases its cotransport activity, becoming more efficient in moving solutes from intracellular into extracellular spaces. Oppositely, NKCC1 becomes less active, reducing the influx of solutes from the ECS. The net result is that intracellular osmolarity is reduced so that that water influx is diminished. When neurons are shrunken below their normal resting state, perhaps from an increase in the osmolarity of the ECS, KCC2 and NKCC1 are again regulated oppositely in such a way as to restore osmotic balance. Several families of protein kinases, including WNK and SPAK, that may play a role in regulating transporter activity have recently been identified; the presence of these kinases themselves may be directly modulated by changes in cell volume. These cotransporter-related mechanisms represent another means through which changes in the ECS can modulate excitability.

Pharmacological and Electrophysiological Investigations of the Role of ECS in Human Epilepsy

Rationale

We have recently begun intraoperative experiments on the effects of ECS-altering treatments in consenting patients undergoing surgical treatment for epilepsy. In this context, we have shown the effects of mannitol and furosemide on spontaneous interictal spiking and stimulation-evoked afterdischarge activity. Mannitol is an inert osmolyte – i.e., it is not significantly metabolized in the body and does not participate in pharmacological interactions; its physiological effects are believed to be limited to the changes it elicits in plasma osmolality. Furosemide is a loop-diuretic and an NKCC1 cotransporter antagonist. As mentioned earlier, evidence from in vitro slice studies suggests that furosemide suppresses epileptiform activity, possibly through its effects on the ECS.

Methods

Several aspects of the usual surgical procedure for the treatment of epilepsy offer a fortuitous opportunity to acquire experimental data directly relevant to the study of ECS alterations. After a craniotomy is performed to expose the cortex, a focal area of interictal activity is localized and an array of EEG recording electrodes is placed on the surface of the cortex. These electrodes allow for the recording of spontaneous interictal spiking directly at its site of initiation, as well as from adjacent sites that show no abnormal activity. Electrophysiological data from the EEG electrodes are acquired before and after i.v. administration of furosemide or mannitol, which are commonly administered during neurosurgical procedures to prevent brain swelling. The EEG data are digitized, and automated software is used to quantify changes in spike frequency and amplitude over time. In some experiments, a stimulating electrode is also placed on the cortical surface to test for the effects of mannitol and furosemide treatments on afterdischarge activity. The cortex is initially stimulated for 4 s with currents of increasing amplitudes. A stimulating current is found that is just sufficient to elicit several seconds of afterdischarge activity (i.e., activity that persists following cessation of the stimulus); this current level is defined to be the 'afterdischarge threshold.'

Results

Four patients were given a single 50-g intravenous injection of mannitol while spontaneous interictal activity was recorded (**Figure 1**). In each experiment, the site of the maximal spontaneous interictal activity was localized, and referred to as the 'interictal focus'; recordings from electrodes 1–2 cm away showed significantly fewer or no interictal spikes. Only those EEG events that were ≥ 3 SD from the average EEG baseline value were counted as spikes. Data were analyzed over two time intervals: (i) a first 20-min duration that was a control period prior to mannitol treatment, and (ii) a final 20-min duration at the end of the 1-h recording session (i.e., starting 40-min after mannitol had been administered). After mannitol administration, the average spontaneous interictal spiking frequencies of the four patients was reduced by ~60%. Several minutes prior to the decrease in interictal activity, there was a noticeable change in the morphology of the interictal spikes; their peak-to-peak amplitudes decreased, with a proportion of spikes losing their biphasic structure and becoming broader. Prior to administration of mannitol, the mean interspike interval was 3.5 ± 0.12 s (99% CI = [3.2 s, 4.0 s]). After mannitol treatment, the mean interspike interval was increased to 8.3 ± 0.36 s (99% CI = [6.3 s, 9.2 s]). A *t*-test rejected the null hypothesis that the mean interspike intervals before and after mannitol administration were the same ($P < 0.0001$). A similar study was performed on five patients in which a single intravenous bolus injection of furosemide (20 mg) was administered (data not shown). Within 10–30 min after administration of furosemide, the

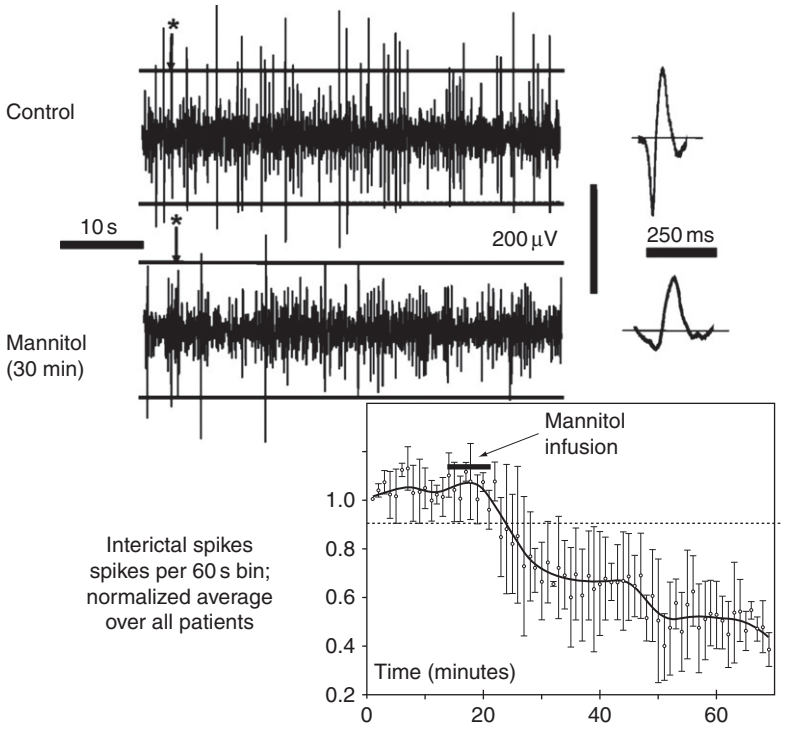


Figure 1 Suppression of spontaneous epileptic spiking activity after mannitol administration. Electrophysiological activity was recorded from EEG electrodes placed on the cortical surface (parahippocampal gyrus) before and after administration of a 50-g intravenous injection of mannitol (data from an individual patient is shown in the top 2 traces, top left). Events that differed from the mean activity by ≥3 SD, indicated by the horizontal lines, were counted as spikes. Mannitol suppressed the frequency of spontaneous activity within 30 min after administration in all patients. Prior to mannitol treatment, all spikes typically had a sharp biphasic waveform (first top right trace). Several minutes prior to the mannitol suppression of the spontaneous activity, many spikes appeared to become broader with diminished peak-to-peak amplitude (second top right trace). The starred arrows on the leftmost side of the traces mark spikes that were plotted at a faster time course (top right traces). A graph of the average number of spikes occurring per minute, averaged over the four patients, is shown bottom right, where a smooth curve was fitted to the data. The black bar indicates the time period over which the mannitol bolus was administered. Error bars show 90% CIs. Reproduced from Haglund and Hochman (2005) *Journal of Neurophysiology* 94: 907–918, with permission from American Physiological Society.

frequency of the interictal spikes was significantly reduced in all five patients, with all spontaneous spiking being abolished during the final 10 min of the recording session in three patients. The average reduction in spiking frequency over these five patients was ~60%. Prior to furosemide administration, the average interspike interval was 9.6 ± 0.65 (SE) s with the 99% confidence interval (CI) of [7.9 s, 11.3 s]. After furosemide treatment, the mean interspike interval was increased to 21.9 ± 3.9 s (99% CI = [11.8 s, 31.9 s]). A *t*-test of the means of interspike intervals before and after furosemide treatment rejected the null hypothesis that the treatment had no effect ($P < 0.0001$).

The effects of furosemide (**Figure 2**) and mannitol treatments on afterdischarge activity were also measured. The duration of afterdischarge activity was defined to be the time during which a train of spikes followed the cessation of the electrical stimulus, where spikes were defined as those EEG events that were ≥3 SD from the mean of the voltage of prefurosemide activity. For each patient ($n = 8$), the minimal current was selected that consistently evoked afterdischarge activity in three consecutive stimulation trials. The furosemide treatment resulted in either a complete blockade (four patients) or in a reduction by >50% (four patients) of the duration and amplitude of the afterdischarge activity. The average reduction in the duration of stimulation-evoked afterdischarge activity over these eight patients was $(85 \pm 14.6)\%$ (mean ± SD). In comparison with the mean duration of the afterdischarge activity before and after furosemide treatment, a Wilcoxon rank-sum test rejected the null hypothesis that the furosemide treatment had no effect ($P < 0.01$). The effects of mannitol were also studied in four patients (data not shown). The mean reduction of afterdischarge duration after mannitol treatments was $(41.8 \pm 8.0)\%$.

Future Goals

Although the roles of the ECS in modulating excitability and epileptogenicity have been investigated over many decades, it has certainly received less attention than

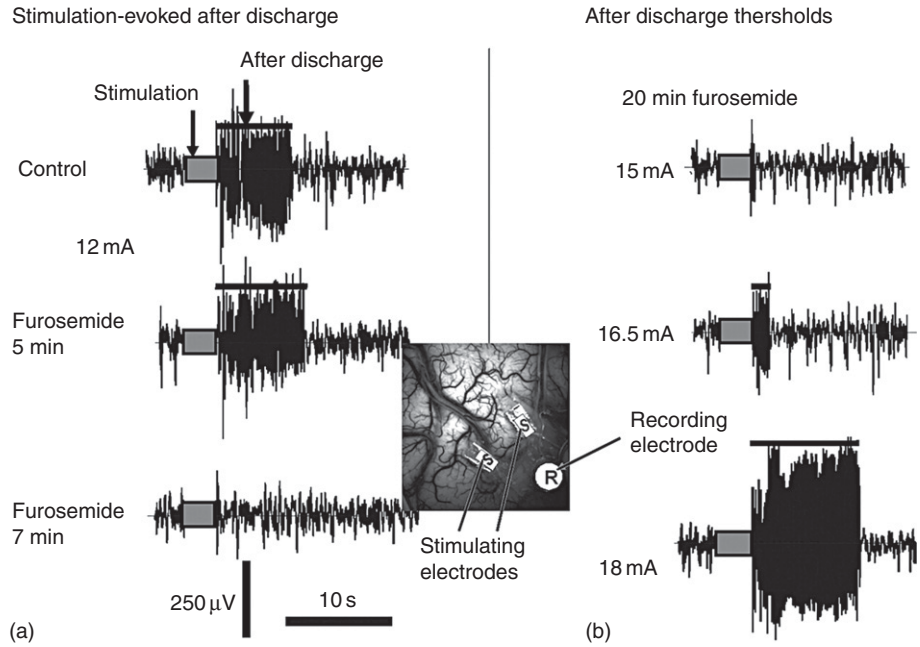


Figure 2 Suppression of electrical-stimulation-evoked epileptic activity in neocortex after furosemide administration. A bipolar stimulating electrode was placed on the cortical surface as shown in the gray-scale image (bottom middle); the distance between the two stimulating electrodes was ~5 mm. A recording electrode was placed within 1 cm of the stimulating electrode. A 4-s stimulus (60 Hz; biphasic; 1 ms per phase) was delivered at various currents; the stimulation duration is represented by gray boxes imbedded in the beginning of the traces. Prior to furosemide treatment, the minimum current necessary to elicit ≥5 s of afterdischarge activity in three consecutive trials was determined; this was defined to be the 'afterdischarge threshold current' (a, top). The black horizontal bars above each trace mark the episode of afterdischarge activity. After furosemide administration, stimulation trials were performed every 2–5 min for the next 40 min. In this patient, the afterdischarge activity was abruptly blocked soon after furosemide treatment (a, bottom). To determine whether the blockade of afterdischarge activity was mediated by an increase in afterdischarge threshold, the stimulation current was incrementally increased (b). It was found that sufficiently large stimulation currents were able to elicit afterdischarge episodes at least as long in duration as those observed prior to furosemide treatment (b, bottom). Reproduced from Haglund and Hochman (2005) *Journal of Neurophysiology* 94: 907–918, with permission from American Physiological Society.

chemical synaptic mechanisms. There is much work remaining to be done in determining the importance of ECS-related mechanisms in modulating excitability and neuronal synchronization. Further elucidation of the regulation and interdependence of the network of ECS-related mechanisms is also required. For example, evidence is accumulating that seizure activity may alter the expression of ion cotransporters and water channels in neurons and glia, and these changes may in turn affect the epileptogenicity of the tissue. There is also some evidence that at least two different splice variants of the NKCC1 cotransporter are expressed in the brain, with functional evidence suggesting that one splice variant is more efficient than the other in transporting ions. It has yet to be investigated whether variations in the expression of these splice variants, or of the protein kinases related to these transporters, play a role in modulating epileptogenicity. Finally, the data presented here support the notion that pharmacologically altered changes in the ECS – e.g., with mannitol or furosemide – can modulate epileptic activity in the human brain. This result suggests that the ECS may be a suitable pharmacological target for the development of novel pharmacotherapeutic treatments of epilepsy.

See also: **Imaging**: Optical Imaging in Epilepsy Research: Overview of Methods and Applications; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Ionic Microenvironment in Living Human Brain and Epileptic Seizures; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties.

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Ionic Microenvironment in Living Human Brain and Epileptic Seizures

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Introduction

Epileptic foci may result from many different pathological conditions. Among the different causes, perturbations of the extracellular microenvironment – including alterations in different ion concentrations – have been postulated to play an important role in epileptogenesis. Ionic gradients are fundamental for the maintenance of normal neuronal function and excitability. Excessive excitation, defective inhibition, energy failure, mechanical trauma, or blood–brain barrier defects drive ion concentrations beyond normal limits. Abnormal ion distributions influence neuronal function, which in turn aggravates abnormal ion distribution. It is important to determine the nature of these pathological ionic distributions, not only as a window into possible underlying causes of the pathology, but also as potential targets for therapeutic strategies.

Background

Extracellular potassium concentration ($[K^+]_o$) is actively maintained within narrow limits in neuronal tissues, a role essentially played by glial cells. The relation of potassium concentration and potassium clearance mechanisms to hyperexcitability of the neurons has been well studied in both animal models and human tissues. It has been known for some time that exposing brain tissues to a high or a low concentration of K^+ can induce epileptiform discharge, and that excess K^+ is released from neurons during seizures. Ion-selective microelectrodes revealed that early during an ictal discharge, $[K^+]_o$ increases to 7–12 mM and stays more or less steady at this elevated level during the tonic phase. This ceiling phenomenon has also been observed during electrical stimulation. Rising $[K^+]_o$ depolarizes neurons and moves the membrane potential closer to firing threshold, thereby increasing excitability. Elevation of $[K^+]_o$ also leads to excitation of neuronal networks because of: (1) the reduction of the potassium electromotive driving force; (2) the impairment of γ-aminobutyric acid (GABA) receptor-mediated activity; and (3) the perturbation of the extracellular space.

A decreased K^+ driving force causes a reduction of outward (hyperpolarizing) K^+ currents. Such changes bring the membrane potential closer to spike threshold and can potentially activate voltage-regulated conductance. Reduction of the K^+ driving force also decreases the repolarizing conductance of action potentials (mediated by both I_A and I_C) and increases action potential duration; at synaptic terminals, this change might enhance the influx

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A decreased K^+ driving force causes a reduction of outward (hyperpolarizing) K^+ currents. Such changes bring the membrane potential closer to spike threshold and can potentially activate voltage-regulated conductance. Reduction of the K^+ driving force also decreases the repolarizing conductance of action potentials (mediated by both I_A and I_C) and increases action potential duration; at synaptic terminals, this change might enhance the influx

of Ca^{2+} into the synaptic terminals and increase transmitter release. An indirect excitatory action of increased $[K^+]_o$ involves the impairment of postsynaptic $GABA_A$ receptor-mediated inhibition by inducing a depolarizing shift in the fast Cl^--mediated IPSP reversal potential.

The various mechanisms that glial cells possess to clear excessive extracellular K^+ also render these cells susceptible to osmotically induced swelling when $[K^+]_o$ becomes too high. Glial cell swelling will consequently lead to a reduction in the extracellular space. Reductions in extracellular space have a number of excitatory actions. Reduction of $[K^+]_o$ causes a transient neuronal hyperpolarization, which is followed by a repolarization and subsequent depolarization period. Epileptiform field potentials appear in the late repolarization or early depolarization phase. Reduction of $[K^+]_o$, as well as associated neuronal hyperpolarization, reduces the efficacy of the Na^+–K^+–ATPase pump. Inhibition of the pump causes intracellular K^+ loss, neuronal depolarization, and initially spontaneous discharges. Na^+–K^+–ATPase pump inhibitors also facilitate the voltage-dependent Ca^{2+} conductance, and thereby neuronal excitability. Reduction of $[K^+]_o$ below mean resting level increases EPSP amplitude, which indicates an enhanced transmitter release. At subnormal levels of $[K^+]_o$, the enhancement of transmitter release may be related to the hyperpolarization of the presynaptic terminals and consequent increases of presynaptic spike amplitude.

Cl^- homeostasis is essential for many cell functions including volume regulation and cell signaling. The regulation of extracellular chloride concentration ($[Cl^-]_o$) has a decisive role in neuronal function, especially by setting the magnitude and direction of the Cl^- flux gated by GABA receptors. The intra-as well as extracellular Cl^- concentration of neurons determines their response to the neurotransmitters glycine and GABA. Glycine, $GABA_A$, and $GABA_C$ receptors are ligand-gated Cl^- channels, and their activation leads to a passive influx or efflux of chloride, depending on the electrochemical potential for Cl^-. Activation of these channels therefore leads to an excitatory, or to the more commonly observed inhibitory, response. Alterations of Cl^- homeostasis and GABAergic signaling might contribute to human epilepsy.

Methods

Despite the many studies on the mechanisms of epileptogenesis based on experiments in human tissue in vitro, no data are available regarding $[K^+]_o$ and $[Cl^-]_o$ in human epileptic tissue in vivo. Microdialysis can be used to monitor changes in the composition of the extracellular fluid in living tissue, providing a means for measuring the concentrations of small molecules such as neurotransmitters, hormones, ions, or peptides. It is a rapidly developing tool for monitoring physiological and pathophysiological changes in human brain. Microdialysis is based on the diffusion of water-soluble substances through a permeable membrane. Depending on the permeability of membrane, the molecular weight of these substances can vary between 5000 and 20 000 Da. A microdialysis device consists of a small polycarbonate tube (length of 10–30 mm) through which flows the so-called perfusion fluid. When the tube is placed into an environment of interest (e.g., the brain), substances from the extracellular fluid diffuse into perfusion fluid. This fluid, the dialysate, is then collected and subsequently chemically analyzed. Concomitant electrophysiological recordings and microdialysis of human brain tissues during epilepsy surgery can reveal the relationship of different substances, such as neurotransmitters and ions, to epileptiform discharges. By using cerebral microdialysis in the human brain, we investigated the basic $[K^+]_o$ and $[Cl^-]_o$, as well as their kinetics, after epicortical electric stimulation in patients with medically intractable epilepsy.

For intraoperative microdialysis, a frame was mounted on the operating table, allowing micromanipulators to be fixed directly over the craniotomy for an exact positioning of the dialysis catheters in the brain tissue. The catheters were guided by metal cannulae that were mounted on plates with 6-mm distance between centers of the catheters. In order to restrict the respiratory and pulsatory movements of the brain, pressure feet were guided by micromanipulators to rest on the cortical surface. This procedure prevents disruption of the cortical tissue by movement against the fixed catheters. When the arachnoid was found to be too rough for easy penetration of the dialysis tubes, it was penetrated by a mandril (pushed through the metal cannulae for not more than 0.5 mm).

For intraoperative microdialysis, CMA 70 Microdialysis Bolt Catheters (CMA Microdialysis AB Solna, Sweden) were used. The membrane is made from polyamide, and the shank from polyurethane. The cutoff permeability of the membrane is 20 kDa. The perfusate was moved by battery-supplied pumps and collected in microvials from CMA, sealed by rubber stoppers on both sides. The flow rate was 2.0 µl min^{-1}, and the sample period was 5 min (for 10 periods). $[K^+]_o$ and $[Cl^-]_o$ were measured at room temperature. Cerebral microdialysis was performed with simultaneous recording of electrocorticographic (ECoG) activity in selected targets in the patients' brains. Epicortical stimulation was applied bipolarly via two electrodes on a conventional strip electrode. The first trains of stimuli (50 Hz, 300 µs, 10 mA, 5 trains, intertrain interval 1 min) were delivered at the start of dialysis period 4 (P4) – that is, 20 min after catheter insertion. The second set of stimulus trains, with an increased stimulation intensity (50 Hz, 300 µs, 5 trains, 15 mA), was applied after another 15 min. The levels of potassium and chloride ions were measured

directly by inserting ion-selective microelectrodes into the microvials.

For preparation of ion selective microelectrodes (ISM), double-barreled borosilicate glass fiber microelectrodes were pulled on a vertical microelectrode puller to a tip smaller than 2–3 µm. One barrel was exposed to hexamethyldisilazane. The Cl^- exchanger or K^+ exchanger was then introduced into the tip of the silanized barrel until the exchanger column reached 50–70 µm in length from the tip. The silanized barrel filled with Cl^- exchanger was further backfilled with 100 mM NaCl, and the reference barrel was filled with 150 mM Na_2SO_4. The ion-sensing barrel contained K^+ ion-exchanger, and the reference channel, were backfilled with 100 mM KCl and 150 mM NaCl, respectively. Electrical signals from the two barrels were monitored with a high-impedance differential electrometer amplifier. The potential difference between the two barrels is logarithmically proportional to the ion activity at the microelectrode tip. The K^+-sensitive electrodes were calibrated in solutions with different concentrations of KCl, with NaCl added to preserve isoosmolarity. The selectivity coefficient of ISM was introduced in the Nikolski–Eisenman equation. Only electrodes with a slope in the range of 54–60 mV per 10-fold change in $[K^+]$ were used. The same calibration process was done for Cl^- sensitive electrodes, using solutions with different concentrations of NaCl in the presence of Na_2SO_4. Chloride-sensitive electrodes with a slope in the range of 56–60 mV per decade change in $[Cl^-]$ were used. Linear-regression analysis of calibration data on pre- and postruns was performed. The potential measured by ISMs (mV) was transferred into concentration (mM) by direct analysis.

Results

The average $[K^+]_o$ in the samples collected immediately after placing the microdialysis catheters into the cerebral tissues was 4.93 ± 0.25 mM. Within 10 min of probe insertion, $[K^+]_o$ was significantly reduced – to 3.83 ± 0.08 mM – and was stable for the next 10 min (**Figure 1**; $p \leq 0.001$). The values of $[K^+]_o$ obtained in periods 1–3 have been regarded as basal levels. The mean basal levels of $[K^+]_o$ in dialysates of 11 patients were significantly different from each other ($p \leq 0.001$; ANOVA). ECoG spike activity,

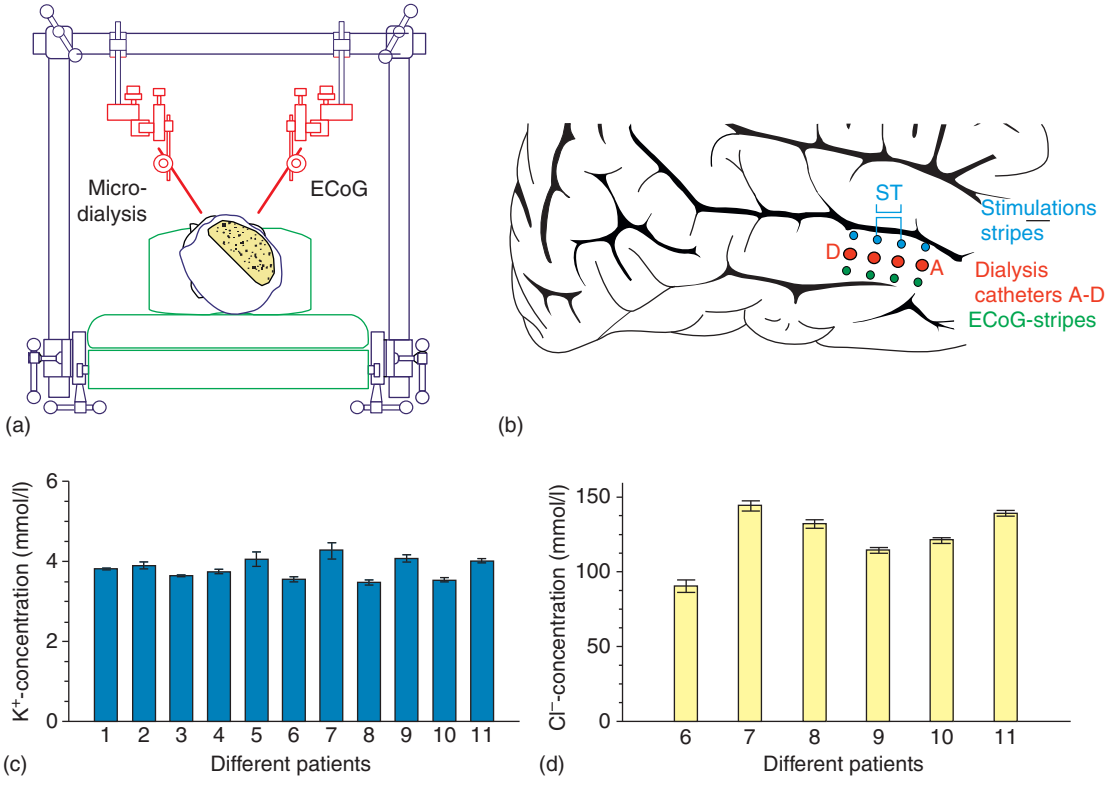


Figure 1 (a) The set-up designed to insert microdialysis catheter and ECoG electrodes during epilepsy surgery. The frame was mounted at the operating table allowing micromanipulators to be fixed directly over the craniotomy for an exact positioning of the catheters and ECoG electrodes in the brain tissue. (b) Schematic of the positioning of dialysis catheters, stimulations stripe, and ECoG electrodes in medial temporal gyrus of epileptic patients during surgery. (c, d) histogram representing baseline levels of extracellular potassium concentration (c; $[K^+]_o$) and chloride (d; $[Cl^-]_o$) in different patients. Dialysates were collected from the medial temporal gyrus of medically intractable epilepsy patients. Results are given as mean ± SEM; n represents the number of samples.

with a rate of 0–3.5 spikes per minute, was observed in most patients (see below for an exception). No correlation was found between the spike frequency and the potassium basal levels. In two patients (patients 3 and 10), $[K^+]_o$ in samples collected at different sites was significantly different ($p \leq 0.003$). In one patient, $[K^+]_o$ ranged between 3.6 and 4.2 mM in different samples collected from various catheters. In the other patient, $[K^+]_o$ varied between 3.7 and 4.1 in different sites. ECoG spike activity recorded close to the area in which different samples were collected did not show any differences in these patients.

Trains of local electrical stimuli applied to the surface of the cerebral cortex increased $[K^+]_o$ in all patients. Application of epicortical stimulation (50 Hz, 300 μs, 10 mA, 5 trains, intertrain interval 1 min) after 20 min of probe insertion induced an increase of $[K^+]_o$ that peaked at 4.55 ± 0.14 mM. The second set of trains, delivered an increased stimulation intensity (50 Hz, 5 trains, 300 μs, 15 mA) 15 min later, increased $[K^+]_o$ to 4.70 ± 0.12 mM.

During microdialysis, ECoG monitoring revealed 0–3 spikes per minute. Epicortical electrical stimulation was accompanied by appearance or enhancement of spike activity recorded by ECoG. Appearance or enhancement of spiking activity lasted 5–10 s after stimulation. In this short period, the repetition rate of spike activity increased to 0.5–4 spikes per second. No correlation was noted between the frequency of spike activity induced by electrical stimulation and the level of potassium enhancement. By 5 min after the stimulation, $[K^+]_o$ returned to the baseline level.

According to the velocity, as well as spatial distribution, of $[K^+]_o$ relaxation to the prestimulation levels, three different types of responses were defined (**Figure 2**).

(i) Slow return to baseline: In three patients, the enhanced $[K^+]_o$ elicited by electrical stimulation gradually decreased and reached the baseline levels within 10–15 min. In all the cases, the slopes of $[K^+]_o$ recovery after the two stimulus trains (delivered at P4 or at P7) were similar.

(ii) Fast return to baseline: In five patients, a transient elevation of $[K^+]_o$ induced by electrical stimulation recovered toward the basal values within 5 min of the last stimulation. In these cases, the dynamics of $[K^+]_o$ recovery was similar after the first (P4) and the second (P7) train of stimuli.

(iii) Fast and slow return to baseline: In three patients, measurement of $[K^+]_o$ in different samples, simultaneously collected in different catheters (i.e., at different sites) revealed a mixture of fast and slow recovery of $[K^+]_o$. In these cases, the elevated $[K^+]_o$ in samples collected by one or two catheters reached the baseline values within 5 min. However, recovery of $[K^+]_o$ in samples from the other catheters, located 6–12 mm apart, was prolonged to 10–15 min.

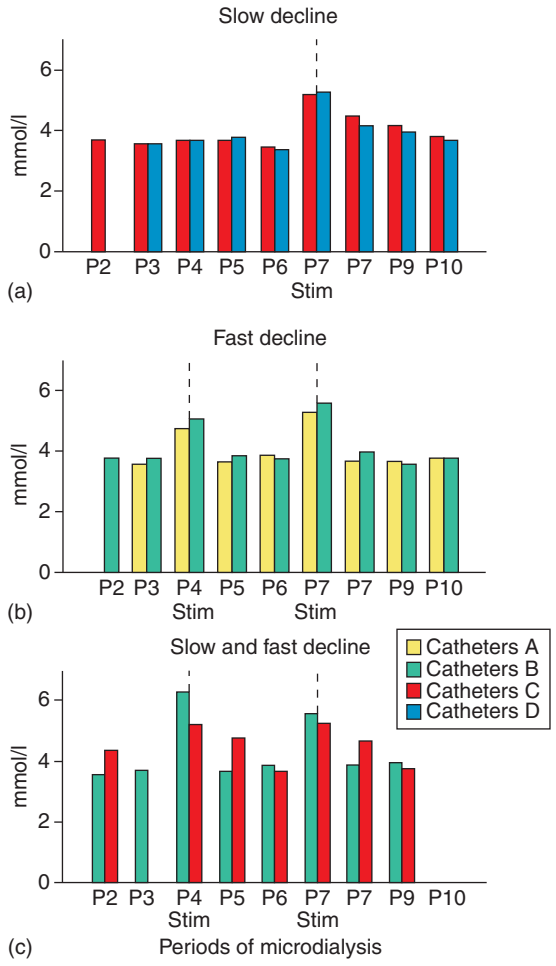


Figure 2 Different types of $[K^+]_o$ reduction to the prestimulation levels after application of epicortical electrical stimulation to the medial temporal gyrus of medically intractable epilepsy patients. According to the velocity of $[K^+]_o$ reduction to the prestimulation levels, three different types of responses, including slow decline (a) and fast decline (b) at any catheter site, as well as slow and fast declines at adjacent catheter sites (c), were observed. Epicortical electrical stimulations (stim) were applied in period 4 (P4, 20 min after probe insertion) and period 7 (15 min later).

No correlation was found between the basal level of $[K^+]_o$ and the speed of potassium recovery to baseline.

Extracellular chloride concentration was measured in the medial temporal gyrus of six epilepsy patients undergoing surgery therapy. The mean basal value of $[Cl^-]_o$ in all patients was 122.9 ± 2.6 mM. However, the average baseline levels of $[Cl^-]_o$ in various patients differed significantly from each other ($p \leq 0.001$). In all patients, 20 and 35 min after catheter insertion, trains of local electrical stimuli (50 Hz, 300 μs, 10 mA and 15 mA) were applied epicortically. These stimuli did not change the $[Cl^-]_o$ baseline values in any cases.

In four patients, $[Cl^-]_o$ ranged between 125 and 154 mM (134 ± 1.8 mM) in dialysates from different catheters (different sites). In these cases, $[Cl^-]_o$ in different samples obtained from four catheters did not differ significantly. Furthermore, in this group of patients, $[Cl^-]_o$ was stable (with <10% deviation) during 45–50 min of microdialysis. The mean chloride concentration of CSF in these patients was 121 ± 1.2 mM. In one patient, $[Cl^-]_o$ was between 110 and 117 mM, a concentration that was significantly lower than in the previously mentioned cases. In this patient, $[Cl^-]_o$ in the dialysate from different cannulae was similar and stable during the microdialysis period (mean chloride concentration of CSF was 120.2 ± 2.6 mM). In another patient, $[Cl^-]_o$ ranged between 65 and 110 mM. In this case, $[Cl^-]_o$ was significantly different from the samples obtained via different catheters ($p \leq 0.001$); $[Cl^-]_o$ was stable in dialysates from three catheters, but was gradually increased by 45% in samples at one position during 50 min of microdialysis. ECoG spike activity recorded close to the sample area in this patient was dramatically reduced during microdialysis. The ECoG recordings in other patients, with higher $[Cl^-]_o$, revealed less spike activity.

Ion concentration data presented here should not be treated as equivalent to 'real' extracellular concentration, because full equilibration does not occur at the site of the membrane. The method presented here shows accuracy rates of >90% for potassium at concentrations between 4 and 8 mM, and accuracy rates of >85% for chloride at concentrations between 100 and 150 mM. In addition to these studies on human epileptic tissue, experiments were performed in an in vivo animal model with the identical microdialysis probes, and ISM revealed similar accuracy rates. Another important concern in this study is the influence of catheter placement on changes of ion content in the extracellular space. The content of potassium may be increased by cell injury. It is a clinical practice to wait ≥1 h before collecting the first microdialysate sample in the intensive care unit. However, in the present study, 5–10 min after catheter insertion (P0), a steady basal level of $[K^+]_o$ was observed. Nearly the same time interval (10–15 min) has been observed between catheter placement and reduction of potassium concentration to the steady level in rats. Histological investigations in rat neocortical tissue revealed that the catheter placement within the brain resulted in minimal cell injury near the catheter.

In our experiments, the depth of the catheter within the cortex was not measured. This factor, as well as the variability in location of probes across the cortical surface, may affect the consistency of equilibration across the dialysis membrane. In most cases, however, two microdialysis catheters with identical membrane lengths, perfusion fluid, and flow rates inserted in two adjacent areas of human frontal cortex, showed equivalent results.

In the majority of patients in our study, $[K^+]_o$ and $[Cl^-]_o$ measured in microdialysate derived from two or four different sites in the temporal lobes were similar and stable during the whole microdialysis. However, $[K^+]_o$ in three patients and $[Cl^-]_o$ in one patient were different from the samples collected from different catheters. No significant differences were found when $[K^+]_o$ and $[Cl^-]_o$ were measured with these same probes in serum and CSF samples of the respective patients. These findings indicate a nonhomogeneous spatial distribution of K^+ and Cl^- in some epileptic patients. Interestingly, measurement of antiepileptic drugs in the same samples in which K^+ and Cl^- were measured revealed considerable variances in drug concentrations in different sites of the temporal lobes. In patients with uniform spatial distribution of K^+ and Cl^-, no significant differences were observed in antiepileptic drug concentrations among different samples collected by different probes. Previous electrophysiological studies of human brain tissue obtained from patients undergoing neurosurgical treatment for medically intractable epilepsy revealed a heterogeneous spatial distribution of spontaneous rhythmic sharp waves. Furthermore, optical monitoring of activity with voltage-sensitive dyes and fast imaging (in vitro) indicates that localized and spatially inhomogeneous neuronal depolarizations underlie spontaneous sharp field potentials.

Directions for Future Research

There is a vast and growing literature dealing with the role of various messenger substances in epileptic brain tissues, and much less attention has been paid to electrolytes in recent years. Yet, it has long been known that moderate shifts in ion distributions can alter neuronal excitability. Normal limits of different ion concentrations in healthy human brain (with noninvasive methods) need to be measured, and comparisons made with measures from epileptic tissue. Furthermore, the effects of altered levels of ions on normal and abnormal brain functions need to be precisely defined. Determining these effects will improve our understanding of basic mechanism of epileptogenicity, which in turn may help in the treatment of refractory epileptic patients.

See also: **Imaging**: Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In vitro; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties.

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Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition

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Introduction

Ion concentration gradients provide the basis for neuronal activity, and neuronal activity in turn leads to changes in extracellular ion concentrations which are very significant during seizures and seizure-like events. These changes are due to transmembrane ion fluxes mediated by voltage and ligand gated ion channels. Ligand gating may occur from the intracellular or/and from the extracellular side of the membrane. Transmembrane transporters contribute only marginally to fast changes in extracellular ion concentrations, since transport protein density and transport capacity are much smaller than those of ion channels. In the long run, of course, the transporters must sustain the transmembrane ion concentration gradients. The transmembrane ion concentration gradient for potassium, and to a lesser extent for sodium and other ions, sets the resting membrane potential negative to −50 mV. This resting membrane potential level, in turn, determines the availability of voltage gated ion channels and thereby strongly influences neuronal excitability. For example, at a membrane potential of −30 mV the voltage-gated Na channels are all in an inactivated state, and neurons are therefore no longer excitable.

The difference between resting membrane potential and the equilibrium potential for a given ion sets the driving force for transmembrane ion flux. For example, at a resting potential of −50 mV, the driving force for Ca influx into the cell is close to 200 mV due to the equilibrium potential for Ca ions beyond +120 mV. Changes in transmembrane ion concentration gradients therefore affect membrane potential and the transmembrane ion currents induced by opening of voltage- and ligand-gated ion channels. During and following periods of increased neuronal activity, ion transporters must restore the normal transmembrane ion concentration gradients by using (directly or indirectly) ATP. The brain's dependence on this function accounts for most of the 15% of the whole organism's oxygen consumption.

Background

The extracellular space is narrow, although very tortuous. It accounts for about 15% of the overall brain volume. This space is filled with proteins and electrolyte. The colloid osmotic pressure is low. The electrolyte

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The extracellular space is narrow, although very tortuous. It accounts for about 15% of the overall brain volume. This space is filled with proteins and electrolyte. The colloid osmotic pressure is low. The electrolyte

contains significant concentrations of the amino acids GABA (20 μM), taurine (20 μM), and glutamine (500 μM – 2 mM). GABA and taurine may activate high affinity $GABA_A$, $GABA_B$, and glycine receptors, providing for tonic inhibition. Glutamine is generated in glutamine synthetase-containing astrocytes from glutamate, and is exported into the extracellular space; neurons then take up glutamine for GABA and glutamate synthesis. Lack of glutamine contributes to hepatic coma. The extracellular space also contains glucose necessary for brain metabolism. The pH of the extracellular space is 7.4.

The electrolyte composition in the extracellular space is similar, but not identical, to that of cerebrospinal fluid. The potassium concentration ($[K^+]_0$) is between 2.7 and 3.2 mM at rest, the sodium concentration ($[Na^+]_0$) is about 155 mM, the chloride concentration ($[Cl^-]_0$) 135 mM, the calcium concentration ($[Ca^{2+}]_0$) 1.6 mM, and the magnesium concentration ($[Mg^{2+}]_0$) about 1–1.2 mM. The bicarbonate concentration is about 21 mM. There is iso-osmolarity between the interstitial and intracellular space, and the sum of cation concentrations equals the sum of anion concentrations.

Salts are not fully dissociated in the extracellular electrolyte (as described by their activity coefficients). Unfortunately, we do not know all ion interacting partners, so that our view of ionic concentrations is based on comparison to calibration solutions. In the case of calcium, this point is particularly important, as Ca binding proteins exist in the extracellular space and bicarbonate can interact with Ca to form an almost insoluble complex. The 1.6 mM Ca concentration given earlier compares to a calibration solution containing also 21 mM bicarbonate.

The transmembrane ion movement through ion channels is determined by the driving force and the conductance. The relevant formula is $Ix = gx\ (E_m - E_x)$, with I being the transmembrane current carried by a defined ion x, E_m being the actual membrane potential, gx the conductance for the ion x, and E_x being the equilibrium potential for the ion species x. The resultant ion changes in the extracellular space depend not only on the transmembrane ion fluxes themselves but also on the size of the extracellular space, and on regulatory processes such as diffusion and transport. An open blood–brain barrier can be a confounding variable in trying to measure extracellular ion concentrations; in such conditions, the Ca and Mg binding of plasma proteins will lower apparent concentrations of these ions, while the higher and more variable K concentration in plasma will affect baseline extracellular K concentrations. In fact, in many conditions with epilepsy, for example, temporal lobe epilepsy, tumors, inflammations, hypoxia, and ischemia, the blood–brain barrier is not tight.

As indicated earlier, the magnitude of ion shifts is influenced by extracellular space (ES) size changes. Shrinkage of the ES due to water uptake into cells (and subsequent cell swelling) will increase baseline ion concentrations, while widening of the ES (e.g., due to increases in colloid osmotic pressure as a consequence of protein exsudation from the plasma into the interstitial space) will lower baseline concentrations. During seizure-like events, shrinkage of the ES by up to 30% has been reported. This change would elevate Na and Cl concentration to near 200 mM, while K, Ca, and Mg concentration would be much less affected. For quantitative estimates of the transmembrane ion fluxes, therefore, ES size changes must be taken into account.

The intracellular space is, of course, shared between neurons and the different types of glial cells. This space is always larger than the extracellular space, and hence the ionic changes are usually thought to be smaller than indicated by ion concentration changes in the extracellular spaces – but this is not necessarily the case. Assume for a moment that extracellular Na decreases by 10 mM, but the ES shrinkage is 30%. As a result of the ES change, baseline sodium would increase to near 200 mM. For that change to occur, 60 mM Na would have to move across membranes into the cell. If the interneuronal volume fraction is 40%, then the increase in intracellular Na concentration would amount to about 25 mM; however, due to the extracellular space shrinkage, the Na concentration declines only by 10 mM (instead of the projected 60 mM). Thus, the ES size change compensates, in part, for the large transmembrane movements.

Shrinkage of the ES has a further consequence. It increases the extracellular resistivity, thereby augmenting extracellular potentials and potential gradients generated by glial cell and nerve cell transmembrane currents. This influence is particularly important in conditions where (e.g., due to trauma or hypoxia) the ES is already reduced, or in areas such as rodent hippocampal CA1 pyramidal cell layer where the ES size is about 10% and thus smaller than elsewhere in cortical structures. Indeed, ionic changes and extracellular space size changes are not uniform in cortical structures. The largest ionic changes are usually seen in layer IV/V of neocortex and in the cell layers of the hippocampus (granule and pyramidal cell layers). At those sites, ES shrinkage during seizure-like events and/or prolonged repetitive stimulation are also maximal, while at remote sites (such as in superficial cortical layers or in stratum moleculare of the hippocampus) a widening of the extracellular space has been seen.

Methods

Changes in ion concentrations, inside or outside neurons, can be measured with ion selective microelectrodes. These electrodes are filled at their very tips with a resin which can resolve the activity of specified ions (e.g., K, Ca,

or Na). Within the electrode (above the tip), this resin is superfused by a defined solution containing the ion of interest. When the electrode tip is immersed into an ionic solution, the ion for which the resin is selective will move into the resin and create a 'border potential.' The difference between the border potential inside the electrode (at the resin–electrolyte border) and outside the electrode tip (at the border between the resin and the outside medium) responds in a Nernstian manner to a change in ionic concentration (provided the measurements are done within the range of concentrations at which the ion-sensing microelectrode is selective). Such an electrode measures field potentials also. Therefore, a second electrode is required to measure the local field potential, and that field is then subtracted from the signal of the ion selective microelectrode. In practice, we use double barreled microelectrodes (one barrel filled with resin, the other to measure field potentials), the sensitivity of which is determined before and after the experiment. For such measurements, specialized amplifiers with a high input impedance, negative capacitance compensation, and DC potential correction are required.

Changes in ES size can be measured by monitoring the apparent increase in concentration of a substance which can not enter cells by transport processes or movement through ion channels. In practice, we use tetrapropylammonium or tetraethylammonium. These substances may be applied locally by pressure pulses or ionophoresis, or may be present in the ES due to experimental perfusion. An increase in concentration of such a marker substance is inversely correlated with the decrease in ES size.

Results

Changes in Extracellular Na Concentration

Repetitive stimulation, seizures, and seizure-like events usually lead to biphasic changes in extracellular sodium concentration ($[Na]_0$), with an initial decline and a subsequent increase above baseline. Such alterations have been seen in different species and cortical regions. Similar changes in $[Na]_0$ were also observed when AMPA or kainate were focally applied. The second, overshoot component of this response is probably due to shrinkage of the ES. The consequences of these changes for Na-dependent uptake processes – for glutamate and GABA or glucose into glial cells – are probably minimal, as the transmembrane Na gradient will not be strongly affected. In neurons, the Na equilibrium potential might decline by about 20 mV (from 60 to 40 mV), thereby reducing driving force for Na inward currents. This change will also reduce Na-dependent secondary transport for glucose and amino acids into neurons. In addition, the intracellular Na accumulation will activate the electrogenic sodium–potassium pump, which will transport excess Na out of the cell to shift the membrane potential in the hyperpolarizing direction. Activation of the sodium–potassium pump thereby contributes to seizure termination. In addition, intracellular Na accumulation may lead to activation of potassium channels of the slick or slo family.

Changes in Extracellular Cl Concentration

As is the case with Na concentration changes, Cl signals are also usually biphasic in response to repetitive stimulation, seizures, and seizure-like events. Following an initial decrease in Cl concentration, there is a small overshoot (by about 10 mM). Based simply on the decrease in ES size, one would expect a larger increase (overshoot) in Cl concentration. Consequently, some 15 mM of Cl will accumulate within neurons, shifting the E_{Cl} in a depolarizing direction to levels beyond or equal to the membrane potential. This intracellular Cl accumulation contributes to the depolarizing shift of the equilibrium potential for $GABA_A$-mediated synaptic potentials. A reduced electrochemical gradient for Cl will also increase the contribution of the transmembrane bicarbonate gradient to the $GABA_A$-mediated synaptic potentials. As glucolysis and respiration are increased during seizures, the bicarbonate equilibrium potential may be shifted in a depolarizing direction, thus accounting for depolarizing inhibitory potentials during seizures.

Changes in Extracellular Potassium Concentration

Increases in extracellular potassium concentration as a function of action potential generation were first noted by Frankenhaeuser and Hodgkin in 1956; they saw that hyperpolarization after an action potential (AHP) became smaller with each action potential in a train of action potentials. It was, therefore, no surprise that changes in extracellular potassium in the central nervous system occur during motor system activation, during sensory stimulation and during different states of mentation. These changes do not exceed 2 mM above baseline. During pathophysiological states, much larger changes in potassium concentration have been noted. In particular, during seizures in adult neocortex and hippocampus, potassium can increase to near 12 mM, a level that is surpassed during seizures in developing cortex and hippocampus. In adult tissue of monkeys, cats and rodents, this ceiling level of 12 mM is usually preserved, unless blood flow or metabolism is disturbed. If K accumulates to between 15 and 18 mM, it is likely that spreading depression (SD) will be induced, which is frequently observed in cortical structures during early postnatal development, but also after brain trauma and subarachnoid hemorrhage. The increased probability for induction of spreading depression during ontogenesis may be due to delayed

maturation of the NaK ATPase, delayed maturation of glial cell properties, and a delayed maturation of the blood–brain barrier. Disturbances in ion regulation due to compromised blood flow, disturbances in mitochondrial function, or reduced availability of ATP later in life will increase the likelihood of the induction of spreading depression. If blood flow is interrupted or synthesis of ATP is stopped, then a rapidly spreading depolarization develops which – if not rapidly reversed – leads to cell death. During SDs, potassium increases to about 60 mM, while Na and Cl (and extracellular Ca) concentrations decrease. The ion movements across the membrane lead to a strong reduction of the extracellular space size.

Elevation in K has a number of different effects. In most situations, potassium accumulation leads to depolarization of neurons. In axons, the hyperpolarization mediated by voltage activated K currents will be reduced, leading not only to prolonged action potentials but also to changes in firing rate. In presynaptic terminals, elevation in K causes augmented spontaneous and evoked transmitter release. Postsynaptically, in neurons, the $E_{(EPSP)}$ is shifted in a depolarizing direction, and the reduction in outward currents will contribute to increased excitability. $GABA_B$-mediated inhibitory currents decline in amplitude; indeed, part of the depolarization observed during repetitive stimulation is due to the activation of $GABA_B$ receptors. K accumulation also has effects on chloride transport. The KCC2 transporter reverses transport direction when K increases to beyond 5.5 mM, so that instead of Cl extrusion, Cl is transported into the neuron – thereby shifting Cl equilibrium potential in depolarizing direction and impairing the efficacy of synaptic inhibition. The depolarizing effect of K accumulation onto neurons is mostly mediated by two-pore-domain potassium channels but Kir channels will also contribute.

K accumulation in the ES also leads to depolarization of passive (or simple) astrocytes. These astrocytes are also described as glutamate transporter cells (they express high levels of Na-dependent glutamate transporters) and are usually strongly coupled by gap junctions and form an electrical and metabolic syncytium. Depolarization of astrocytes by local K accumulation will spread within the glial syncytium, leading to facilitated K uptake at sites of maximal K accumulation and facilitated K release at remote sites. At sites of maximal K accumulation, this K uptake contributes to shrinkage of the ES, whereas at remote sites, spatial K buffering can lead to increases in the ES size. The spatial K buffering is associated with generation of slow field potentials, which are (at least in part) due to glial cells.

The spatial K buffer mechanism may contribute to vasodilation with subsequent increased blood flow. This effect is due to an increase of K conductance in Kir channels which are expressed in smooth muscle cells. These cells have a rather depolarized membrane potential which is paradoxically shifted in the hyperpolarizing direction when potassium concentration increases. The widening of the ES at sites of K release, presumably at astrocytic endfeet, may contribute to increased blood flow by reducing transepithelial pressure.

Depolarization of astrocytes has effects on Na-dependent glutamate and GABA uptake, as the transmembrane electrochemical Na gradient necessary for effective uptake is reduced in depolarized glial cells. This effect will further lead to the accumulation of transmitters in the extracellular space. Whether glutamate transport can be reversed under conditions of anoxia and spreading depression depends on the transmembrane Na gradient; in such conditions, as the Na concentration gradient declines the transport may be slowed (but not reversed).

It is generally believed that K accumulation in the extracellular space is due to K release from neurons, mediated by voltage-dependent K conductances. That this assumption is not really true is shown by experiments in which A-type and delayed rectifier K currents are blocked by TEA and 4-AP – leading to increased extracellular K accumulation. Most of the K release is therefore due to K movement through two pore domain (2P) K channels, that are sensitive to quinine, quinidine, and bupivacaine. These agents reduce stimulus induced K signals considerably. Interestingly, blockade of 2P K channels or of Kir channels augments iontophoretically induced K signals, presumably due to reduced potassium uptake into glia and prevention of spatial K buffering. Down-regulation of glial Kir 4.1 channels in neuronal tissue is an early reaction after lesions to the brain and after opening of the blood–brain barrier. This down-regulation causes a facilitated accumulation of potassium in the extracellular space, and leads to early transformation of physiological responses into epileptiform events during normal processing of information in the brain.

Experiments on mature neocortex have shown that K accumulation does not trigger seizure activity. However, the situation is less clear in the hippocampus, where both in vivo and in vitro studies have shown that elevation of extracellular K concentration can trigger generation of seizure-like events. This sensitivity to extracellular K was also seen in human tissue from drug refractory patients. In such patients, elevation of K, when combined with bicuculline, could trigger seizure-like events in neocortex. Elevations in extracellular potassium concentration may support seizure generation by associated shrinkage of the extracellular space and a resultant increase in extracellular resistivity. Transmembrane currents will then cause larger extracellular potentials. Potential gradients in the order of $8\,mV\,mm^{-1}$ have been shown to affect neuronal excitability. Spatial buffering of potassium causes elevation of potassium at sites which are not yet recruited into

seizures. Spread of potassium by diffusion and spatial K buffering seems to underlie the slow spread of low Ca-induced seizure-like events in rat hippocampus. The speed of spread in this condition compares to spreading velocities of Jacksonian march, of experimentally induced seizures caused by lowering Mg concentration, and by the application of 4-aminopyridine. It may well be that K accumulation, by reversing Cl transport and by increasing the driving force for generation of EPSPs, is involved in recruiting new tissue into a seizure. This effect may be enhanced by ephaptic interactions due to shrinkage of ES and build-up of large field potential gradients.

Changes in Extracellular Mg Concentration

The interstitial Mg concentration is probably close to 1 mM, and changes in extracellular Mg concentration are difficult to detect due to the lack of appropriate sensors. In favorable conditions, small decreases in Mg concentrations have been observed. Mg is intracellularly bound to ATP but is free when ATP is consumed. Hence, the intracellular magnesium concentration may fluctuate, and in some condition may be as large as 1 mM. Such an intracellular level would result in a Mg equilibrium potential close to 0 mV, and provide a driving force for Mg ions to move in the inward direction. The major pathway for Mg ions to enter cells is through NMDA receptors. Indeed, measurement of Mg concentration changes in response to iontophoretic application of NMDA under conditions of zero Ca (when measurement of Mg loss is facilitated) indicates that Mg enters neurons. This observation would suggest that the Mg blocking site within the NMDA channel is deep within the pore. Larger changes in Mg concentration, in both plasma and CSF, may occur during eclamptic seizures, when a sudden and large increase in blood pressure opens the blood–brain barrier. Lowering Mg concentration causes facilitated transmitter release, due to the removal of the Mg block on voltage-dependent Ca channels. In addition, the activation of NMDA type glutamate receptors is facilitated by lower Mg. Both effects cause a rapid accumulation of Ca within neurons. Lowering Mg concentration will also affect surface charge screening, thereby augmenting neuronal excitability. Dependent on the animals' age and the subunit composition of the channel, Mg regulates activation of A-type potassium currents.

Lowering Mg concentration has also been investigated and found to trigger seizure-like events in juvenile hippocampus and in juvenile and adult neocortex. However, the concentrations required are probably lower than achieved under physiological conditions. Whether Mg can decline during eclampsia to levels sufficient to trigger seizures is unclear. However, normalizing Mg is as effective as phenytoin in blocking seizures under these conditions.

Changes in Extracellular Calcium Concentration

Ca concentration declines whenever neurons are activated. The decreases are about 0.1–0.2 mM during physiological activity. However, repetitive electrical stimulation of the thalamus or the cortex can induce decreases by upto 0.4–0.6 mM. As is the case with changes in potassium concentration, the amplitude of decreases in calcium concentration is layer-dependent in cortical structures. Maximal Ca decreases occur in layer II and IV/V of cat somatosensory cortex. In the hippocampus, decreases in Ca concentration are largest in the pyramidal cell layer, followed by changes in stratum granulare of the dentate gyrus. During seizures, Ca has also been shown to decline, and these decreases are generally similar to the decreases seen with repetitive stimulation. However, in some species, decreases in Ca can be much larger; for example, in the neocortex of the baboon *Papio papio*, decreases in Ca concentration reach about 1 mM during seizures induced by photic driving – changes only observed during spreading depression and anoxic depolarization in other animals.

The unique cellular arrangements in the hippocampus permit separation of pre- and postsynaptic Ca signals. Repetitive stimulation of the Schaffer collaterals causes Ca concentration decreases due to activation of P/Q type Ca channels (N type Ca channels inactivate relative rapidly during repetitive stimulation). K channel blockers such as TEA and 4-AP augment presynaptic Ca entry. Antidromic stimulation permits analysis of postsynaptic Ca entry; P/Q type and also L type Ca channels are activated during antidromic stimulation. NMDA receptors mediate postsynaptic – and when present – also presynaptic Ca entry.

While Ca declines monotonically to a new steady state level during the application of NMDA, glutamate or aspartate, Ca only transiently declines when AMPA and kainate receptor agonists are applied; under this latter condition, the initial decline in Ca concentration is followed by an overshoot. The reasons for these kinetic differences are not fully understood. The overshoot in Ca concentration during kainate or AMPA application, however, is removed when the Na–Ca exchanger is blocked.

The decrease in Ca concentration increases neuronal excitability by effects on surface charge screening and facilitation of action potential generation. In addition, the decrease in Ca concentration facilitates activation of NMDA receptors. It is expected that decreases in Ca concentration also lead to reduced transmitter release. Studies in the hippocampus have revealed that during washout of Ca, stimulus-evoked synaptic inhibition disappears before synaptic excitation; the loss of synaptic inhibition was noted at about 0.9 mM extracellular Ca, while postsynaptic excitatory potentials were lost at about 0.2 mM. The release of transmitters such as glutamate and GABA depends on the fourth power of Ca entry.

In disynaptic pathways, therefore, the effects of lowering Ca concentration will be cumulative. The loss of feed-forward and feed-back inhibition will therefore be particularly sensitive to decreases in extracellular Ca concentration.

Surprisingly, lowering Ca can also directly activate nonselective cation currents, as originally reported for dorsal root ganglion cells and more lately for central neurons. Whether this effect involves a unitary class of channels or includes different ion channels is presently unclear. The activation of these channels requires only relatively small decreases in extracellular Ca concentration.

Lowering of Ca, similar to lowering of proton or Mg concentration, can increase neuronal excitability by effects on surface charge screening; it can trigger epileptiform activity in rodent hippocampus, provided that K is elevated to 5 mM or more. This effect is not seen, however, in human hippocampus from patients with epilepsy.

Since decreases in extracellular Ca concentration are due to Ca inward currents, effects of changes in extracellular Ca concentration on neuronal activity are not always easy to predict. The net consequences for excitability depend on the intracellular Ca kinetics and the different effects which Ca can have on activation of ion channels, pumps, secretion, etc. With regard to effects of intracellular Ca accumulation, it must be noted that Ca can activate cation channels, and different types of K channels. However, in some cells, Ca may also inhibit activation of K channels. Recent data suggest that certain types of voltage gated Ca channels co-localize with either BK or SK type Ca-activated potassium channels, thus permitting generation of locally limited domains of intracellular Ca concentration changes. Intracellular Ca changes may have effects on metabolism as well. Three enzymes in the tricarboxic acid cycle respond to increases in intramitochondrial Ca concentration. However, larger increases in intracellular Ca concentration lead to mitochondrial depolarization, with potentially impaired synthesis of ATP and increased production of radical oxygen species.

Alterations in pH

Usually, seizure-like events are initially accompanied by alkalosis and then by acidosis. Part of the acidosis may be due to shrinkage in ES size, but also to lactate accumulation in the tissue. While acidosis reduces neuronal excitability, alkalosis increases neuronal excitability. The increase in excitability can, in part, be attributed to reduced surface charge screening. Changes in bicarbonate concentration in babies have been suggested to contribute to generation of febrile convulsions.

Alterations in pH strongly affect seizure susceptibility, and alkalosis in babies may even trigger seizures – perhaps due to the fact that inhibition is less stable and not fully developed, that the blood–brain barrier is still incomplete, and that elevated K can easily be modified by neuronal excitability.

Directions for Future Research

Obviously, changes in extracellular ion concentrations and changes in transmembrane ion concentration gradients have profound effects on neuronal activity, and may often be relevant for seizure induction and seizure propagation. When seizures spread, the 'front' of seizure spread is preceded by an avalanche of strongly increased inhibition. Recruitment of 'normal' brain regions into seizure activity then depends on the break-down of this inhibition. The precise mechanisms by which this break-down occurs are not yet fully understood, but changes in transport efficacy for Cl, as well as changes in bicarbonate concentration, may be involved. In order to maintain, and particularly to recover, relatively normal ion concentration gradients after a seizure or a seizure-like event, the nerve cells must increase production of ATP so that the ion pumps can work efficiently. This demand for ATP requires increases in blood flow and more delivery of oxygen and glucose to sites of ictogenesis. The roles played by ionic changes in neurovascular coupling are far from being understood. The same applies to the precise mechanisms by which neuronal metabolism is adapted to the needs for ATP synthesis in very active neurons. Another still-to-be-resolved question is how ionic changes are altered when the blood–brain barrier is open, or when seizure activity develops in an area with compromised blood–brain barrier. Particularly important are the effects of alterations in glial cell properties on the kinetics of ionic changes in the nervous system since these kinetics may influence the conditions under which seizure progresses into status epilepticus and/or induces damage to the nervous system.

See also: **Models**: Seizure Models in Acute and Organotypic Slices; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Ionic Microenvironment in Living Human Brain and Epileptic Seizures; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; **Pharmacoresistance**: Mechanisms of Pharmacoresistance.

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Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties

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Introduction

Epileptic seizures are generated by positive feedback excitation. Positive feedback can be mediated through reverberating excitatory synaptic pathways, and also by intrinsic neuronal membrane processes.

Mammalian brains are inherently capable of generating epileptiform seizures. The difference between epileptic and healthy brains is that the epileptic brain can generate a seizure without obvious provocation or in response to an innocuous stimulus, whereas only excessive stimuli or exogenous toxins induce seizures in the healthy brain. This general insight raises several questions. What enables seizures to arise? What prevents them from happening in healthy individuals? What terminates a seizure after it started? This article addresses the first of these problem areas.

Traditionally, an imbalance between excitatory and inhibitory synaptic transmission has been blamed for epileptic seizures. Yet simple forward transmission of excitation, no matter how powerful and unopposed, will not produce a tonic or tonic–clonic seizure. True epileptic discharge must be sustained by some form of reverberating or self-repeating excitation. Excitation can become self-sustained by the action of recurrent synaptic excitation, but brain tissue can generate seizure-like discharges even when all synapses are incapacitated. The most striking examples of nonsynaptic seizure generation occur in slices of brain tissue deprived of calcium. In low extracellular Ca ($[Ca^{2+}]_o$), synapses cease to function; yet Ca-deficient preparations produce 'spontaneous' electrical activity resembling synchronized epileptiform discharges. Moreover, in at least some forms of epilepsy in animals, it has been shown that during a seizure interstitial calcium concentration sinks to such low levels that chemical synapses could not transmit. Still, the seizure proceeds unabated until it is extinguished by the mechanisms that usually terminate it. The conclusion is inescapable – that nonsynaptic mechanisms can, at least sometimes, support ictal events.

This article discusses the effect of ion fluxes and ion concentration changes on ion channels, as these relate to epileptic seizures. Malfunctioning synapses probably play the primary roles in many forms of clinical epilepsy. Defective voltage-gated ion channels can, however, also become epileptogenic. Moreover, ion imbalance affects the intrinsic properties of brain cells and thus shapes the progression of seizures – either in addition to, or independently from, classical synaptic processes.

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Background

Observations in Live Brain Tissue: Ion Concentration Changes Modulate Ion Channel Function

During seizures, ion fluxes result in ion concentration changes that modulate the functioning of the very ion channels through which the ions flowed in the first place.

Intensely stimulated neurons lose K^+ and gain Na^+, Ca^{2+}, and Cl^-. They also discharge HCO_3^- and produce excess CO_2 and acid metabolites. These fluxes result in temporary shifts between intra- and extracellular ion concentrations and pH, as well as cell swelling and consequent shrinkage of the interstitial space (IS). Each of these changes feeds back to influence intrinsic membrane functions.

Elevated Interstitial Potassium

As a rule, the opening of K^+ channels and the resulting outward K^+ current tend to hyperpolarize neuron membranes. If, however, during high frequency firing the efflux of K^+ exceeds the rate of its clearing from interstitial fluid, the elevated ratio of extracellular over intracellular potassium concentration, $[K^+]_o/[K^+]_i$, makes the membrane potential less negative, and the neuron more excitable, at least as long as the imbalance is not too great. Excessive depolarization inactivates Na^+ channels and renders neurons unexcitable. This extreme case is evident only during spreading depression (SD).

It has been known since at least 1929 that solutions of potassium salts introduced into the brain, or into one of its ventricles, can induce epileptiform convulsions in animals. Combining this observation with the ionic theory of action potentials, several authors have proposed, hypothetically, that accumulation of K^+ in cerebral IS is the immediate cause of epileptic seizures.

During normal function, $[K^+]_o$ in the cerebral interstitium is kept within narrow limits by reuptake of K^+ into neurons mediated by the 3Na/2K ATP-fueled ion exchange pump, and by uptake of excess K^+ into glial cells, mainly astrocytes. However, during strong repetitive electrical stimulation, and also at the start of a tonic seizure, $[K^+]_o$ rises from its normal 2.8–3.5 mM to 7–12 mM, and usually, settles at this elevated 'ceiling' level for the duration of the excited state. Thus, excessive excitation elevates $[K^+]_o$, which favors more excitation and more release of K^+, and this circular sequence has the makings of positive feedback. High $[K^+]_o$ stimulates both reuptake into neurons and uptake into glial cells, and the elevated quasi-steady ceiling of $[K^+]_o$ is attained when the release of K^+ from neurons is matched by its enhanced clearing from the IS. After termination of neuronal firing, $[K^+]_o$ decreases gradually and usually sinks below its resting level (the 'undershoot'); this undershoot probably contributes to postictal depression.

Figure 1 illustrates the rise of $[K^+]_o$ during a seizure-like event induced by electrical stimulation. In the hippocampal formation, cell bodies of the principal neurons (granule cells in the dentate gyrus (DG) and pyramidal cells in Ammon's horn) are packed tightly in a compact layer, while branching dendrites, axons and glial processes intertwine in the neuropil of adjacent layers with relatively few interneuron cell bodies. For the experiment of Figure 1, a main input pathway (perforant path) to the DG was stimulated repeatedly for 10 s. In the layer containing the dendritic trees (stratum moleculare), $[K^+]_o$ rose to a 'ceiling' and remained at that steady level until the end of the stimulation. By contrast, in stratum granulosum, among the neuron cell bodies, after about 3.5 s $[K^+]_o$ rose to a new, even higher level, while the baseline (DC) potential

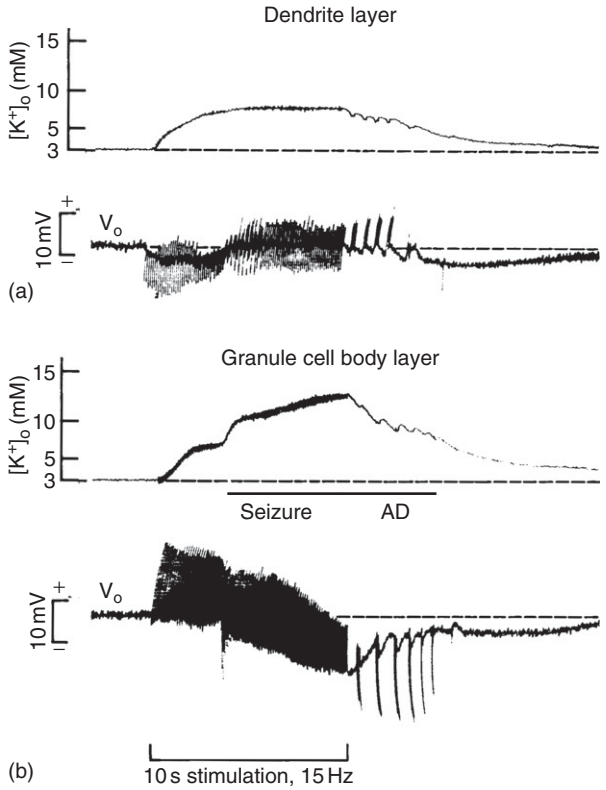


Figure 1 Interstitial potassium concentration ($[K^+]_o$) and DC voltage (V_o) in dentate gyrus of an anesthetized rat during electrical stimulation of an afferent (perforant) path and during clonic afterdischarge (AD). About 3.5 s after the start of stimulation, the responses attained a seizure-like character. At that moment the already elevated $[K^+]_o$ in the cell body layer (stratum granulosum) increased and V_o shifted in the negative direction. In the dendrite layer (stratum granulosum), $[K^+]_o$ remained flat and V_o shifted to a slightly more positive level during tonic seizure-like discharge. Parts (a) and (b) were recorded in the same experiment with 80-min interval. Modified from Somjen GG and Giacchino JL (1985) Potassium and calcium concentrations in interstitial fluid of hippocampal formation during paroxysmal responses. *Journal of Neurophysiology* 53: 1098–1108, with permission from American Physiological Society.

shifted in the negative direction and the nature of the electrical responses changed abruptly. Although not apparent at the slow recording speed shown here, oscilloscope recordings of these and many similar traces showed that the neuronal discharge no longer followed beat-for-beat the electrical stimuli, but acquired seizure-like characteristics. The train of stimuli was followed by afterdischarge (AD), resembling clonic seizure activity. It appears that the rise in $[K^+]_o$ and the associated depolarization were maximal near the site of action potential initiation (in stratum granulosum), not where most of the synapses are located (in stratum moleculare).

Current Source Density Distribution during Seizure Discharge

The difference between the behavior of the neuronal somata and the apical dendrites during paroxysmal firing was also evident in the experiment shown in **Figure 2**. Preparation and stimulation were similar to those in **Figure 1**, but the recording was made with a probe containing a straight row of miniature nonpolarizable contacts. Extracellular current source density (CSD) was computed for the baseline (DC) voltage shift, by sampling the voltages in between spike discharges. (A brief description of CSD analysis is given in Current Source Density Analysis section) CSD indicates the direction in which the predominant extracellular current flows in the IS and also the sites of 'sinks' and 'sources,' indicating where current enters or leaves cell membranes, but it does not resolve the movement of individual ions. The stimulation provoked seizure-like activity and AD, which was followed by SD. During the seizure, a sustained current flowed from the layer of apical dendrites (the source) into the layer of neuron somas (the sink). During SD, the dominant current reversed and a much larger sink appeared in the dendrite layer. Most excitatory synapses in the hippocampal formation are located on apical dendritic branches and excitatory synaptic currents generate sinks in the dendritic layer. Thus, the CSD associated with an excitatory synaptic potential (EPSP) is oriented similarly to that of the SD but its polarity is opposite to the DC component of the current associated with the tonic seizure.

Thus, the sustained firing during a hippocampal seizure appears to be maintained by inward current in or near the cell somata, and not by synaptic currents. This observation then brings up the problem of which ion channels are involved in this epileptogenic current flow – a question to be discussed in Recent Results – Computer Simulation of Nonsynaptic Epileptogenesis.

Low Calcium

In 1901, Regoli and Sabbatani applied citrate and oxalate to the exposed cortex of experimental animals and saw the eruption of epileptiform convulsions. They correctly inferred that the effect was due to the binding of Ca^{2+} in the tissue and not to the toxicity of the anions. Since then, it has been shown that exposing brain tissue slices to low calcium bathing solutions induces epileptiform electrical activity in the tissue. Deleting Mg^{2+} from the solution has a similar effect. The mechanism of the two effects is, however, only partly identical. Both divalent cations screen negative charges on the outer surface of cells, thereby augmenting

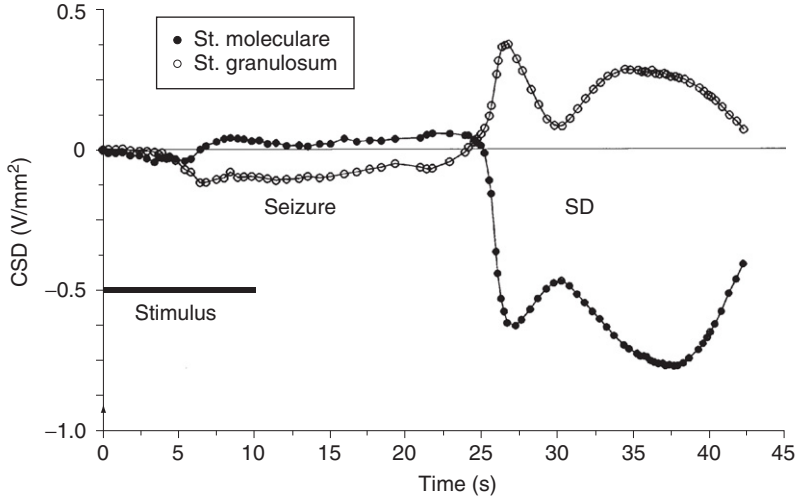


Figure 2 Current source density (CSD) in dentate gyrus of an anesthetized rat during stimulation-induced seizure and spreading depression (SD). The stimulation was similar to **Figure 1**. Recording with a multisensor microprobe. CSD was computed as the second spatial derivative of V_o corrected for tissue resistance variations. Baseline V_o (sustained potential shift) was sampled between synaptic potentials and spike discharges. Current sink is plotted as negative; source as positive. Modified from Wadman WJ, Juta AJA, Kamphuis W, and Somjen GG (1992) Current source density of sustained potential shifts associated with electrographic seizures and with spreading depression in rat hippocampus. *Brain Research* 570: 85–91.

the transmembrane voltage (V_m). Decrease in the extracellular concentration of either of the two divalent cations diminishes charge screening and increases the excitability of nerve and muscle cells. But, beyond this similarity, the two divalent cations have very different effects.

The outer (plasma) membrane, as well as the membranes of several major internal organelles, of brain cells are richly endowed with a variety of voltage and/or ligand controlled calcium channels and transporters. Inward current of Ca^{2+} into cells, like that of Na^+, tends to depolarize membranes. However, as there are many more Na^+ ions than Ca^{2+}, the direct effect of Ca-currents on the membrane potential is relatively small. On the other hand, variations of Ca^{2+} concentrations in- or outside cells modulate the functioning of a number of other ion channels. Furthermore, Ca^{2+} ions regulate a number of other molecular processes. Ca^{2+} entry into presynaptic terminals is required for the release of synaptic transmitters, which is why depletion of $[Ca^{2+}]_o$ blocks synaptic transmission.

Depolarization opens voltage-dependent Ca channels, and the channels controlled by NMDA receptors are also permeable to Ca^{2+}. Thus intense excitation causes Ca^{2+} influx into cells, depleting interstitial Ca^{2+} concentration, $[Ca^{2+}]_o$. Since the majority of Ca^{2+} permeable channels are located in dendrites and presynaptic terminals, the decrease in $[Ca^{2+}]_o$ is maximal in dendritic (neuropil) layers, at least in the hippocampal formation.

Like high $[K^+]_o$, low $[Ca^{2+}]_o$ can also create positive feedback excitation. Low $[Ca^{2+}]_o$ promotes excitation through three different mechanisms. One is the loss of surface screening, mentioned earlier. Another is depression of the slow hyperpolarizing afterpotential or afterhyperpolarization (AHP), which is generated by a Ca-dependent K-channel. In neurons where this channel is present, the slow AHP prevents high frequency firing. When the slow AHP decreases, a depolarizing afterpotential (ADP) emerges, which facilitates high frequency burst-firing. The third important effect is that Ca^{2+} ions at physiological concentration limit the conductance of certain nonspecific cation channels. Lowering $[Ca^{2+}]_o$ opens these cation channels, producing an inward current and depolarization.

Low Magnesium

Less is known about the behavior of Mg^{2+} ions during seizures. It is, however, well established that bathing freshly isolated brain tissue slices or slice cultures in Mg-free ACSF causes the appearance of seizure-like spontaneous electrical discharges. Also, severe Mg deficiency can cause clinical seizures.

As already mentioned, Mg^{2+} is a synergist of Ca^{2+} in the screening of outer charges on cell membranes, and therefore decrease in $[Mg^{2+}]_o$ enhances excitability of nerve and muscle cells. In synaptic transmission Mg^{2+} acts, however, as an antagonist of Ca^{2+} because in physiological concentration Mg^{2+} limits Ca^{2+} influx at presynaptic nerve terminals. Hence, a decrease in $[Mg^{2+}]_o$ augments the release of synaptic transmitters. Finally, normal Mg^{2+} levels tend to keep NMDA-receptor controlled channels closed, and a decrease in $[Mg^{2+}]_o$ enhances the NMDA component of glutamate-induced EPSPs. This last effect is believed to be the most important cause of low-Mg^{2+}-induced seizures.

Ph and Seizures

As a rule, an increase in extracellular proton concentration, $[H^+]_o$, (i.e., a decrease in pH_o, acidosis) depresses neuron excitability and a decrease in $[H^+]_o$ (increase in pH_o, alkalosis) enhances excitability. There are exceptions: specialized acid-sensing neurons, mainly in the respiratory nuclei, are excited by acid pH. The pH of cerebrospinal fluids is slightly more acidic than that of blood plasma and the pH of interstitial fluid in the central nervous system is slightly lower yet.

During intense electrical stimulation and seizure discharge, pH_o becomes transiently alkaline and then more acid. The acidification can persist for minutes after cessation of firing. A host of biochemical reactions produce, consume, and buffer protons. Furthermore, the transmembrane fluxes of H^+ and HCO_3^- mediated by numerous channels, ion exchange, and transport mechanisms can influence pH_o. In spite of much research, there is as yet no consensus concerning the details of the mechanisms mediating the pH changes associated with neuron firing. For our discussion, it is sufficient to remember that the initial alkaline shift can augment excitability, while the subsequent acidification dampens excitation and so can assist seizure termination and can contribute to postictal depression.

Cell Swelling

During intense excitation, both neurons and glial cells gain some solutes and lose others, but uptake exceeds release; therefore, cells accumulate osmotic equivalents accompanied by uptake of water, and swell. Astrocyte plasma membranes contain aquaporin water channels, and water flows more swiftly across their surface than across neuronal membranes. Cell swelling tends to restrict the size of ISs, which in turn favors intercellular electrical interactions known as 'ephaptic' communication. Effective ephaptic transmission of excitation is the likely explanation of the synchronization of epileptiform discharges in the absence of synaptic transmission, such as that occurs in low Ca^{2+} condition.

Brain cells swell when extracellular osmotic concentration is lowered. The clinical equivalent is water poisoning. For example, stokers' cramps are seizures induced by salt loss by sweating followed by excessive water intake. Besides

enhanced ephaptic effects, there is an added factor favoring neuron excitation. Hypotonia augments synaptic transmission, apparently by increasing the release of transmitter from presynaptic terminals. Iso-osmotic lowering of $[Na^+]_o$ has a similar but weaker effect. Placing brain slices in strongly hypotonic solutions can induce seizure-like discharges due to the dual effect of enhanced synaptic as well as ephaptic excitation. Whether these effects are relevant for epileptic seizures is not clear. During seizures induced under isotonic condition, water moves from the IS into cells (drawn by the movement of solutes) without a change in total water content of the tissue, and $[Na^+]_o$ decreases only moderately. The shrinking of the IS is much less severe during seizures than during SD.

Methods Frequently Used in the Study of Nonsynaptic Seizure Mechanisms: A Nutshell Summary

The Induction of Acute Seizures

To study their mechanisms, seizures have to be provoked 'on demand.' The customary means to induce epileptiform discharges in the brains of anesthetized animals include electrical stimulation and convulsant drugs. Strong alternating current of the right frequency applied by electrodes attached to the skull can provoke tonic–clonic convulsions. In the clinic, this is known as electroshock or electroconvulsive therapy (ECT). Better suited to microelectrophysiological analysis are seizures limited to a readily accessible target area. The hippocampal formation is especially prone to experimentally triggered seizures, and it is also the main focus in temporal lobe epilepsy. Repetitive stimulation of the main input pathways to this area can trigger paroxysmal firing of the form illustrated in **Figures 1** and **2**.

Convulsant drugs fall into two broad categories: antagonists of synaptic inhibition and stimulants of neuron excitability. Typical examples of the former include strychnine and bicuculline and of the latter include picrotoxin and 4-amino-pyridine.

As described already in Observations in Live Brain Tissue: Ion Concentration Changes Modulate Ion Channel Function, brain tissue slices kept alive in vitro generate seizure-like discharges when the concentration of K^+ in the bathing solution is raised, or that of Ca^{2+}, Mg^{2+}, or H^+ is lowered.

Ion-Selective Microelectrodes

Ion-selective microelectrodes (ISMEs) are, in principle, similar to pH electrodes. The invention of these ultrafine probes in the early 1970s made it possible to record in live tissues the changes in the concentration of certain ions while these changes happen. ISMEs are made of double barreled glass capillaries, drawn to a common tip a few micrometers across. One of the twin tips is filled with a blend of chemicals that functions as an ion selective membrane, admitting some and rejecting other ions, and generating a voltage that is a function of the concentration of that ion. The other tip contains a physiological salt solution and it serves as the reference electrode. The most successful ISMEs are highly selective to K^+ (see **Figure 1**), Ca^{2+}, or H^+. There is also an anion-sensitive type in use, which, however, does not distinguish Cl^- from HCO_3^-. The scientific world is eagerly awaiting the invention of a truly selective liquid membrane for Mg^{2+}.

Current Source Density Analysis

External electrical signals, including the electrocardiogram and the EEG, are generated when electric current, carried by ions, flows in IS. In technical jargon, an 'open field' is needed to record extracellular electric signals. An open field results when electric charge enters the IS at some limited area, and leaves it at some distance. A region where positive charge flows from the inside of cells into their surround is called a 'source,' and where it disappears into cells is the 'sink.' In fact, of course, positive and negative ions always move in opposite direction in electrolyte solutions, and instruments register only a transient excess of one. An excess of negative ions leaving the IS also creates a 'source,' just like an excess of positive ions entering.

In practice, CSD distribution is computed from voltage recordings made with a regular array of multiple extracellular microelectrodes, spaced as closely as possible within the target tissue. The electrical signals of interest include the currents associated with action potentials, synaptic potentials, or (most importantly for our discussion) the sustained potentials shifts marking seizure-like events (**Figure 2**). The locations of sources and sinks of current are detected as the maxima and minima of the second spatial derivative of the voltage gradient.

There are limitations to the usefulness of this technique. Meaningful CSD maps can be derived only from tissues where assemblies of similar electrically active cells are lined up in parallel, so that they can generate open voltage fields. Such areas in the central nervous system include the cerebellar cortex and the cerebral cortex, but the hippocampal formation probably has the optimal cytoarchitecture for CSD analysis. Here as elsewhere, however, the CSD map shows only the origins and the direction of the *net* current without revealing its ionic composition or the membrane ion channels that generate it.

Computer Simulation of Ion Currents that Generate Seizures

To test mechanistic hypotheses of seizure generation, it would be necessary to register simultaneously the

operation of the many biophysical mechanisms that interact during seizure discharges. Currently available techniques, such as patch-clamp recording, cannot provide the necessary data, but computer simulations can help. Simulations cannot discover what *is*, but they can exclude what is not possible.

The simulations of biological events involve digital computation of analog processes. Nonetheless, the verisimilitude of model neurons has improved over time, and a wide variety of realistic ion channels have been successfully incorporated in the membranes of model neurons and these can generate life-like virtual membrane ion currents.

Seizures are among the phenomena that lend themselves to computer simulations. The majority of published computer-based studies have examined synaptic interactions among populations of neurons and some models had incorporated arrays of thousands of cells. In most studies, however, ion concentrations in and around cells were assumed to remain constant. There have been only few attempts to incorporate ion concentration changes, and their inevitable functional consequences.

The following section of this article presents the salient data and conclusions obtained from simulations by a computer model consisting of a single neuron, the IS surrounding it, and a 'glia-endothelial' compartment enveloping the interstitium. Ion channels that are known to be present in hippocampal pyramidal neurons and astrocytes have been incorporated in the membranes of the model neuron and glia. Activation, deactivation, and inactivation of the ion channels were based on published reports of voltage-clamp experiments using live cells. The flux of ions across the neuron and glial membranes and the resulting concentration changes, and the effect of these ion changes on membrane channels, were continuously computed. A model neuron could imitate the morphology of a real neuron (e.g., with branching dendrites), but the model used to produce **Figures 3–5** was simplified; it had a soma, an unbranched apical dendrite and a short basal dendrite. The simplified cell replicated the behavior of the more realistically-shaped model, as well as that of a 'live' neuron. If undisturbed, it maintained a stable resting potential; when stimulated, it fired action potentials and, with appropriate adjustment of parameters, it produced tonic or clonic seizure-like after discharges, or SD.

Recent Results – Computer Simulation of Nonsynaptic Epileptogenesis

Computer simulation reveals the ion currents that could underlie nonsynaptic seizure-like discharge. Seizure-like discharge was produced when ion imbalance resulted in the activation of slowly inactivating inward current(s).

Simulated Elevation of Interstitial Potassium

Seizure-like AD is enabled by elevated $[K^+]_o$, which can maintain the activation of a slowly inactivating Na^+ current.

A computer model of a single 'pyramidal neuron' can generate seizure-like self-sustained and self-limiting discharge. The model used to produce **Figures 3–5** is described in Computer Simulation of Ion Currents that Generate Seizures.

Figure 3 illustrates the simplest type of simulated paroxysmal AD. The model neuron was stimulated by depolarizing current injected into the neuron soma. During stimulation, the neuron fired action potentials at an increasing rate (**Figure 3(a)**). The acceleration of firing was related to a depolarizing shift of the equilibrium potential of potassium, E_K (the blue line in **Figure 3(a)**). E_K is a function of the logarithm of the ratio $[K^+]_o/[K^+]_i$. The positive shift of E_K curtailed the hyperpolarizing afterpotentials, which are generated by the K^+ currents that follow each spike. Suppression of the AHPs enabled an increase in firing frequency. Following termination of the stimulus, the neuron continued to fire in the absence of an extrinsic stimulus, until the firing frequency decreased and finally ceased. This model AD simulates neuron behavior during a brief tonic seizure.

Figure 3(b) shows the trajectory of $[K^+]_o$ under three different settings for the volume of the 'glia-endothelial' compartment. The black line represents the condition of **Figure 3(a)**. In **Figure 3(c)**, the loss of K^+ from the neuron cytosol is plotted, and in **Figure 3(d)** the accumulation of K^+ inside the 'glia' is plotted. In these three trials, only the relative volume of the 'glia' was varied. The larger this buffer volume, the faster was the clearing of K^+ from the IS and the shorter the AD. The largest glial volume (10 times that of the neuron) is, of course, anatomically absurd. In intact brains, however, astrocytes are connected to one another by gap junctions, greatly increasing the effective volume of dilution for K^+ uptake. The interconnected glial network moves excess K^+ from regions of high $[K^+]_o$ toward regions of low $[K^+]_o$, a process known as 'spatial buffering.' Moreover, the glial network can 'siphon' K^+ ions from the IS around neurons toward the peri-endothelial space around small blood vessels, and the excess K^+ can be temporarily removed by the blood stream. Both the spatial buffer and siphoning mechanisms depend on uptake of K^+ into glial cells – which is equivalent to K^+ being diluted into a large volume. **Figures 3(b)–3(d)** demonstrate that glial buffering of $[K^+]_o$ modulates seizure-like discharge. This effect is particularly interesting, and potentially important, in light of the increasing evidence that in epileptogenic glial scars, the buffer function is defective.

Figure 4 is from another simulation trial, in essential features similar to that of **Figure 3**. **Figure 4(a)** is an expanded plot of the membrane potential of the model

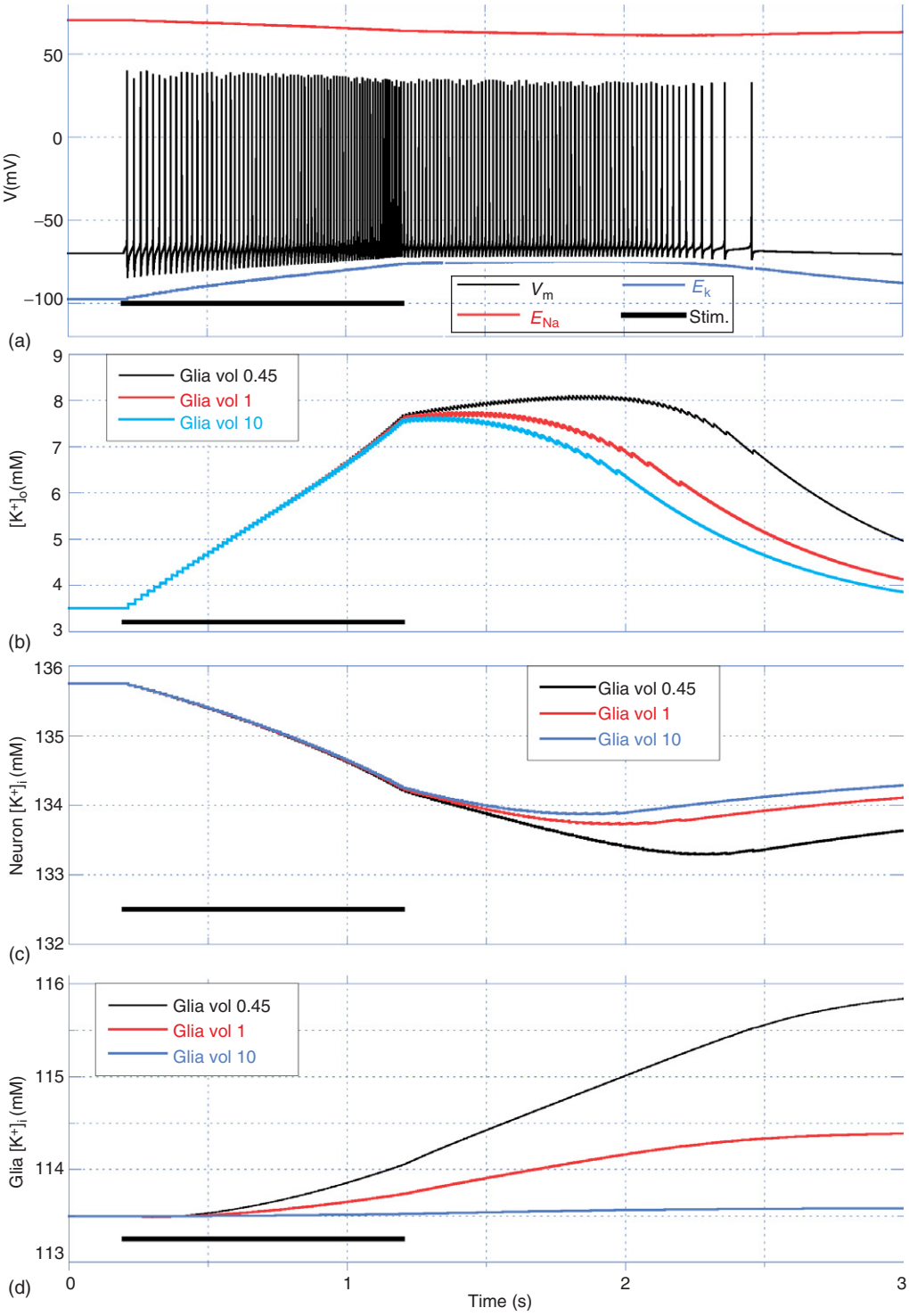


Figure 3 Computer simulation of seizure-like afterdischarge (AD) in a model neuron surrounded by interstitial space and enveloped by a glia-endothelial buffer compartment. (a) Membrane voltage (V<?tfqs-$_m$) of the model neuron and equilibrium potentials of potassium (E<?tfqs-$_K$) and sodium (E<?tfqs-$_{Na}$). The horizontal bar (Stim.) shows the time while a depolarizing current was injected into the neuron soma. (b) Interstitial potassium concentration ($[K^+]_o$ during stimulation-induced firing and AD of the model neuron with three different settings of the volume of the glia–endothelial comportment. Glia volume is indicated relative to the volume of the neuron. Interstitial space was 0.2. (c) Cytosolic potassium concentration ($[K^+]_i$) in the model neuron during the same three trials as in (b). (d) Cytosolic potassium concentration in the glia-endothelial buffer compartment during the same three trials as in (b). Reproduced from Kager H, Wadman WJ, and Somjen GG (2007) Seizure-like afterdischarges simulated in a model neuron. *Journal of Computational Neuroscience*<?tfqs- 22: 105–128, with permission from Springer-Verlag London.

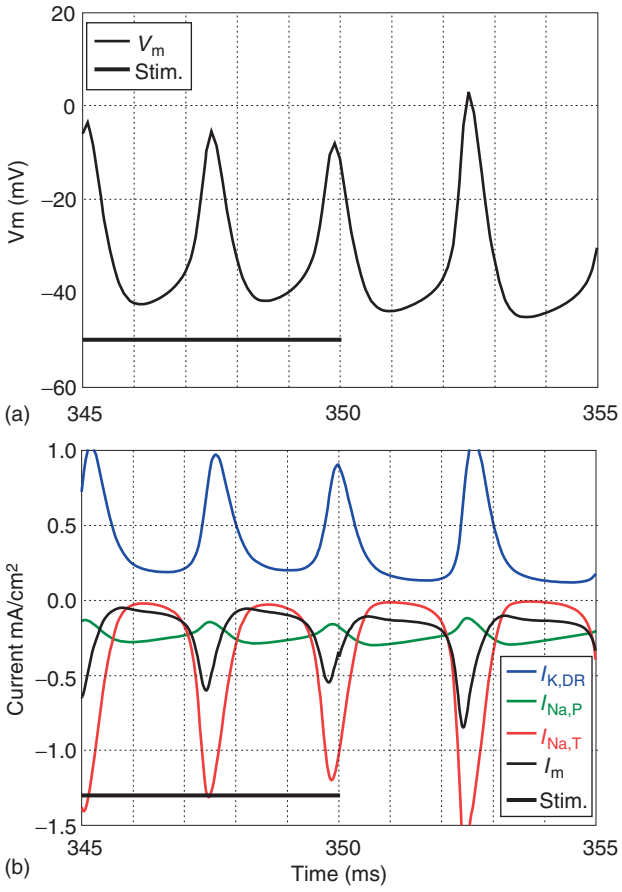


Figure 4 The currents generating the firing of the model neuron during stimulation by a depolarizing current and at the start of the afterdischarge. The stimulus was shorter than that for **Figure 3**, and calcium currents and Ca-dependent K-current were absent, increasing the firing rate. (a) Membrane potential of the neuron soma. Stim.: stimulation. (b) The main soma membrane currents generating the discharge. $I_{K,DR}$, delayed rectifier potassium current; $I_{Na,P}$, persistent (slowly inactivating) sodium current; $I_{Na,T}$, transient (Hodgkin-Huxley-type) sodium current; I_m, aggregate (net) membrane current. Reproduced from Kager H, Wadman WJ, and Somjen GG (2007) Seizure-like afterdischarges simulated in a model neuron. *Journal of Computational Neuroscience* 22: 105–128, with permission from Springer-Verlag London.

neuron at the end of the stimulation and the start of the AD. **Figure 4(b)** illustrates the voltage-dependent ion currents that cooperatively generate the firing of action potentials. The red line represents the 'classical' rapidly inactivating (transient) Na^+ current ($I_{Na,T}$) that is responsible for the steep upstroke of action potentials. The blue line is the delayed rectifier K^+ current ($I_{K,DR}$). The green line represents a slowly inactivating, persistent Na^+ current ($I_{Na,P}$), which has been shown to exist in many types of central neurons. The black line is the aggregate membrane current (I_m), which is the sum of all currents (both those plotted and those operating in the background and not shown here).

Action potentials are triggered when voltage-dependent fast inward currents exceed outward currents. This state initiates positive feedback because depolarization activates more inward current, which favors more depolarization. In cells whose repertory of voltage-gated currents is limited to $I_{Na,T}$ and $I_{K,DR}$, firing ceases in the absence of an extrinsic stimulus because $I_{Na,T}$ is rapidly inactivated, and the membrane is repolarized by the $I_{K,DR}$, which outlasts $I_{Na,T}$. Firing continues after the end of the stimulation in **Figure 4** because of the presence of the small but persistent inward current of $I_{Na,P}$. As $I_{K,DR}$ subsides, $I_{Na,P}$ continues to flow. Thus, an aggregate I_m is maintained, leading to $I_{Na,T}$ reactivation, generating the first action potential of the AD.

For the scenarios of **Figures 3 and 4** to be played out, it is necessary that $[K^+]_o$ should rise to a high enough 'threshold' level during the stimulation. When $[K^+]_o$ was artificially 'clamped' at or near its resting level, the same stimulation did not trigger an AD – because V_m repolarized and therefore $I_{Na,P}$ became deactivated when the stimulating current was withdrawn.

Seizures Influenced by Ca^{2+} Currents and Ca^{2+}-Dependent K^+ and Nonspecific Cation Current

In the simulations of **Figure 3**, the Ca^{2+} currents $I_{Ca,T}$ and $I_{Ca,L}$ and the Ca^{2+}-dependent K^+ current $I_{K,SK}$ were set to a low maximal conductance, and for **Figure 4** they were altogether absent. However, after appropriately adjusting the maximal conductance of $I_{Ca,T}$ and $I_{K,SK}$ in the dendritic membrane, the apical dendrite generated rhythmic 'calcium spikes' in response to stimulation, and the firing pattern of the neuron changed from 'regularly spiking' to firing in spike bursts. When $[K^+]_o$ was allowed to rise sufficiently, then stimulation was followed by AD in a burst pattern, simulating a clonic seizure (**Figure 5**).

Seizures Promoted by $[Ca^{2+}]$-Sensitive Nonspecific Cation Current

The channels that mediate nonspecific cation currents, I_{NSCC}, form an extensive family with widely diverging properties and are expressed in many cell types. They have in common the property that their activation tends to depolarize membranes. For the present discussion, of special interest is an I_{NSCC} identified in many central neurons, including hippocampal pyramidal cells. It is not voltage dependent, but it is activated by low $[Ca^{2+}]_o$ and it is permeable to monovalent cations – but not to Ca^{2+}. It has been suspected to facilitate seizures and SD. In simulations, we have shown that, indeed, in the presence of a

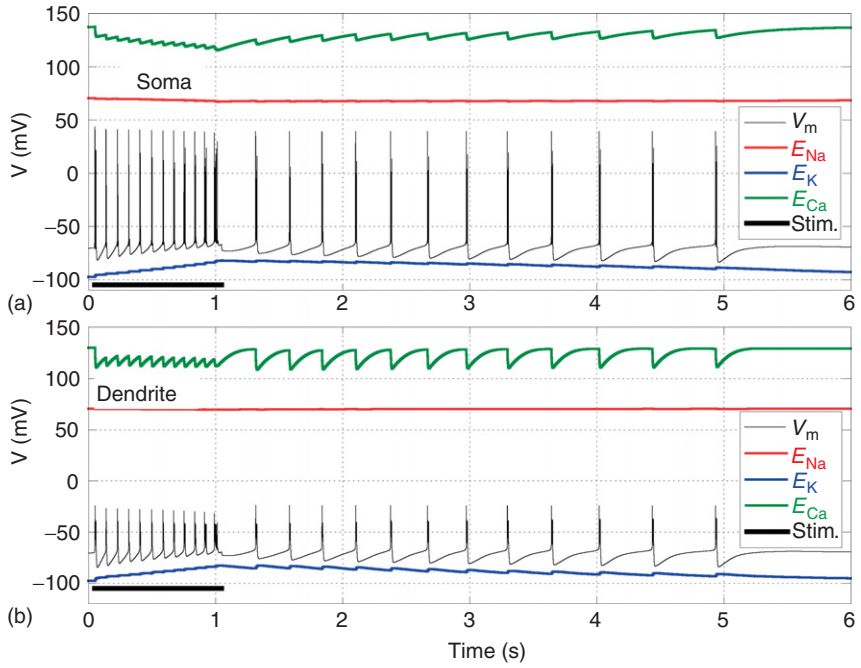


Figure 5 Simulated burst-firing and clonic afterdischarge of the model neuron. Burst-type firing was enabled by setting voltage-dependent calcium currents and calcium-dependent potassium currents to a higher value than those for **Figure 3**. (a) Membrane potential and equilibrium potentials of sodium, potassium, and calcium in the soma of the neuron, during and after injection of a depolarizing current into the soma. (b) The same variables computed for an apical dendrite segment. Reproduced from Kager H, Wadman WJ, and Somjen GG (2007) Seizure-like afterdischarges simulated in a model neuron. *Journal of Computational Neuroscience* 22: 105–128, with permission from Springer-Verlag London.

low $[Ca^{2+}]_o$-activated I_{NSCC}, a model neuron could generate seizure-like AD under conditions where no AD occurred in the absence of the I_{NSCC}.

An other I_{NSCC} was shown to be activated by increase of intracellular free Ca^{2+}, $[Ca^{2+}]_i$. Since during intense firing $[Ca^{2+}]_o$ decreases while $[Ca^{2+}]_i$. increases, in neurons that are endowed by both type of Ca^{2+}-sensitive I_{NSCC} the effect of the two are, presumably, additive.

Conclusions and Challenges

A number of central neurons naturally fire spontaneously. Paroxysmal firing underlying epileptic seizures is different in that it disrupts the normal function of a neuron population. Eruption of seizure discharge requires self-re-excitation.

Figure 6 is a simplified conceptual scheme summing up the parallel positive feedback loops that can support paroxysmal firing independently from synaptic interactions. Depolarization activates $I_{Na,P}$, which enhances depolarization. When the neurons fire repetitively, $[K^+]_o$ increases and $[Ca^{2+}]_o$ decreases. Elevated $[K^+]_o$ favors more depolarization directly and also indirectly because it potentiates the activation of $I_{Na,P}$. Low $[Ca^{2+}]_o$ enhances neuron firing by diminishing neuron surface charge screening and also by activating I_{NSCC}. Such feedback cycles must be initiated by a stimulus that depolarizes a population of neurons. This initial stimulus could be mediated, for example, by excessive

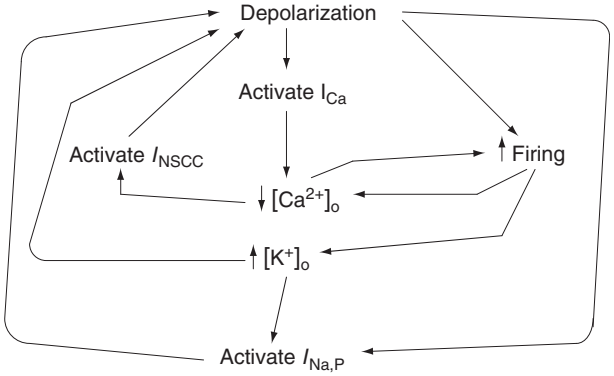


Figure 6 Conceptual chart of parallel positive feedback cycles that can sustain seizure discharge in the absence of synaptic input. Neuron depolarization causes firing, which raises $[K^+]_o$ and lowers $[Ca^{2+}]_o$. High $[K^+]_o$ augments depolarization. Low $[Ca^{2+}]_o$ augments excitability and so enhances firing; it also activates Ca-sensitive nonspecific cation current (I_{NSCC}), which also augments depolarization. Depolarization also activates persistent sodium current ($I_{Na,P}$), which is also augmented by high $[K^+]_o$, and also aids depolarization. Not represented in the diagram to avoid clutter are the processes that limit and ultimately extinguish seizures.

synaptic excitation, or else by head trauma, or electrical stimulation as in ECT.

During seizures, $[K^+]_o$ rises to a more or less steady ceiling level where the release of excess K^+ into the IS is balanced by its removal by augmented re-uptake into neurons and uptake into glial cells. Limitation of the rise of $[K^+]_o$ enables reactivation of $I_{Na,T}$ so that paroxysmal firing can continue. If the ceiling of $[K^+]_o$ is breached, runaway depolarization can persistently inactivate the transient Na^+ channel and SD is ignited. In hippocampal formation and neocortex, tonic seizure discharge is maintained by persistent inward current centered around the neuronal somata, while during SD the inward current in apical dendrites exceeds that flowing into the soma membrane. This spatial separation depends on the distribution (across membrane regions) of ion channels with diverse characteristics. This separation is, however, theoretically not required, and single-compartment computer models can generate either seizures or SD.

In healthy brains, positive feedback that could cause an ictal event is kept in check by inhibitory synapses and by intrinsic negative feedback represented, for example, by various K^+ and Cl^- conductances. Discussion of these control mechanisms fall outside the scope of this chapter.

Computer simulations, such as the ones illustrated in Figures 4–6, can distinguish between the possible and the impossible, but they cannot identify what actually exists in real life. In the simulations, either $I_{Na,P}$ or I_{NSCC} could enable seizure-like discharges. When both were active, seizures were facilitated. Importantly, in live brain cells both can be present. Other slowly-inactivating inward currents could also take their place or augment their actions. Future research must identify which of these currents have roles in clinical epilepsies.

See also: **Imaging**: Functional Imaging of Neuronal Network Activity in Living Human Brain Tissues In vitro; **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Role of Gap Junctions in Epileptic Synchronizations; **Pyramidal Cells**: Intrinsic Mechanisms Contributing to Hyperexcitability of Human Epileptogenic Cortex.

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Nonsynaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations

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Introduction

Epileptic seizures represent periods of electrical hyperactivity and hypersynchrony in cortical structures. The mechanisms by which cortical neurons become abnormally synchronized are critical to a thorough understanding of how epileptic seizures are generated and propagated. Although chemical synapses clearly play an important role in electrical seizure activity, and their alterations are likely important in the formation of the reorganized neural circuitry that defines the epileptic brain, it is highly likely that nonsynaptic mechanisms of neuronal interaction also play critical roles in the synchronization of electrical activity during seizures.

Electrical mechanisms of neuronal communication have been viewed as important contributors to the synchronization involved in seizure-like activity for many decades. Electrotonic coupling through gap junctions represents a mechanism of electrical synaptic transmission, and it has been proposed to synchronize electrical activity in a wide variety of situations where rapid synchronization is critical to normal neuronal function. The influence of electrical field effects, or ephaptic interactions, which arise through electrical current flow in the extracellular space, has also been debated for many years, but these effects are known to occur between hippocampal pyramidal cells under a variety of circumstances where synchronization and hyperactivity are present. These two mechanisms of electrical interaction are quite different: electronic coupling is based on a membranous structure (i.e., the gap junction) involving connexon channels, mediated by connexin proteins, between cells; electrical field effects, or ephaptic interactions, arise when the extracellular space is small and neurons are arranged in parallel, so that the electrical current flow between neurons can cause abnormal depolarizations during synchronous activity. These two forms of electrical interaction have a very fast mechanism of action, and so they are thought to play an important role in synchronizing action potentials, although they may also contribute to other forms of synchronization.

Another nonsynaptic mechanism involves activity-dependent changes in the concentration of extracellular and intracellular ions. When intense electrical activity occurs, as is expected during seizures, the ionic currents across the membrane cause a change in the extracellular concentration of ions such as potassium and calcium. These changes in ion concentrations can cause a slow form of synchronization, particularly since reduced extracellular calcium would also lower action potential threshold, whereas increases in the concentration of extracellular potassium would depolarize the membrane.

These two mechanisms – electrical interactions and ionic shifts – can work in concert to cause increased excitability. This article will focus on electrical forms of neuronal communication but will also consider ionic interactions.

Background

Electrotonic Coupling Through Gap Junctions

Probably one of the oldest and most controversial issues in neuroscience is the degree to which neuronal communication in the mammalian nervous system involves chemical versus electrical mechanisms. There is no question that chemical synapses – whether they be fast synapses involving transmitters such as glutamate and GABA, or neuromodulatory mechanisms invoking slower processes such as seen with serotonin and neuropeptides – play an important role in both seizure generation and epileptogenesis. During the 1970s and 1980s, however, a wide range of studies suggested that hippocampal pyramidal cells, dentate granule cells, and neocortical pyramidal cells are also electrotonically coupled through gap junctions. One form of evidence in support of this hypothesis was that low molecular weight tracers, such as Lucifer yellow and neurobiotin, can sometimes pass from one neuron to another. This method of analysis has been criticized because some neurons (and other cells) thought to be electrically coupled through gap junctions do not necessarily pass the tracer, whereas tracer has been found in cells that had previously been thought not to be electrically coupled to the injected neuron. Studies in the 1980s, using intracellular recordings with either indirect or direct methods, provided evidence that the fast prepotentials or spikelets sometimes recorded in hippocampal pyramidal cells are due to electronic coupling and reflect the transmission of action potentials from one cell to another (as opposed to representing dendritic action potentials of the recorded cell). This hypothesis, that pyramidal cells could be coupled through gap

junctions, was viewed skeptically and is still criticized by many researchers, because it has been difficult to find morphological evidence of gap junctions between these neuronal types.

Electrical Field Effects

Another mechanism of electrical interaction involves the effects of electrical fields, generated by a population of synchronously active neurons, on adjacent neurons that are not electrically active. Because the electrical field effects, or ephaptic interactions, arise when the extracellular space is small and neurons are oriented in parallel, the structure that has attracted the most attention with regard to this mechanism is the hippocampus. It has long been known that the hippocampus generates particularly large electrical field potentials during synchronous firing, primarily because of the tight packing and parallel polar orientation of hippocampal pyramidal cells (and dentate granule cells). There is a large negativity (the 'population spike') recorded at the cell body layer during synchronous action potentials generated by hippocampal neurons; this negative extracellular field causes a fast transmembrane depolarization that can be detected when electrodes are positioned intracellular and extracellular, so as to record the transmembrane potential (i.e., intracellular voltage minus extracellular voltage in the immediately adjacent extracellular space). The field-effect depolarizations, which can occur either in hippocampal slices or in intact animals, are generally about half the amplitude of the extracellularly recorded population spikes, and so are often many millivolts. These electrical field effects may be particularly important, since they can act together with electrotonic coupling (via gap junctions) to synergistically cause synchronization of action potentials of hippocampal pyramidal cells and granule cells.

Ionic Mechanisms

Electrographic seizures represent extremely intense activity and synchronization, so that the concentration of extracellular and intracellular ions changes significantly. When extracellular potassium increases, and extracellular calcium concentration decreases, neurons become more excitable. A variety of studies in the 1970s and 1980s used ion-selective electrodes to show that major shifts in these ions occur during seizures, and these shifts can dramatically alter the electrical activity of neurons in the hippocampus and neocortex. Because this mechanism acts on a slow time scale, it is unlikely that it is involved in synchronization of action potentials on a millisecond basis; however, these ionic shifts presumably play an important role in slow changes in excitability.

Models of Seizure Activity Based on Nonsynaptic Mechanisms

During the early 1980s, several research groups showed that bathing hippocampal slices in extracellular solutions with little or no extracellular calcium leads to hyperexcitability and hypersynchronous activity (**Figure 1(a)**). In such low-calcium solutions, chemical synaptic transmission is blocked (since calcium is required for transmitter release). Under these conditions, extracellular stimulation of pyramidal cell axons (i.e., antidromic stimulation) causes prolonged afterdischarges and can lead to spontaneous bursts of action potentials with large-amplitude population spikes. Furthermore, it has been shown that hippocampal slices with reduced extracellular calcium and elevated potassium, in the presence of pharmacological antagonists that block glutamatergic and GABAergic receptors, also generate profound seizure-like activity in areas such as the CA1 and the dentate gyrus. During these periods of electrical activity, fast prepotentials or partial spikes can occur within one cell when extracellular recordings show that the population is generating action potentials (**Figure 1(b)**). Furthermore, differential recording across the membrane (i.e., transmembrane recording of intracellular minus extracellular potentials) reveals large field-effect depolarizations, which also contribute to the synchronization (**Figure 1(c)** and **1(d)**). These data have suggested that both electrotonic coupling and electrical field effects play an important role in synchronizing action potentials. Although this change is most profound in the CA1 area of the hippocampus, it can also be detected quite readily in the dentate gyrus, in the CA3 pyramidal cell region, and in medial entorhinal cortex (the field potentials are smaller and the synchronization is less profound in CA3 and entorhinal cortex, presumably because the cells are less densely packed).

Methods

The work in our laboratory, and from several other groups, which has provided the basis for the experiments described earlier, has primarily been done on hippocampal and neocortical brain slices studied in vitro. The early experiments used focal field potential recordings and sharp intracellular recordings to analyze population activity in relation to single-neuron responses. The data obtained from these in vitro preparations have been related to field-potential recordings and traditional electroencephalograms (EEGs) from both animals and human patients with chronic epilepsy. Much of the in vitro work has, in turn, been analyzed with computational models aimed at trying to determine the cellular mechanisms that most likely contribute to electrical and ionic nonsynaptic interactions. More recently, various imaging techniques

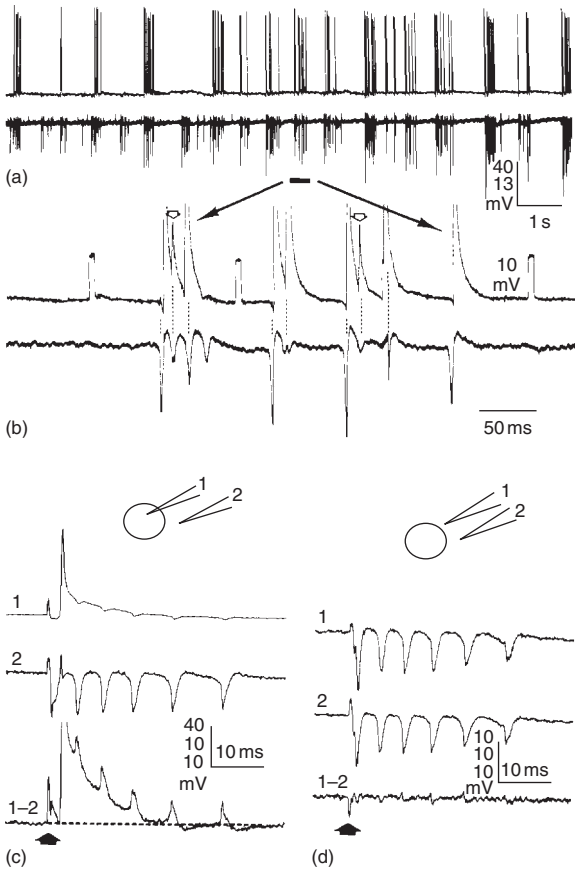


Figure 1 Spontaneous synchronous burst discharges after blockade of chemical synapses in a hippocampal slice with evidence for synchronization from nonsynaptic mechanisms, such as electrotonic coupling and electrical field effects between CA1 pyramidal cells. (a) Spontaneous bursts of synchronous action potentials were recorded from a hippocampal slice bathed in a solution containing 0.5 mM Ca^{2+} and 2.3 mM Mn^{2+} for several hours. Intracellularly recorded action potentials can be seen in the top trace, and these action potentials occurred synchronously with spontaneous bursts of population spikes seen in the extracellular recordings in the lower trace. Part of this record, as shown by the dark bar, is expanded in (b). The open arrows indicate fast prepotentials or spikelets that occurred during small population spikes in the extracellular recordings. In (c), an extracellular recording electrode was positioned close to the intracellular recording site (see inset) to allow differential recording of the voltage across the membrane of the CA1 pyramidal cell (i.e., intracellular minus extracellular), as shown in the bottom trace. Trace 1 shows the 'single-ended' intracellular recording (i.e., referenced to ground) as is traditionally performed in this type of experiment. Similarly, Trace 2 shows the extracellular recording referenced to ground. The lower trace (1–2) is the differential recording, which shows the field-effect depolarizations that occurred in the recorded CA1 pyramidal cell during a brief burst of action potentials in the hippocampal network. In (d), the intracellular electrode was withdrawn from the pyramidal cell, as shown in (c), and now both electrodes (1 and 2) were used to record the extracellular field potential. The differential recording in this condition (1–2) shows little or no voltage change, thus indicating that electrode 2 in (c) recorded the actual extracellular voltage changes immediately outside the pyramidal cell. The key point is that the actual transmembrane voltages 'seen' by the CA1 pyramidal cell are the differential recording illustrated at the bottom of C, not the single-ended recording shown in Trace 1 of (c). Reproduced from Taylor CP and Dudek FE (1982) Synchronous neural afterdischarges in rat hippocampal slices without active chemical synapses. *Science* 218: 810–812.

and gene-targeting strategies (including knockout mice) have been used to analyze specific roles of different types of gap junctions. These studies have yielded new insights into how nonsynaptic mechanisms contribute to seizure activity.

Recent Results

Fast Oscillations at Seizure Onset

As described earlier, early studies in hippocampal slices showed that synchronous action potentials can occur when chemical synapses are blocked. The fast nature of the synchronization, and the waveform of the events, strongly suggested that some form of electrical interaction was responsible. The available evidence for electrotonic coupling and electrical field effects further supported this perspective. This issue has recently become more important in the field of epilepsy research, since a variety of studies have detected very fast oscillations (in the range of 100–500 Hz) in extracellular field-potential recordings in the hippocampus of patients undergoing surgical resection for intractable epilepsy and in animal models with spontaneous seizures (such as the kainate-treated rat). These studies have shown that seizure onset is often, although not always, associated with these very fast oscillations – which likely represent the synchronized activity of action potentials in individual pyramidal cells (**Figure 2**). Although other mechanisms could conceivably generate these fast oscillations, the fact that action potentials have a duration of only about 1 ms makes electrical coupling the most likely candidate for the synchronization mechanism. Investigators have searched for the mechanism(s) that is upregulated in epileptogenic tissue to make these events so prominent and have proposed that electrotonic coupling via gap junctions is increased between injured neurons. Another hypothesis is that the epileptogenic tissue has reduced extracellular space and that higher extracellular resistance makes the electrical field potentials larger – leading to larger effects of ephaptic interactions. Other changes could indirectly lead to an enhancement of very fast oscillations through electrical mechanisms. One prime example is a potential decrease in GABA-mediated inhibition. Since $GABA_A$ receptor activation essentially shunts the membrane, one might expect that a simple reduction in this shunting effect could lead to enhancement of electrical interactions. A critical question is whether these forms of electrical interaction can be shown to mediate fast

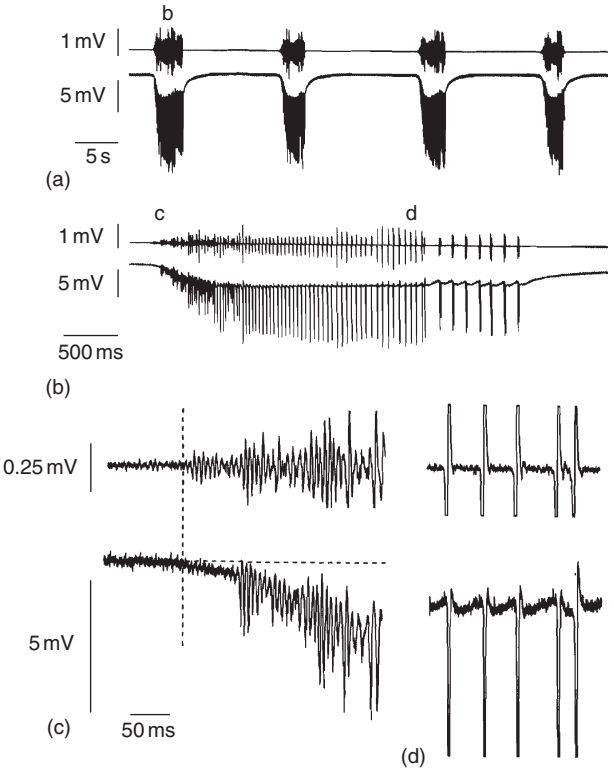


Figure 2 Combined multiple-unit and field-potential recordings of prolonged bursts generated in the CA1 area of a hippocampal slice bathed in a low Ca^{2+} solution with 5 mM extracellular K^{+}. The multiple-unit recordings are shown in the top traces, while the bottom traces illustrate the field-potential recordings. (a) Spontaneous bursts occurred repetitively under these ionic conditions. As expected, the increased multiple-unit activity occurred during the negative field potentials that had large superimposed population spikes. (b) The activity in (a) was expanded temporally (see the burst labeled 'b' in (a)) to show the correspondence between the field potential and the multi-unit recording. (c) The onset of the burst in (b) is shown at higher temporal resolution and gain. The recording shows that the small high-frequency oscillations at the onset of the burst occurred synchronously with the action potentials in the multi-unit recording. (d) Later in the burst, large population spikes were recorded with both electrodes. Reproduced from Patrylo PR, Kuhn AJ, Schweitzer JS, and Dudek FE (1996) Multiple-unit recordings during slow field-potential shifts in low-$[Ca^{2+}]_o$ solutions in rat hippocampal and cortical slices. *Neuroscience* 74: 107–118.

oscillations directly and, in particular, if these mechanisms could be altered in a manner that could block seizure onset.

Gap Junction Communication Among Cortical Pyramidal Cells

A variety of recent studies have begun to focus interest on the role of specific connexins in electrotonic coupling in the hippocampus and neocortex. Considerable debate has existed on whether multiple types of connexins mediate electrotonic coupling among neurons, but recent freeze-fracture immunogold labeling has provided extremely strong evidence that connexin-36 – and not connexin-32 or 43, or any other connexin identified to date – mediates electrotonic coupling among neurons in the mammalian brain. Interneurons are electrotonically coupled through gap junctions containing connexin-36, but it is currently not clear about the degree to which gap junctions on pyramidal cells also use connexin-36. Previous computer modeling studies suggested that gap junctions are more likely to be present on axons than on dendrites, and recent studies with thin-section electron microscopy and freeze-fracture immunogold labeling suggest that gap junctions are on both pyramidal and granule cell axons. This finding leads to the issue of the functional role of fast prepotentials or spikelets often seen in cortical neurons. The classical view was that these fast prepotentials represent the passive spread of active action potentials in cell dendrites. However, most recent studies strongly indicate that action potentials are initiated near the soma (at the axon hillock) and backpropagate into the dendrites. The combination of the presence of gap junctions, computer modeling studies, and previous electrophysiological research strongly suggests that fast prepotentials are electronic coupling potentials.

Challenges for the Future

Unlike typical chemical synaptic transmitter systems, or even voltage-dependent ion channels, gap junctions and electrical field effects are difficult to block with high selectivity. Recently, a number of compounds have become available to use as gap junction blockers. Probably the most widely used gap junction blocker has been carbenoxolone, but recently mefloquine has been used to suppress transmission between cells electrically coupled via gap junctions. Unfortunately, these compounds may not be selective, and there is concern that experiments with these drugs could involve other mechanisms. Nonetheless, numerous studies have provided evidence that gap junction blockers tend to suppress synchronized electrical activity. A number of studies have also shown that treatments that increase the size of the extracellular space, such as bath application of mannitol and sucrose, suppress synchronized electrical activity when chemical synapses are blocked (and when they are not blocked); furthermore, dilution of the extracellular fluid to decrease the osmolality can induce seizure activity, suggesting that cell swelling promotes seizures. These mechanisms of altering electrical interactions suggest a potential role for them in seizure activity. A major challenge for the future is to develop more specific treatments to affect these types of electrical interaction. Identification of molecules with selective actions on gap junctions and on ephaptic mechanisms could be extremely useful, not only for

identifying the role of nonsynaptic interactions in the generation of fast oscillations and synchronization of action potentials, but also for potentially treating patients with epileptic seizures.

See also: **Non-Synaptic Mechanisms**: Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Role of Gap Junctions in Epileptic Synchronizations; **Oscillatory Activity**: Cellular and Network Oscillations in Epilepsy; Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures; Neuronal Networks Generating Pathological High Frequency Oscillations; **Plasticity**: Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus.

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Role of Aquaporins in Nonsynaptic Mechanisms of Epilepsy

Z Zador, X Yao, and G T Manley, University of California, San Francisco, CA, USA

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Introduction

Nonsynaptic transmission refers to the mechanisms of neuroexcitation that continue to operate during the complete blockade of synaptic function. The ionic shifts that take place outside of the cell during nonsynaptic transmission occur in extracellular space (ECS) that typically comprises 20% of brain volume. The volume of ECS is not static and can be altered with water flux in and out of brain cells. For example, rapid uptake of water by swelling astrocytes expands the cellular volume, thereby decreasing the surrounding extracellular space. By manipulating the hydration status of patients suffering from epilepsy, it has been shown that dehydration can reduce seizure incidence, possibly by osmotic cell shrinkage and concomitant expansion of the ECS. In contrast, excessive hydration resulted in more frequent seizure events. Subsequent experiments in brain slices demonstrated that altering ECS volume by osmotic manipulation affected the incidence of neuronal bursts, even in the case when synaptic transmission was blocked. Taken together, these studies suggest that changes in ECS volume due to osmotic water movement play a role in the excitability of nervous tissue by modulating nonsynaptic mechanisms.

The glial water channel AQP4 is abundantly expressed in the central nervous system (CNS). AQP provides a

identifying the role of nonsynaptic interactions in the generation of fast oscillations and synchronization of action potentials, but also for potentially treating patients with epileptic seizures.

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The glial water channel AQP4 is abundantly expressed in the central nervous system (CNS). AQP provides a

highly efficient transmembrane route for water transport driven by osmotic gradient or hydrostatic pressure. The deletion of AQP4 in mice has been shown to attenuate neuronal excitability in the retina, inner ear, olfactory mucosa, and cerebral cortex. Recent data indicate that AQP4 may be involved in the nonsynaptic mechanism of seizure development and propagation. Multiple seizure models reveal higher seizure threshold, prolonged seizure duration, and slowed potassium kinetics in AQP4-deficient mice.

Evidence to date suggests that AQP4 may modulate the excitability of the nervous tissue through the following mechanisms. The deletion of AQP4 was shown to cause ECS expansion in brain, thus allowing ions and neurotransmitters to distribute in a larger volume and facilitate their diffusion. In addition, AQP4 colocalizes with the inwardly rectifying potassium channel Kir4.1 in Muller cells of the retina and astrocyte end feet. It has been proposed that AQP4 and Kir4.1 form an ion–water transport cluster, which facilitates the clearance of potassium accumulated during neuronal activity. Based on the available literature, in this article, we dissect these potential mechanisms by which AQP4 may contribute to nonsynaptic mechanisms of epilepsy.

Table 1 Tissue expression aquaporin family members

		AQP
Kidney	Proximal tubule	1,7
	Limb of Henle	1
	Collecting duct	2,3,4
Lung	Alveolar epithelia	5
	Alveolar endothelia	1
	Airways	3,4
Gastrointestinal tract	Hepatocyte	8,9
	Cholangiocyte	1
	Gall bladder	1,8
	Pancreas	1,3,8
	Colon	3,4,8
Blood/CVS	Erythrocyte	1
	Leukocyte	1,9
	Endothlia	1
	Heart	1,4
Reproduction	Testis	1,2,7,8,9,11
	Uterus	1
	Placenta	1,8
Other	Salivary glands	1,4,5
	Skin	1,3
	Bladder	3
	Fat	7

Background

Aquaporins

Water transport and homeostasis are essential for the function and survival of all living cells. Although water can freely diffuse through cell membranes, in some cell types water transport is facilitated by transmembrane water channels called aquaporins (AQPs). At present, 13 members of the AQP family have been identified and they are widely expressed in nearly all mammalian tissues (**Table 1**). The first member, channel-like integral protein of 28 kDa (CHIP28) now named aquaporin-1 (AQP1), was isolated and cloned from red blood cells. The AQP1 channel has high selectivity and permeability for water ($p_f \sim 2 \times 10^9$ water molecules per second) where the flux is driven by osmotic and/or hydrostatic forces. The remarkable water selectivity is explained by high resolution X-ray structure of a single channel unit (**Figure 1**). An AQP1 monomer consists of six tilted transmembrane α-helices, forming a barrel-like structure that harbors a central aqueous pathway of 3 Å, which allows the passage of a single file of water molecules (hour glass topology). In the cell membrane, AQP1 functions as a tetramer, with each of the subunits constituting an independent water selective pore. Many of the subsequent members of the family that were identified demonstrated similar principals of subunit structure and tetramerization. One important exception is AQP4. Freeze fracture electron microscopy of cell membranes rich in AQP4 revealed the formation of higher-order square arrays of particles consisting of hundreds of AQP4 tetramers. These structures were found to represent the previously described orthogonal array particles (OAPs). Whether AQP4 is organized in these OAP structures to better facilitate transmembrane water flux or to provide efficient anchoring of these water transport clusters remains an open question.

Multiple unexpected roles of AQPs have also been demonstrated. The deletion of AQP1 resulted in slowed tumor growth due to reduced angiogenesis and cell migration. Members AQP 3, 7, and 9 expressed in skin, adipose tissue, and the CNS, respectively, were found permeable to glycerol and lactate (aquaglyceroporins), and subsequently shown to play a role in cell energy-metabolism. The AQP with the highest water permeability, AQP4, was shown to facilitate glial scar formation and cell migration in the CNS, and there are also reports suggesting its role in intercellular adhesion.

Aquaporins in the Central Nervous System

AQP1 and AQP4 help regulate water homeostasis in the central nervous system. The movement of water in and out of the brain is facilitated by the polarized expression of these two channels at key locations of water transport, such as CSF–brain- and blood–brain barrier (BBB). AQP1 is expressed in the choroid plexus, where it facilitates the formation of cerebrospinal fluid (CSF). In this process,

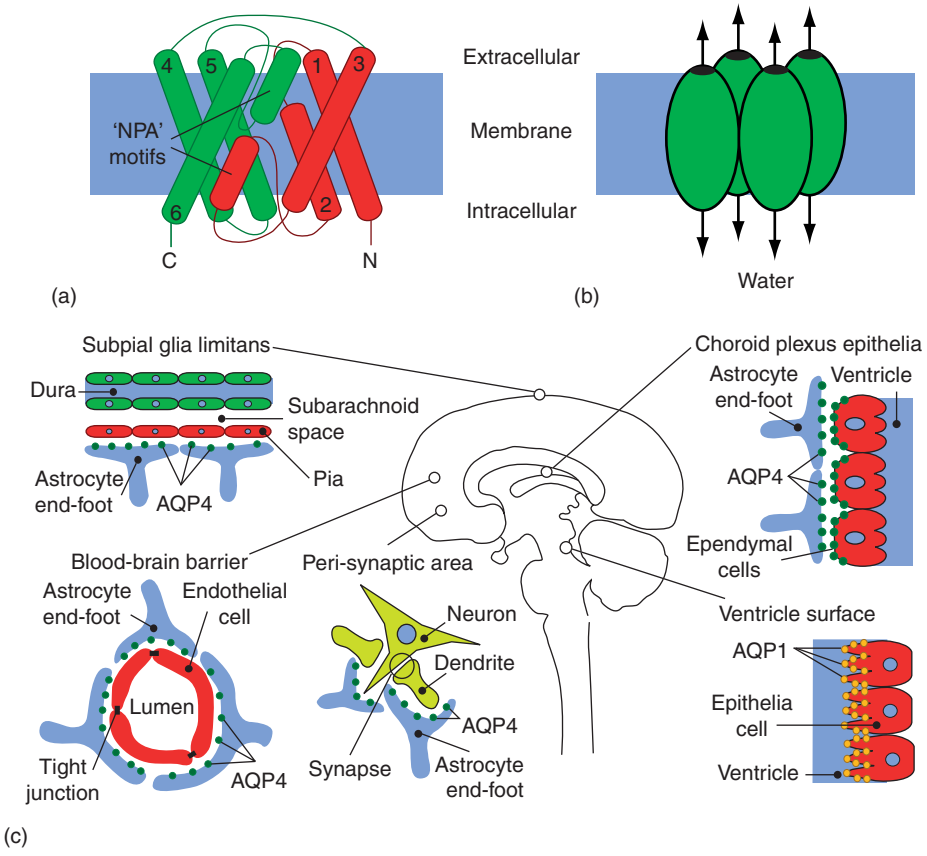


Figure 1 (a, b) Structure of AQP1 monomers and their organization into tetramers. (a) Model showing the six transmembrane α-helices of AQP4 numbered from 1 to 6, forming the highly selective water pore. The conserved 'NPA' motifs are indicated. (b) Tetrameric organization of AQP1 monomers in the plasma membrane. Each AQP1 unit functions as an independent channel. (c) AQP expression at water trafficking interfaces in the CNS. Glial end-feet facing CSF–brain and the blood–brain barrier show polarized expression of AQP4. Ependymal cells a show basolateral localization of AQP4. Apical processes of the choroids plexus cells are rich in AQP1.

active transport mechanisms create an ionic gradient between the choroid epithelial cell plasma membrane and the CSF space, providing an osmotic drive for water flux through AQP1 (Figure 2). The deletion of AQP1 resulted in an approximately 20–25% decrease of CSF production. AQP4 is also expressed at the CSF–brain barrier, where it is localized to the basolateral surface of ependymal cells lining the ventricles, and the subpial processes of astrocytes called the glia limitans. The functional significance of AQP4 in the transepemdymal reabsorption of CSF was demonstrated by the accelerated expansion of the cerebral ventricles and the elevated intracranial pressure in a mouse model of obstructive hydrocephalus. The abundance of AQP4 in the perivascular astrocyte processes suggests a role in water transport at the BBB. AQP4 is the predominant water channel in astrocytes. The deletion of AQP4 results in a sevenfold decrease in astrocyte osmotic water permeability. The intracellular uptake of fluid in cytotoxic edema (seen in stroke and experimental water intoxication) is facilitated by AQP4, whereas the reabsorption and clearance of extracellular edema (extravasated from leaky cerebral vessels associated with tumors and abscesses) were impaired by the AQP4 deletion. These findings demonstrate the important roles of AQP1 and AQP4 in brain water transport in health and disease.

Nonsynaptic Mechanisms of Neuroexcitation

The function and role of neuronal synapses have been widely studied in epilepsy, but processes independent of active chemical synapses – termed nonsynaptic transmission – have also gained considerable recognition. The significance of nonsynaptic transmission was first suggested by the generation and the spread of spontaneous electrical bursts elicited by antidromic stimulation in hippocampal slices. Subsequent experiments have further demonstrated that both ephaptic transmission (electrical current flow through the extracellular space) and ionic concentration changes (most importantly $[K^+]_0$ and $[Ca^{2+}]_0$) contribute to the nonsynaptic spread of neuroexcitation. The brain extracellular space (ECS) contributes the medium for these ionic movements, and accordingly, changes in ECS parameters are expected to modulate nonsynaptic transmission.

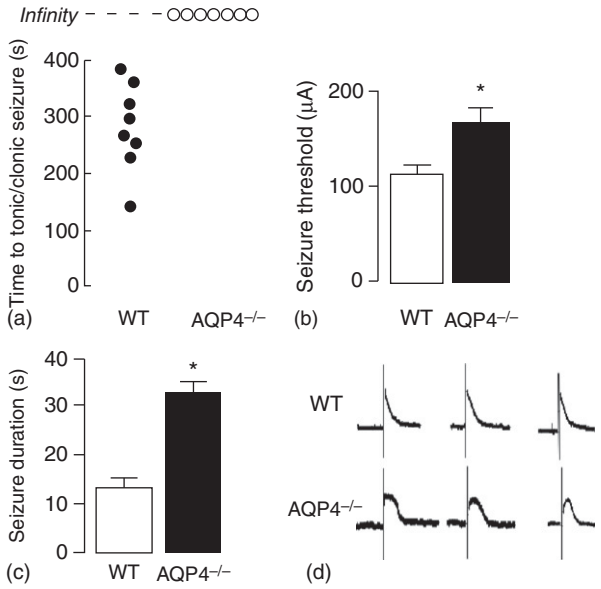


Figure 2 The deletion of AQP4 lowers seizure threshold and prolongs seizure duration. (a) AQP4 deficient mice show decreased seizure susceptibility in response to 40 mg kg^{-1} PTZ. Lowered electrographic seizure threshold (b), prolonged seizure duration (c) and slowed potassium uptake (d) in AQP4$^{-/-}$ mice.

To investigate the impact of ECS changes on neuroexcitation, dynamic changes of ECS volume were correlated with neuroexcitation. With the use of electrical resistance as a measure of ECS size, a small but significant (2%) increase in tissue resistance was recorded preceding seizure generation. Subsequent studies with cortical surface photobleaching have also demonstrated the contraction of ECS prior to the generation of seizures. Additional direct in vivo measurement of ECS volume, using the tetramethylammonium (TMA) method, has demonstrated elevated neuroexcitation in relation to contracted ECS size in the CA1 region of the hippocampus. In vitro alteration of the osmolarity of the bathing solution is known to cause changes in cell volume, which consequently modifies the ECS size and alters neuroexcitation. When manipulating the ECS size by exposure to varied osmotic environments, contracted ECS was shown to facilitate neuronal synchronization through enhancement of spatial effectiveness of nonsynaptic transmission, while ECS expansion eliminated synchronization. ECS expansion does not alter the excitability of the individual neurons; that is, resting membrane potential and action potential threshold of individual neurons are not altered during iso- and hyposmotic challenges. This observation suggests that synaptic and nonsynaptic mechanisms can operate independently.

Methods

We have conducted extensive research into testing different phenotypes of mice lacking AQP4. In initial experiments, isolated brain membrane vesicles and astrocyte cultures from AQP4-deficient mice were found to have significantly slowed swelling in response to an osmotic challenge, demonstrating the dominant role of AQP4 in facilitating water flux in brain tissue. The AQP4-deficient mice show no appreciable alteration in the baseline phenotype: they develop normally and show no gross difference in CNS anatomy. However in pathological states such as cerebral edema from tumors, inflammation, ischemia, and trauma, these mice have phenotypes that demonstrate the central role of AQP4 in brain water homeostasis.

AQP4-deficient mice have been tested using numerous models of neuroexcitation, such as cortical spreading depression, chemically or electrically evoked seizures, and light and auditory evoked potentials. Phenotypes were characterized in cell cultures and live animals, using whole cell patch clamp recordings, field recordings, and in vivo measurement of extracellular potassium kinetics with potassium sensitive electrodes.

Fluorescent methods for measuring diffusion in brain ECS environment have also been developed (**Figure 3**). The basic principle of FRAP (fluorescence recovery after photobleaching) was used in two different applications to assess macromolecular diffusion in brain ECS. Membrane impermeant, noninteracting fluorescently labeled dextrans were loaded into brain ECS via topical application or by microinjection delivery. With dedicated optics (immersion objective or microfiber optics), a bleach pulse was applied to the brain volume loaded with the fluorophore. Following this very brief (ms length) bleaching stimulus, the fluorescently labeled molecules from the surrounding unbleached areas diffuse into the bleached volume, giving a recovery signal (**Figure 3(a)**, right). The fluorescent signal is recorded by the optics and displayed as a recovery curve (**Figure 3(a)**, left). The speed of diffusion in brain is subsequently compared with free diffusion in a control solution to yield a measure of relative diffusion in the ECS.

Recent Results

AQP4 Modulates Neuroexcitation

The first finding to suggest a role for AQP4 in modulating neuroexcitation was its wide expression in electrically excitable tissues. AQP4 is most abundant in support cells such as astrocytes in the brain and spinal cord, Muller cells of the retina, and the Claudius and Hensen cells of the inner ear. At these sites AQP4 was found to co-localize with the inwardly rectifying potassium channel Kir4.1, which led to the idea that water flux and potassium flux might be coupled. Supporting this hypothesis, multiple models using AQP4 deficient mice demonstrated attenuated neuroexcitation and slowed potassium kinetics. Increased auditory brainstem evoked response threshold

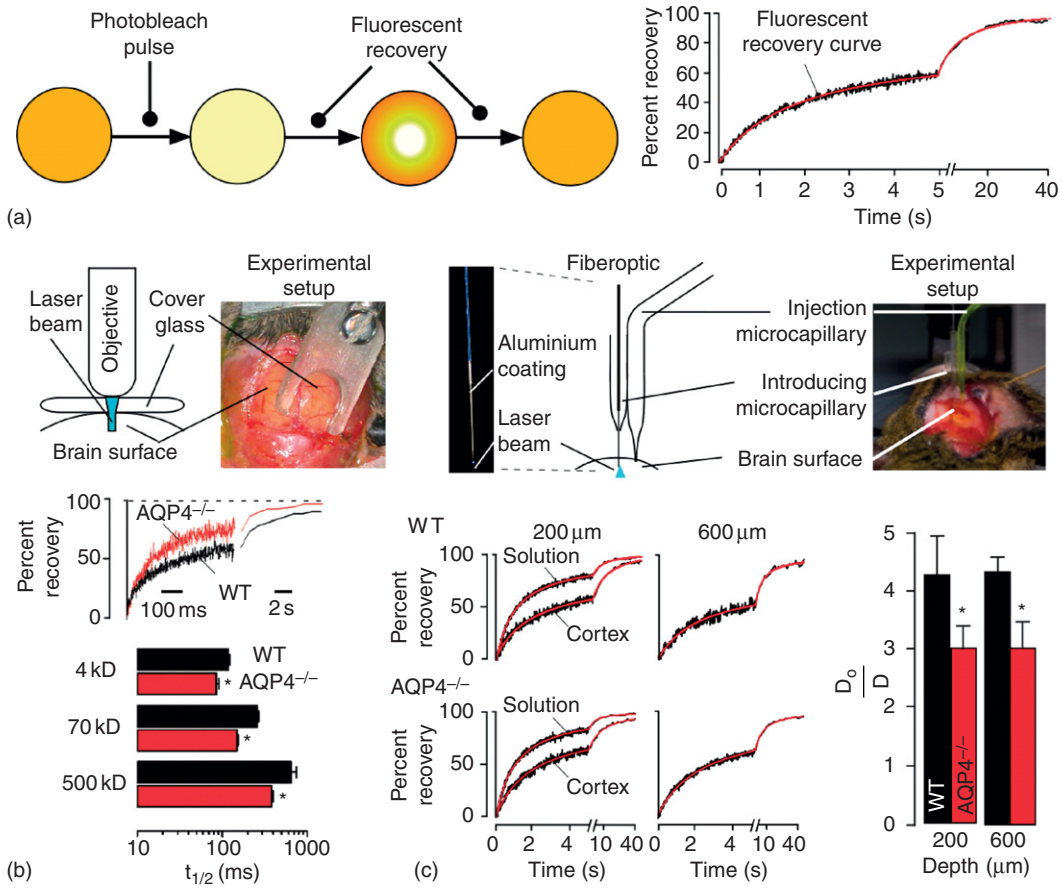


Figure 3 Principle of fluorescence recovery after photobleaching (FRAP). A high intensity laser pulse is applied to bleach a volume of fluorescent dye in brain ECS. Fluorescent molecules are replenished via diffusion from surrounding brain regions. The recovery of the fluorescent signal gives a fluorescent recovery curve reflecting the speed of diffusion. (b) Cortical surface photobleaching. *Top*: bleaching and signal acquisition is preformed on the brain surface through a thin cover glass. *Bottom*: original data showing decreased halflife for the diffusion curves from AQP4$^{-/-}$ mice, reflecting enhanced ECS diffusion. (c) Microfiberoptic epifluorecent photobleaching. *Top*: Mirofiber-optic is used to for delivering bleach pulse and acquisition from deep brain structures. *Bottom*: Original data showing enhanced diffusion at indicated depths of the cerebral cortex.

and reduced retinal potentials were seen in AQP4-deficient mice. These results indicate a role for AQP4 in modulation of neuroexcitation.

AQP4 Modulates Ion Fluxes in Seizures

Similar to the previous models of neuroexcitation, phenotypic analysis of AQP4-deficient mice was conducted using both chemical and in vivo electrical stimulation and electroencephalographic (EEG) recording (**Figure 2**). The AQP4-deficient mice proved to be less susceptible to seizures, requiring a higher dose (50 mg kg^{-1} vs. 40 mg) of the chemoconvulsant pentylenetetrazol (PTZ) or higher intensity electrical stimulation (167 ± 17 µA vs. 114 ± 10 µA) to produce seizures. Once initiated, AQP4-deficient mice showed significantly prolonged seizure duration compared to wild type controls (33 ± 2 s vs. 13 ± 2 s). The possibility that the slowing of potassium kinetics could account for the seizure phenotype of AQP4-deficient mice was tested directly. Parallel measurements of ECS potassium using ion sensitive microelectrode demonstrated similar baseline potassium values and peak potassium values during seizure discharge; however, there was a significant prolongation in potassium recovery to baseline in the AQP4-deficient mice ($t_{1/2}$ = 6.6 ± 0.7 s vs. 0.5 ± 0.1 s in wild type). The development of a new long wavelength potassium sensor dye also helped to reveal significant slowing of potassium uptake during cortical spreading depression in APQ4-deficient mice. It was proposed, based on these studies, that AQP4-dependent water transport was coupled to potassium flux through Kir4.1.

To test whether there was a functional association between AQP4 and Kir4.1, freshly isolated CNS astrocyte and Muller cells were subjected to whole cell and single channel patch recordings. During these experiments, seizure equivalent conditions of elevated potassium ($[K^+]_0$ = 20 mM) were reconstituted. Surprisingly,

whole cell patch of AQP4-deficient cells did not show significant differences in barium-sensitive Kir4.1 current or current-voltage curves. Single channel recordings failed to reveal differences in Kir4.1 unitary conductance, voltage-dependent open probability or voltage–current relationship. However, more recent results have provided evidence demonstrating a functional association of AQP4 and Kir4.1. The protein Dp75 is responsible for anchoring AQP4 and Kir4.1 at the end feet of the Muller cell, and its deletion was shown to reduce AQP4 expression, producing a partial AQP4 knockout while leaving Kir4.1 levels unchanged. Freshly isolated Muller cells of Dp75-deficient mice, exposed to an elevated potassium ($[K^+]_0 = 50$ mM), showed decreased potassium currents compared to wild-type controls. It is possible that in the earlier experiments with the AQP4-deficient cells, they were exposed to an insufficient transmembrane $[K^+]$ gradient (20 mM). These conflicting results suggest that further studies are needed to define the functional role of the AQP4–Kir4.1 ion–water transport complex. It should also be noted that during seizure events, the ceiling level of potassium reaches about 12 mM, which is considerably less than the 20 or 50 mM used in these experiments. Therefore, the functional significance of the Kir4.1-AQP4 interaction remains uncertain in seizures.

AQP4 Modulates ECS Parameters in Seizures

Another potential mechanism by which AQP4 may affect neuroexcitation is via modulating nonsynaptic transmission through changing ECS parameters such as diffusion hindrance and ECS volume fraction. The expression of AQP4 was demonstrated to affect both baseline as well as dynamic changes in ECS. Mice lacking AQP4 were shown to have enhanced macromolecular diffusion as demonstrated by cortical surface photobleaching and mirofiber-optic epifluorescent photobleaching (**Figure 3**). These results have been interpreted to reflect an expanded ECS volume fraction in the AQP4-deficient mice. Although the differences in ECS diffusion were as subtle as ~20%, based on in vitro results from brain slices, they may well account for the alterations seen during neuroexcitation. Experiments using hypersmotic media to modulate ECS volume fraction have demonstrated that increasing osmolarity by as low as 5–30 mOsm kg^{-1} inhibits high $[K_0]$ induced seizure events in brain slices. The correlation established between ECS volume fraction and ECS osmolarity dictates that ECS volume fraction is increased to 21.5–23.6%, from the physiological value of ~21% – which corresponds to the range of potential differences between AQP4-deficient and wild-type mice based on measurements of ECS diffusion. Confirmation of these differences will require direct measurement of ECS parameters.

AQP4 function may also affect nonsynaptic transmission by modulating dynamic changes of ECS volume. The relevance of ECS volume fraction changes in neuroexcitation has been demonstrated by multiple studies. Experiments with hippocampal slices showed a subtle but progressive increase of tissue resistance indicative of ECS volume contraction that preceded seizure initiation. The subsequent synchronized neuronal firing has been shown to be associated with reduced ECS volume fraction, as assessed by direct measurement with the TMA method, changes in the intrinsic optical signal, and reduced diffusion assessed by cortical surface photobleaching. ECS contraction in acute seizures has also been studied with diffusion weighted imaging (DWI) that shows reduced diffusion indicating cell swelling. In vitro data suggest that these dynamic changes in cell size may be dramatically altered by the lack of AQP4. As previously discussed, osmotic cell swelling is greatly reduced in AQP4-decicient astrocytes. We have also seen similar reduction in cell swelling in astrocytes exposed to an elevated potassium ($[K^+]_0 = 50$ mM). These data suggest that AQP4-dependent astrocyte swelling may contribute to ECS contractions and subsequently modulate concentration of ions and neurotransmitters during synchronized neuronal activity.

Future Goals

Based on the current data, AQP4 may modulate nonsynaptic transmission via its effects on the ECS and potassium flux, two mechanisms that most likely coexist during seizure events (**Figure 4**). The main challenge is to further explore these interrelated mechanisms. Further experiments, examining potassium conductance in AQP4-deficeint astrocytes exposed to potassium concentrations ranging from near physiological levels to the higher concentrations observed during CSD, are needed to resolve the current conflicting data regarding the functional interaction of AQP4 and Kir4.1. The biophysical association between water and ion fluxes also needs to be studied with respect to the effects on the intra- and extracellular compartments. For example, potassium fluxes driven by the existing electrochemical gradient may subsequently create an osmotic drive for water flux that requires AQP4 to facilitate transmembrane water transport. This question may be addressed using an in vitro cellular system where AQP4 and Kir4.1 are substituted with different members from their respective families to determine whether this is a general mechanism of coupled ion–water transport, or a mechanism specific to the AQP4–Kir4.1 interaction.

Capitalizing on the available evidence, the significance of AQP4 in the treatment of epilepsy will be explored along several avenues. If water transport through AQP4

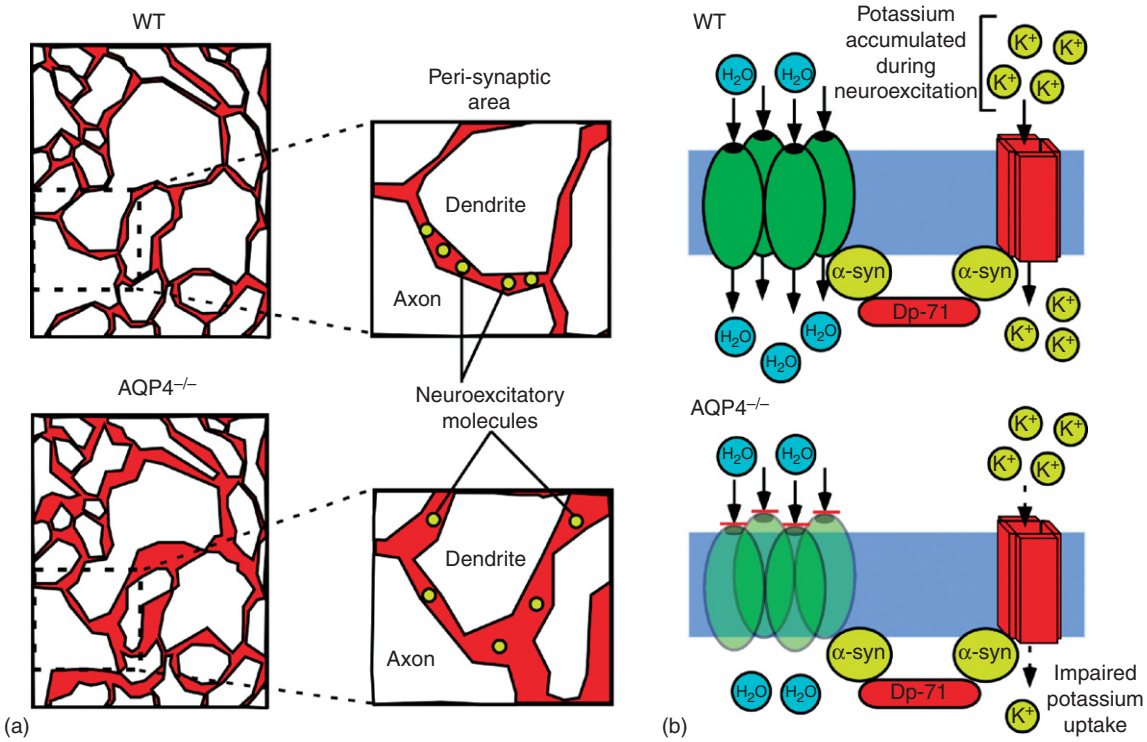


Figure 4 (a) AQP4 modulates ECS diffusion. Neuroexcitatory molecules released during neuronal firing may distribute into a larger volume due to the expanded ECS of AQP4$^{-/-}$ mice, resulting in lower seizure threshold. (b) AQP4 modulates ion fluxes. Potassium is elevated during neuroexcitation and also contributes to seizure progression. The functional coupling between Kir4.1 and APQ4 contributes a water–ion trafficking cluster facilitating potassium uptake into astroglia. Note the molecular scaffolding contributed by members of the dystroglycan complex (α-syntrophin and Dp71). The deletion of AQP4 results in slowed potassium kinetics, which translates to prolonged seizure duration in AQP4$^{-/-}$ mice.

facilitates more efficient transcellular potassium currents, modulation of AQP4 expression may enhance potassium buffering during seizures. Several studies have identified compounds that appear to upregulate AQP4; although the time window of their effect on AQP4 expression may preclude their use for the acute treatment of seizures, their utility for chronic modulation of AQP4 expression should be examined. The inhibition of AQP4 could potentially expand baseline ECS volume, contributing a larger distribution space for potassium and neurotransmitters released during neuronal firing. Exploration of this effect, however, will require the development of inhibitors of AQP4, which to date have not been successful. When available, AQP4 inhibitors could be administered to determine whether they can achieve chronic ECS expansion and increase seizure threshold. The continued exploration of the role of AQP4 in the nonsynaptic mechanism of epilepsy will likely lead to a better understanding of the complex interrelationship of ion and water transport, cell swelling, and the ECS in health and disease.

Finally, the function of AQP4 in ECS dynamics should be tested. A series of ultrastructural studies in cortical spreading has shown cell swell during cumulative neuronal firing, followed by a subsequent recovery to baseline as the neuronal activity terminates. These changes in cell volume translate to dynamic changes in ECS; as cells initially swell, ECS contracts and subsequently recover to baseline. Such ECS changes may impact on neuronal excitation by altering the volume or distribution of ions and neurotransmitters. We speculate that by facilitating transmembrane water flux, and thus cell volume changes, AQP4 is likely to enhance ECS dynamics and again modulate neuroexcitatory processes. Further experiments and mathematical modeling of these phenomena will help to relate ECS dynamics to the characteristic changes in extracellular potassium concentration during neuroexcitation.

Acknowledgements

Supported by NIH NS050173 and the UCSF Brain and Spinal Injury Center.

See also: **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Role of Astrocyte Dysfunction in Epilepsy; **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in

Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties.

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Role of Gap Junctions in Epileptic Synchronizations

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Introduction

Intercellular communication through gap junctions (GJs) in brain is increasingly recognized as playing an important role in neuronal synchrony and seizure generation, participating in both normal and pathological conditions. In this article, we describe the effect of GJ blockers on epileptiform activities generated in vivo and in vitro in an animal model of generalized absence epilepsy, and in vitro in neocortical tissue excised from patients presenting with partial epilepsy.

GJs are formed of specific proteins named connexins (Cx) or pannexins. GJ channels consist of two hemichannels (connexons), each made up of six Cx surrounding a central aqueous pore that permits the passage of ions, second messengers, and metabolites (up to 1 kDa). Cx 26, 29, 30, 32, 36, 37, 40, 43, 45, 46, and 47 are expressed in the brain, with marked variation according to the developmental stage and cell type. GJs have been described between neurons or between glial cells, rarely between glia and neurons. Cx43 is the major Cx in astrocytes while Cx36 and Cx45 are detected only in neurons. In the rodent neocortex and in the nucleus reticularis thalami (nRT), GABAergic interneurons are interconnected via electrical synapses and express Cx36, while very few GJs exist between pyramidal cortical neurons.

Background

It has been shown in several in vitro and in vivo models of epilepsy that blocking or enhancing GJ function respectively decreases or increases epileptiform neuronal synchronization. For example, carbenoxolone (CBX: the succinyl ester of glycyrrhetinic acid), a GJ blocker, has an inhibiting or delaying action on epileptiform activities in various models of epilepsy when locally applied in vitro or injected i.p in vivo. Recent studies in mouse knockouts for the Cx36 gene (the major neuronal Cx) did not reveal any obvious developmental or behavioral abnormalities, but showed a decreased intensity of epileptiform discharges induced by convulsant drugs, a result consistent with reduced electrical coupling between interneurons. In addition, neuronal synchronization reflected in field potential/EEG activity at various frequencies – gamma rhythms or high-frequency oscillations in the ~200-Hz

Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties.

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domain (ripples) – were also affected in these mice, both in the cortex and hippocampus. It has also been postulated that changes in Cx expression may play a role in epileptogenesis. Elevated levels of the astrocytic Cx43 have indeed been reported in the human epileptogenic tissue.

Over the last few years, several studies have highlighted a paradoxical role for GABA receptor-mediated mechanisms in initiating and maintaining epileptiform synchronizations; in particular, GABA-mediated synchronous activities can be instrumental in triggering epileptiform discharges. These epileptiform events continue to occur during application of glutamatergic receptor antagonists, and are abolished by application of the $GABA_A$ antagonist bicuculline. In this context, investigators have speculated that GJs between GABAergic cells may represent an important mechanism for such epileptiform synchronization. This hypothesis has been supported by the observation that CBX abolishes the synchronized negative-going field potentials generated in rodent CA3 pyramidal neurons during application of the K^+ channel blocker 4-aminopyridine (4AP), and also blocks the 4AP-induced neocortical seizures in rats in vivo. Such GABA receptor-mediated synchronous events can also be recorded in the presence of 4AP in neocortical slices obtained from patients undergoing surgery for epileptic disorders such as temporal lobe epilepsy (TLE) or focal cortical dysplasia (FCD).

Seizures are generated in the thalamocortical system in typical absence epilepsies. We investigated in vitro the role of GJs in thalamocortical synchronizations using thalamocortical slices from Genetic Absence Epilepsy Rats from Strasbourg (GAERS), a well-established rodent model of absence epilepsy, and in Non-Epileptic Rats (NER). We tested whether epileptiform activities recorded in thalamocortical slices prepared from GAERS or NER were equally sensitive to GJ blockers. We also examined whether CBX had an antiepileptic effect on spontaneous spike-wave discharges (SWD) recorded in the GAERS in vivo.

Finally, we sought evidence for a functional role played by GJs in the human neocortex during generation of synchronous GABA receptor-mediated potentials, as recorded in neocortical slices obtained from TLE patients during bath applications of 4AP and of glutamatergic receptor antagonists. In addition, we tested the effects of GJ blockers on the spontaneous synchronous activities occurring in some FCD neocortical slices in the absence of any pharmacological manipulation.

Methods

Tissue

Samples of human neocortex were obtained from the anterior part of the second temporal gyrus in the course of neurosurgical interventions for medically intractable seizures associated with TLE, or from FCDs in temporal or frontal external cortices. The tissue used for the in vitro electrophysiological investigations was part of a block of brain tissue removed for strict therapeutic reasons. Informed consent was obtained according to the guidelines of the ethics committees of the Hôpital Sainte-Anne (Paris, France) and of the Montreal Neurological Institute and Hospital (Montreal, P.Q., Canada).

In vivo studies were performed in GAERS and in NER aged 3–4 months. In vitro experiments were performed on brain slices from GAERS and NER, both young (aged 20–25 days) and adult (aged 40–50 days). All animal experiments were performed in accordance with the rules of the European Community Council Directive of November 24, 1986 (86/609/EEC), and the French Department of Agriculture.

Slices

The techniques used for preparing and maintaining human or rat brain slices in vitro were similar to those previously reported. For studies on rat tissue, combined thalamocortical slices (500 μm thick) were obtained using procedures described previously (see Further Reading section).

Slice Preparation Recordings

Extracellular field potentials were recorded with electrodes filled with artificial cerebrospinal fluid (ACSF) positioned under visual control in the supragranular neocortical layers (supposedly layer III) in human slices, or in neocortex or ventrobasal thalamus (VB) in rat thalamocortical slices. To test the effect of GJ blockers (CBX, octanol, and quinidine) on neuronal excitability, constant current stimuli (0.02–1 mA, 80 ms) were delivered every 5 s to the infragranular cortical layers and the fiber responses were measured in supragranular layers; measures were made before drug application, 60 min after application of GJ blockers, and 60 min after drug washout. Slices obtained from TLE patients were continuously perfused with a medium containing 4AP and ionotropic glutamate receptor antagonists. FCD slices were kept either in normal ACSF or in 4AP-containing ACSF. The degree of phase unlocking (more details in the Further Reading section), as assessed in measurements of synchrony recorded from two sites, was determined during 10 min blocks under control condition, 20 min after GJ blocker application, and 60 min after GJ blocker washout.

ECoG Recordings in GAERS and NER

Adult GAERS and NER were implanted with four epidural electrodes over the frontoparietal cortex. All the

animals were allowed 1 week recovery and handled twice a day. All GAERS displayed spontaneous SWD (Figure 1 (a)). After a reference ECoG recording period of 30 min, saline or CBX (50 or 100 mg kg^{-1}) was injected i.p and the recordings were carried on for 90 min. The experimental protocol was as follows: day 1: injection of saline; day 3: injection of CBX 100 mg kg^{-1}; day 5: injection of saline; day 6: injection of CBX 50 mg kg^{-1}. The mean duration, the frequency, and the cumulative duration of SWD episodes were measured during a reference period of 30 min and during three consecutive periods of 30 min after the injection. We also determined the effect of CBX on the EEG power spectra in GAERS and NER, under saline vs. CBX conditions.

Recent Results

Effects of Carbenoxolone Administration *in vivo* in GAERS and NER

Following i.p. CBX injections, the animals' behavior was apparently not impaired, except for a somewhat reduced locomotor activity lasting up to 90 min. At 50 mg kg^{-1} i.p. in GAERS, CBX decreased the SWD cumulative duration nonsignificantly, while at 100 mg kg^{-1} the decrease was highly significant during the first 30-min period (to 41% of the reference period value), due to a reduction of both SWD episode frequency and duration (Figure 1(b)). The frequency and amplitude of spike-waves within a SWD episode were, however, not affected. During the

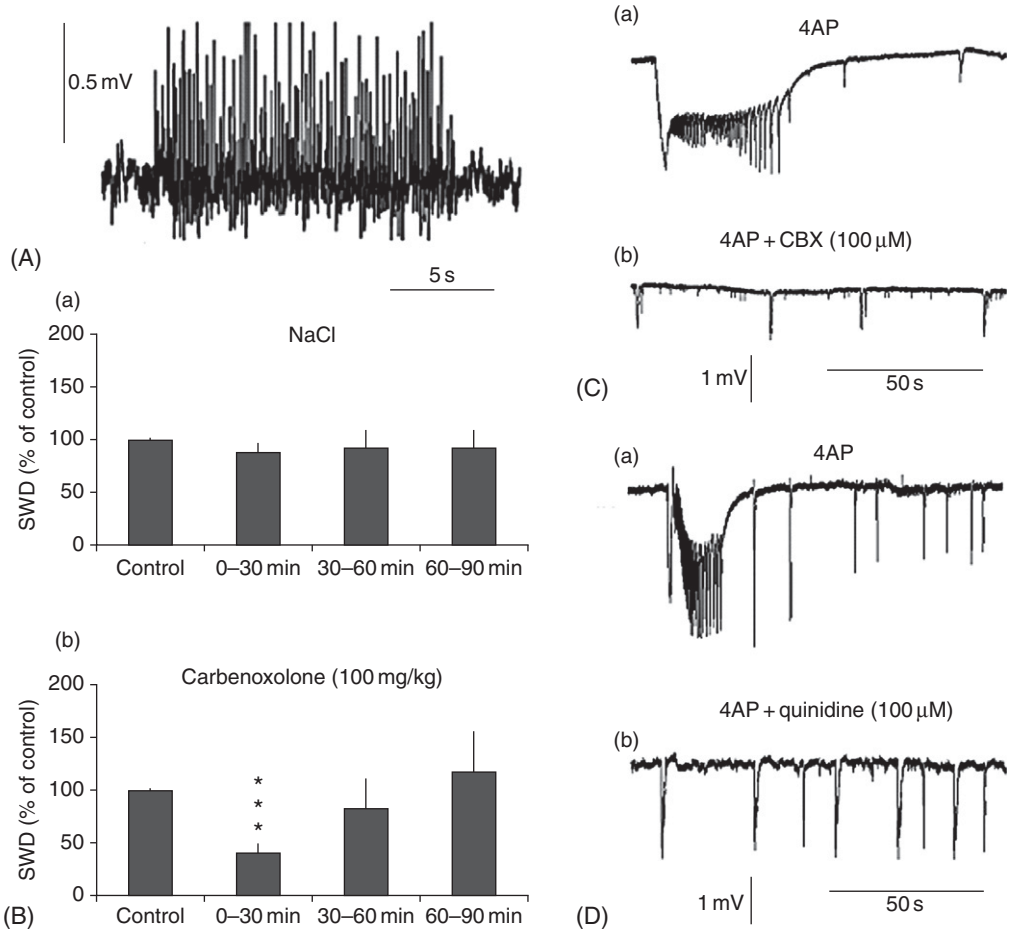


Figure 1 Effects in vitro and in vivo of gap junction blockers on epileptiform activities recorded in genetic absence epilepsy rats from Strasbourg (GAERS). (a) ECoG recordings showing an example of spike-wave discharges (SWD) recorded in freely moving GAERS. (b) Effect of i.p. injections of NaCl (a) and carbenoxolone (CBX) (b) in GAERS on the cumulative duration of SWD. The percentages obtained after CBX injections are compared with those obtained after NaCl injections (***$p < 0.005$). The cumulative duration is significantly decreased during the first 30-min period after CBX injection. (c) Field potential recordings in functional thalamocortical slices. a – ictal-like activity (ILA) induced at the neocortex by bath application of 4-aminopyridine (4AP), triggered by a GABA-dependent negative field potential. b – bath applied CBX (100 μM) abolished the 4AP-induced neocortical ILA after about 30 min while the GABA-dependent negative field potentials remained. (d) a – 4AP-dependent neocortical ILA. b – effect of bath-applied quinidine (100 μM) – a gap junction blocker more specific for the neuronal connexins – on the 4AP-induced neocortical ILA. The GABA-dependent negative field potentials remained but the ILA vanished after about 50 min. Modified from Gigout S, Louvel J, and Pumain R (2006a) Effects in vitro and in vivo of a gap junction blocker on epileptiform activities in a genetic model of absence epilepsy. *Epilepsy Research* 69: 15–29, with permission from Elsevier.

second and third 30-min periods after the injections, no significant effect could be detected. At 100 mg kg^{-1}, CBX induced a significant reduction (to 45% of control) of the spectral power of the frequencies corresponding specifically to the SWD (peak at 6.2 Hz) during the first 30 min. In NER, CBX at 100 mg kg^{-1} did not modify the power of frequencies corresponding to that of SWD, suggesting that CBX had no obvious non-specific effect.

Effects of Carbenoxolone *in vitro*

Effects induced by 4AP on thalamocortical slices

After introducing 4AP at 50 μM in the perfusion medium, synchronous ictal-like activities (ILAs), characterized by an initial tonic phase followed by afterdischarges (superimposed on a characteristic slow field potential shift) were recorded (within 10–40 min) in the neocortex of thalamocortical slices. ILAs were simultaneously observed in the thalamus, we viewed these thalamic events as volume conduction reflections of activities generated at the cortex. ILAs frequency became stable within 60 min, with interevent intervals ranging between 126 and 690 s. Independent briefer and faster activities were recorded in the hippocampus. 4AP induced occurrence of GABA-dependent negative field potentials that were recorded in the somatosensory neocortex but not in the thalamus. These large negative potentials were often observed at the onset of cortical ILAs and appeared as the triggering factor for ILAs in slices from both young and adult animals (**Figure 1(c)**). The $GABA_A$ receptor antagonist bicuculline abolished these GABA-mediated potentials, and also prevented occurrence of ILAs. There was no significant difference between slices from GAERS and NER (from both young and adult animals) regarding the latency of the appearance of the ILAs, their mean duration, their average frequency or their cumulative duration.

Carbenoxolone effects on synchronous activity

The amplitude, and the stimulus intensity threshold, of the fiber and synaptic components of the corticothalamic and thalamocortical field responses were not altered following CBX application, indicating that CBX does not exert its antiepileptic effect via a general, non-specific depressant action on cellular excitability. Bath application of CBX produced a progressive decrease of the frequency of the 4AP-induced ILAs, until they were abolished (**Figure 1(c)**). Quinidine, another GJ blocker more specific for neuronal GJs, also abolished the ILAs in 20–40 min (**Figure 1(c)**). Surprisingly, the 4AP-induced GABA receptor-mediated negative field potentials remained without decrease in amplitude during the 40 min after CBX or quinidine application, even after the disappearance of the ILAs. After 40 min of application, their frequency decreased progressively until they disappeared during the second hour of application; there was no recovery on wash-out.

The decrease in ILAs frequency after CBX was faster in NER slices than in GAERS slices. This difference was significant for the 0–20 min and 20–40 min periods after drug application. In NER, the mean duration of ILAs first increased (during the 20–40 min period after CBX), while in GAERS it decreased progressively. The resulting effect is that during the 0–20-min period after CBX, the cumulative duration of ILAs in GAERS and NER decreased similarly. After a prolonged washout (60–80 min), ILAs could reappear but at a very low frequency.

Effects of Gap Junction Blockers on 4AP-Induced Synchronous Activity in TLE Tissue

Synchronous activities induced by 4AP in human TLE epileptogenic tissue

We never observed spontaneous field potential activity in any of the neocortical slices obtained from TLE patients perfused with physiological ACSF. During superfusion with a medium containing 4AP and ionotropic glutamatergic receptor antagonists, all slices generated spontaneous negative or positive–negative GABA-dependent field potentials (amplitude: 0.2–3 mV; duration: 0.2–0.6 s; intervals of occurrence: 3–27 s). When simultaneous recordings were performed at two sites (distance: 1–5 mm) along an axis parallel to the pial surface (depth: 600–1 000 μm below the pial surface), the field events were phase locked (measured by a phase unlocking index, see Further Reading section for more details). One event (leader) usually preceded the other (follower), with time lags ranging from 25 to 400 ms depending on the distance between the recording sites (**Figure 2**). By measuring the time lags between the synchronous events, an apparent velocity of propagation of 15 mm s^{-1} was calculated. Followers and leaders could sometimes invert their roles.

Effects of gap junction blockers

Bath-application of CBX (0.3–0.5 mM), quinine or quinidine (0.5 mM) had no significant action on the amplitude, or on the stimulus intensity threshold, of the fiber component of the field responses, indicating that these drugs did not exert a marked non-specific effect on neuronal excitability at these concentrations. After bath-application of GJ blockers, the synchronous 4AP-induced negative field potentials remained and their amplitude or shape was not substantially modified during the first 60 min of perfusion – although their frequency could decrease. However, at the largest doses and for more prolonged periods of applications, these could disappear (and their amplitude decreased just before complete disappearance).

The most striking finding was that GJ blockers disrupted the phase locking of the negative field potentials recorded in the cortex at distances of 1–5 mm, at a time

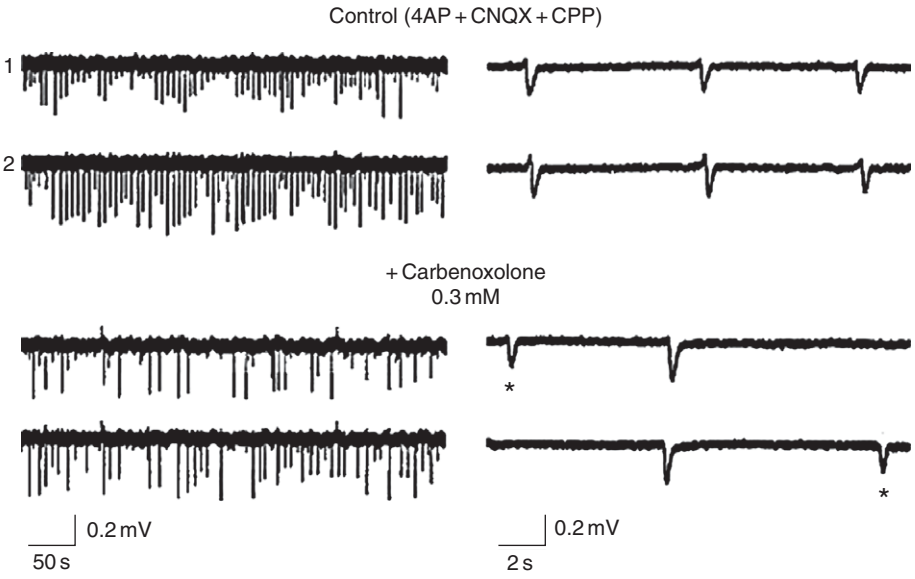


Figure 2 Effects induced by carbenoxolone on synchronous negative field potentials recorded in neocortical slices obtained from patients suffering from temporal lobe epilepsy during 4AP and ionotropic glutamatergic receptors antagonists applications. The spontaneous field events recorded from two sites located 2 mm apart along an axis parallel to the pia (depth: 700 μm) are displayed at two different time bases. These events occurred in synchrony in control conditions (upper right panel). About 30 min after further addition of carbenoxolone (0.3 mM), their frequency was decreased. This effect was accompanied by a disruption in their synchronization (asterisks in the right lower panel). Modified from Gigout S, Louvel J, Kawasaki H, *et al*. (2006b) Effects of gap junction blockers on human neocortical synchronization. *Neurobiology of Disease* 22: 496–508, with permission from Elsevier.

point after application at which their frequency was not reduced. After 20 min of application, CBX (at 0.1, 0.3, or 0.5 mM) produced a significant increase in the phase unlocking index, indicating disruption of phase locking. Quinidine or octanol produced the same effect. Recovery of phase locking was observed after washout of CBX at 0.3 mM, but not at 0.5 mM. Apparent velocities of propagation for the spontaneous activities were not modified by quinidine 100 μM, carbenoxolone 300 μM or octanol 0.5 mM.

Effects of Carbenoxolone on the Synchronous Activity Generated in Focal Cortical Dysplasia Tissue

The only neocortical slices in which spontaneous field potential activities were observed in normal ACSF were prepared from tissue removed from patients suffering from FCD. These events were generated at different sites in the slice and were characterized either by simple field potentials consisting of 1–3 fast negative transients, or by the rhythmic sequence of fast transients riding on a slow negative shift with duration up to 8 s. These events occurred in synchrony when simultaneously recorded from two sites along an axis parallel to the pia (distance up to 3 mm) with time delays between 10 and 50 ms (apparent velocities of propagation of 45–142 mm s^{-1}). Application of NMDA receptor antagonists reversibly abolished, within 5–10 min, these spontaneous events. Single-shock electrical stimuli induced prolonged after discharges (duration of 1–4 s) which were also abolished within 5–10 min by NMDA receptor antagonists.

These spontaneous synchronous activities were markedly reduced or abolished by CBX applications (0.3 mM), within approximately 30 min. These effects were characterized by disruption of phase locking between activities recorded at two different neocortical sites (as identified by analyzing the interevent histograms or the mean phase unlocking measure). The effects induced by CBX were partially reversed during prolonged washout (over 60 min). The stimulus-induced responses recorded under control conditions in these FCD neocortical slices were also reversibly abolished by CBX applications.

Conclusions and Future Goals

These experiments were performed in a model of generalized absence epilepsy and in human cortical tissue excised from patients suffering from partial epilepsy. In both cases, blockade of GJs decreased the expression of epileptic manifestations. More specifically,

- GJ blockers reduced the frequency of spontaneous SWD in vivo.
- The main effect of CBX on the 4AP-induced epileptiform activities in vitro was to abolish the ILAs, without suppressing or modifying the GABA-mediated potentials which initiated them. This observation indicates

that, surprisingly, CBX does not prevent synchronization of interneurons (which was expected in view of the specific electrical coupling of interneurons in the neocortex) but rather interferes with transfer of interneuronal synchronization into seizure activity in the cortex.

- GJs contribute to the spread and phase locking of synchronous GABA-mediated events in neocortical slices from TLE patients, as seen during blockade of ionotropic glutamatergic transmission.
- GJs play a role in the generation of synchronous activity recorded in FCD slices superfused with normal medium.

Very little is known concerning the expression of the various Cx in cortical tissue excised from patients with epilepsy. It would be of interest to determine the nature of the Cx expressed in this tissue and to quantify their expression using RT-PCR. Comparison with 'control' tissue (although difficult to obtain) would be necessary. It would also be very interesting to examine whether GJs are present between interneurons and pyramidal cells in the human epileptogenic cortex or in rodent models of epilepsy. The available GJ blockers have been criticized for their lack of specificity, and the development of drugs that are able to discriminate between the various Cx would constitute a major advance in deciphering the role of electrical coupling in brain.

See also: **Absence Seizures**: Thalamocortical Synchronization and Absence Epilepsy; **GABA**: Synchrony Through GABAergic Inhibition; **Metabolic Changes**: Glucose Metabolism and $GABA_A$ Receptor Dysfunction in Human Partial Epilepsy; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures.

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OSCILLATORY ACTIVITY

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Cellular and Network Oscillations in Epilepsy

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Introduction

The traditional hallmark of epileptiform discharge is entrained electrical oscillatory activity in a large enough volume of cerebral tissue to be measurable in the context of a possible seizure disorder and cerebral dysfunction. This oscillatory activity seen in seizures is usually characterized by reduced complexity and increased widespread synchrony as compared with 'normal' brain activity. We are trying to understand the mechanisms underlying the various forms of oscillatory activity and their transitions. As such, the big questions are as follows:

1. How do we measure and characterize these oscillatory activities and their transitions?
2. What are the sources of these oscillations: subcellular, cellular (which cells?), local clusters, small or large neural networks? Are there multiple sources of seizure activity? Do seizures tend to arise from a single focus or is a seizure largely an emergent property of a distributed network?
3. How do transitions from interictal to ictal modes occur? Are seizures a manifestation of coupled oscillators? Are transitions to seizure due to internal or external factors?
4. How can one intervene to change these oscillatory modes from an epileptic to a nonepileptic mode?

To answer these questions, we approach the neuronal system as a nonlinear dynamical system where, conceptually, epileptiform seizures are modes of the system's oscillatory activities. Our research includes both *in vitro* and *in vivo* measurements, as well as modeling and analyses of the system dynamics. Understanding the mechanisms of complex oscillations and transitions will be critical for diagnosing and ameliorating the negative effects of seizures. Insight into these mechanisms may also enhance our understanding of the manner in which other transitions in brain activity take place during nonseizure phases and cognitive processing.

Background

Sources and Scales of Oscillatory Activity

Brain oscillations emanate from many sources at many different levels. Although one often thinks of cellular-level sources, in fact there is a rich supply of oscillatory activity within the cell itself (both biochemical and electrical), as well as multiple sources at the whole network level.

Subcellular oscillations

Oscillations are seen within the nucleus and also within subcellular organelles such as mitochondria. Oscillations

of intracellular ionic concentrations such as calcium and pH have been measured, and there are rhythms of molecular processes and intracellular protein cycling at a range of frequencies.

Cellular oscillations

From an electrical perspective, measures of the neuronal membrane potential have shown a wide range of spontaneous oscillatory frequencies (sometimes called 'noise'). These oscillatory activities arise from several sources, including afferent synaptic inputs (both action potential-evoked and spontaneous), intrinsic ionic current fluctuations, and ephaptic (electric field) effects (which are frequently ignored). These spontaneous membrane fluctuations are as yet little studied and their contributions to the extracellularly-measured field fluctuations are not well understood. Furthermore, the contribution of the more numerous non-neuronal cells, such as glia, are even less well-studied, but could be of greater importance than heretofore realized. Cell types, each type with its signature oscillatory activity, make their own specific contribution to the measures of oscillatory activity.

Intercellular connectivity

The interconnectedness of neurons to each other, and to other cell types, is a further factor contributing to the overall measures of brain oscillations. The more a group of cells oscillates together at specific frequencies, the higher are the expected amplitudes of the emanating common signals. There are several factors that will unite a group of cells. The most obvious are chemical synapses. However, direct intercellular connections via gap junctions are particularly evident in cortical and hippocampal interneurons, and there is a growing literature showing a role for gap junctional communication in the genesis of seizures. Another means of synchronizing groups of cells is via synchronous afferent synaptic input, be it excitatory or inhibitory. For example, synchronous inhibitory input into thalamocortical and cortical–thalamic neurons plays a large role in the generation of thalamocortical oscillations in absence seizures. Ephaptic (field) effects can also be a powerful entrainment force, as can be demonstrated by the application of an electrical field to neural tissue via direct or transcranial brain stimulation. Less recognized but of significant importance are the synchronizing actions of changes in extracellular chemical elements such as potassium, pH, glucose, oxygen, and various hormones.

Clusters

An important emerging concept is that of cellular clusters, a group of synchronized cells (usually thought of as neurons, but glia could also be involved), which oscillate together at the same frequency or frequencies. A cluster provides local synchrony for a period of time in a spatial context. The cells involved in a cluster could be coupled together by various mechanisms for brief to prolonged periods, following which individual cellular units of this cluster could become attached to another spatially contiguous cluster. Thus, there might be an ongoing rearrangement of these cellular clusters, giving rise to a constantly changing source of local oscillatory activity. Finally, we conceive that these clusters are physically interdigitated or intermingled.

Networks

From the local clusters of synchronized cellular elements, the next conceptual level is that of the network. Networks may range from connectivity between 'local' units to connectivity across larger brain regions, to the coupling of brain regions (e.g., the limbic system), to connections between distal sites in opposite brain hemispheres. Whatever the scale, the concept of a network is important for the understanding of brain oscillatory activity and its propagation. Brain networks not only comprise several levels of size and complexity but are also likely to be constantly and dynamically re-arranging and regrouping themselves. Moreover, the various subcellular, cellular, and network sources of oscillations are undoubtedly interacting simultaneously across different levels in an ongoing and dynamic manner.

Oscillations and Complexity

Early investigations into chaotic and epileptic brain activity provided convincing evidence that the dynamics seen during various cognitive conditions were significantly more complex than those seen during a seizure. For example, Babloyantz and Destexhe. reported that electroencephalogram (EEG) recordings of human absence seizures showed simple attractor dynamics as compared with sleep brain activity. In general, most such studies have provided strong evidence in favor of the suggestion that epileptic seizures corresponded to a lowering of complexity in brain electrical activity.

Synchrony and the Unbinding Problem

In contrast to these findings indicating the lowering of complexity during seizures, researchers such as Netoff and Schiff have argued that certain types of seizures may actually be associated with an *increase* in complexity in the form of a reduction in synchrony. Moreover, reductions in complexity have been found in response to ordinary sensory stimulation during cognitive processing. For example, by studying the EEG recorded from the olfactory bulb and the prepyriform cortex of the rabbit, Freeman reported that when the rabbit is presented with a familiar odor, the normally high-dimensional chaotic activity suddenly bursts in a regularized pattern. Singer and many other researchers have also suggested that synchrony and other forms of reduction in complexity can explain how the brain might generate coherent representation by spatio-temporal 'binding' of neural activity.

Although transient reductions in complexity may indeed be observed across cognitive conditions, epilepsy has taught us that, in most cases, it is precisely the strong entrainment of activity and reduction in complexity that is most likely to interfere with cognitive processing. It is a defining feature of neural systems that they avoid prolonged simple and entrained activity and that they tend toward complexity. With the exception of conditions such as status epilepticus, even the entrainment seen during seizures is transitory. Although a great deal of the current literature has focused on synchrony and binding, the study of epilepsy suggests that we need to understand how the brain *avoids* binding and urges us to ask what brain anatomical and physiological principles enable complexity?

Even if one assumes that synchrony and the lowering of complexity may be necessary for cognition, the question remains as to what mechanisms ensure the escape from this entrainment. The question is not simple. Ohayon *et al.* have suggested that the tendency toward synchrony and limit-cycle activity is ubiquitous in certain network models and that this problem of locked-in periodic oscillations may worsen in the presence of some forms of plasticity. How does the brain solve this unbinding problem? What are the mechanisms of transition between the various modes of oscillatory activity that avoid 'lockup?'

Transitions, Intermittency, and Modulation

Although the specific nature of the transitions into and out of seizures is a central question in the field, the basic mechanisms of transition and the triggering factors – if any – are often unknown. Lopes da Silva *et al.* have outlined several potential routes to seizures. One possibility is the switching between two pre-existing 'attractor' brain states via a perturbation or noise. In this scenario, the brain might switch from interictal oscillatory activity to seizures following a stimulus, as in the case of certain photosensitive epilepsies. Another potential route involves an increase in susceptibility to seizure brought about by a parametric change in the system, as might happen over time with a hormonal shift or hypoglycemia.

Beyond these two routes there also exists another possibility in which the shift into and out of seizures may occur autonomously of any external stimulus or change in the system. Specifically, *intermittency* is a form of activity wherein a dynamical system may continually alternate between two modes of activity independently of an external perturbation. Velazquez *et al.* have reported intermittency in human partial epilepsy. There are various known forms of intermittency, and various statistical techniques may help identify the type of transition that precedes a given seizure.

The identification of alternate transition categories and an understanding of the mechanisms underlying brain dynamics could play an important role in clinical treatment. In particular, seizures may be modulated or altogether avoided by carefully applying small perturbations to a system, thereby eliminating low-complexity activity. For example, Schiff *et al.* were able to use small electrical brain stimulations to induce a change from lower complexity 'periodic' behavior to high-complexity 'chaotic' responses, suggesting that the technique might be applied to activity in epileptic foci. Other studies have since followed, demonstrating the feasibility of using various forms of stimulation to modulate ictal activity both in models and in vivo.

Methods

Human EEG and Biological Models of Epilepsy

The conventional and still current standard for measuring epileptiform phenomena is extracranial electrographic field recordings – the EEG. In humans, the primary measure is the scalp EEG, which, in cases where the site(s) of origin of the epileptic activity is unclear, is augmented by intracranial recordings. Because seizures are usually spontaneous events, prolonged (days to weeks) recordings are often required before enough seizure activity has been observed for appropriate diagnosis and localization. Until recently, human EEG recordings, including intracranial EEG, were almost always done with filtering of all high-frequency activity above 70 Hz, thus ignoring important data relevant to our understanding of the brain oscillatory activity associated with epilepsy.

There are in vivo and in vitro models of epilepsy and seizures, too numerous to mention. In in vivo models, extra-cranial and/or intracranial EEGs are generally recorded in association with a stimulus (e.g., repetitive subthreshold intracranial stimulation – 'kindling'), introduction of a chemical (e.g., kainic acid, pilocarpine, cobalt, tetanus toxin), or an insult (e.g., cerebral trauma) given to elicit seizures. These seizures can directly follow stimulation, or occur spontaneously, which is more akin to the human situation. In vitro models provide direct access to cerebral tissue for local electrical field recordings, whole-cell or intracellular recording, cellular or local network imaging of ionic changes (e.g., calcium), or electrical changes monitored by voltage sensitive dyes. In vitro models also provide control of the extracellular environment. However, the relation of these in vivo and in vitro models to the oscillations and basic mechanisms of clinical epilepsy is not always clear.

Coupled Oscillators Modeling

To further examine the basic mechanisms of oscillations, there has been extensive use of computational models. For example, the mapped clock oscillator (MCO) first described by Bardakjian and Diamant is quantitative in nature and its parameters can be obtained directly from the measured intrinsic transmembrane voltage waveform

in the biological tissue when a segment of the tissue is isolated by partial or complete cuts from its neighborhood. The model thus allows us to explore multiple critical parameters simultaneously at a level of detail not always possible in biological recordings. When such oscillators are coupled together to form networks, they can qualitatively and quantitatively simulate the electrical oscillations recorded in hippocampal neuronal networks. Such models exhibited spontaneous transitions into seizure-like activities and were used to test approaches to predict and then abolish such activities. More details regarding these coupled oscillator models can be found in Further Reading.

Recurrent and Embodied Network Models

To identify the contributions of network structure to oscillations and transitions, we have also modeled activity propagation in more abstract networks, with units governed by probabilistic activation functions (sigmoid, Gaussian) or simple spiking functions with various architectures. For example, we have examined networks with random connections between cells as well as those with spatially organized local connectivity. These models are helping us explore the relation of structure to oscillations in both intact networks as well as in networks with cell deletions (in order to reflect such conditions as posttraumatic epilepsy). There is also active research being done on a range of network architectures, such as small world networks, which may help explain the difference in forms of oscillation (and susceptibility to seizure discharge) across brain structures.

One issue, however, with many of these models is that they are figuratively and literally disconnected from the environment. As such, a recent step has also been the inclusion of 'embodied networks.' These models consist of networks that are connected to external sensors and transducers (robots), and are evolved with genetic algorithms to maneuver in the environment. These embodied models aim at addressing the increasing recognition that behavioral interaction with the environment is critical to brain development, and has an essential role to play in neural oscillations both in epilepsy and other conditions.

Recent Results

Coupled Oscillators – Biological Data

The concept of coupled oscillators is increasingly recognized as useful for the understanding and modeling of brain activity in general and epileptiform activity in particular. We have demonstrated in the isolated mouse hippocampus that recurrent, spontaneous seizures generate bidirectional epileptiform activity along the septotemporal axis (as measured by multiple electrode recordings and optical imaging), indicating a system of coupled network oscillators (see **Figure 1**). Uncoupling the system of oscillators, by partial or complete cuts, resulted in independent bidirectional activity in the separated tissues. Within the same epileptic burst, low- and high-frequency components originated from different locations. A second seizure model (generated by focal tetanic electrical stimulation) also exhibited bidirectional discharges. Clinical data (unpublished), obtained from adjacent intracranial depth electrodes in the hippocampus of epileptic patients, have similarly revealed bidirectional seizure bursting activity, but at a much larger anatomical scale.

Higher Frequencies and Kindling

As mentioned in the Background section, the importance of high-frequency activity has often been overlooked in human EEG and epilepsy. This focus on low frequency at the exclusion of higher ranges has also extended into various seizure models. For example, it has been known for decades that the changes brought about by the repeated stimulations in kindling can have profound long-term effects on low-frequency oscillations. However, changes in high-frequency components were rarely reported, mostly because of limitations in data acquisition equipment and shortcomings in the prevalent approaches to spectral analysis. By increasing the sampling rates and introducing new forms of analysis, we have shown that there are dramatic changes in power and other characteristics of oscillations across all frequencies during kindled seizures. Specifically, we have demonstrated that there is a significant increase in higher frequency events (>80 Hz) during kindled seizures (**Figure 2(a)**). To identify these changes, we recorded the activity at high sampling rates (2–10 kHz) and developed a calibrated amplitude wavelet transform (CAWT) that normalized the power across frequency bands. The normalization of activity by frequency is important because wavelet and other algorithms tend to highlight long period events, thereby drowning out shorter – but likely just as significant – fast events. This application of normalized wavelet analysis across frequency bands could also help elucidate the importance of such events in other models and brain recordings.

Higher Frequencies and Complexity

Although the existence of hippocampal high-frequency electrical activities (greater than 100 Hz) during the progression of seizure episodes in both human and animal experimental models of epilepsy has been previously documented, this information has not been studied between successive seizure episodes or utilized in the application of seizure classification. We examined the dynamical changes of an in vitro low Mg^{2+} rat hippocampal slice model of epilepsy, at different frequency bands, using wavelet transforms and artificial neural

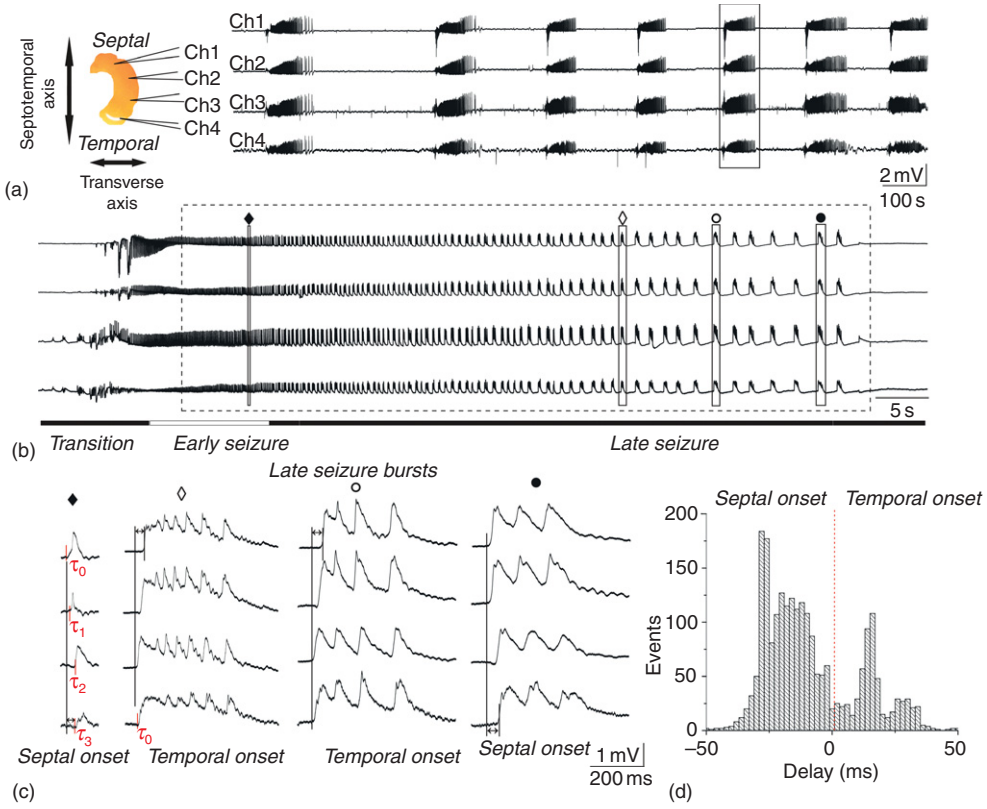


Figure 1 Multisite field recordings illustrate bidirectionality. (a) Four field electrodes in the CA1 subfield (Ch1–Ch4) placed along the septotemporal axis of the hippocampus (left inset-arrows denote direction of two axes) record recurrent, spontaneous seizures from the intact hippocampus. (b) A single seizure (expanded from panel (a)) recorded from all four electrodes shows the three characteristic phases of seizure. The transition phase is characterized by temporal onset during which there is lack of functional coupling, and later during the seizure (early, late phases), epileptiform activity displays bidirectionality and becomes functionally coupled across all four channels. (c) Bidirectionality of single intraseizure bursts (expanded from panel (b)) shows both septal (♦, •) and temporal (◊, ○) onsets. Vertical red lines illustrate beginning of events as represented by their relative times of onset ($\tau_0\tau_0$, τ_1, τ_2, τ_3), and (↔) denotes difference in times between the septal and temporal events. (d) Histogram of the event delays between the two outermost electrodes (Ch1–Ch4) reveals that most epileptic activity in the analysis window is of septal origin, while the average delay between these two channels is irrespective of the site of onset. Adapted from Derchansky M, Rokni D, Rick JT, *et al*. (2006) Bidirectional multisite seizure propagation in the intact isolated hippocampus: The multifocality of the seizure focus. *Neurobiology of Disease* 23: 312–328.

networks (ANNs). By dividing the time–frequency spectrum of each seizure-like event (SLE) into frequency bins, we analyzed their burst-to-burst variations within individual SLEs as well as between successive SLE episodes, using high sampling rates (10 kHz). We demonstrated that the activities of high-frequency oscillations in the 100–400-Hz range increased as the slice approached SLE onsets and in later episodes of SLEs. By examining the time-dependent relationship between different frequency bands, we showed the importance of the higher frequencies for classification of activity. In particular, we demonstrated that activities in the frequency range 100–400 Hz were critical for the accurate classification of electrographic seizure-like episodes – containing interictal, preictal, and ictal states – in brain slices undergoing recurrent spontaneous SLEs. Although preictal activities could be classified with an average accuracy of $77.4\% \pm 6.7\%$ using frequency spectrum in the range 0–400 Hz, we could achieve a similar level of accuracy by using a nonlinear relationship between 100–400-Hz and <4-Hz frequency bands only. We have demonstrated that the decrease in complexity (as measured by the Lyapunov exponent) between interictal and ictal events was significant for the higher frequency bands. These experiments are important in that not only do they examine the change in power across frequencies but also quantify changes in the system's dynamical properties, including complexity (**Figure 2(b)**).

Transitions, Noise, and Intermittency in Coupled Oscillator Models

Coupled oscillator models, based on the biological transmembrane voltage recordings from in vitro hippocampal slices, were used to investigate the following:

1. *The stochastic resonance effects.* That is, we examined the relation of noise to the occurrence of coherent

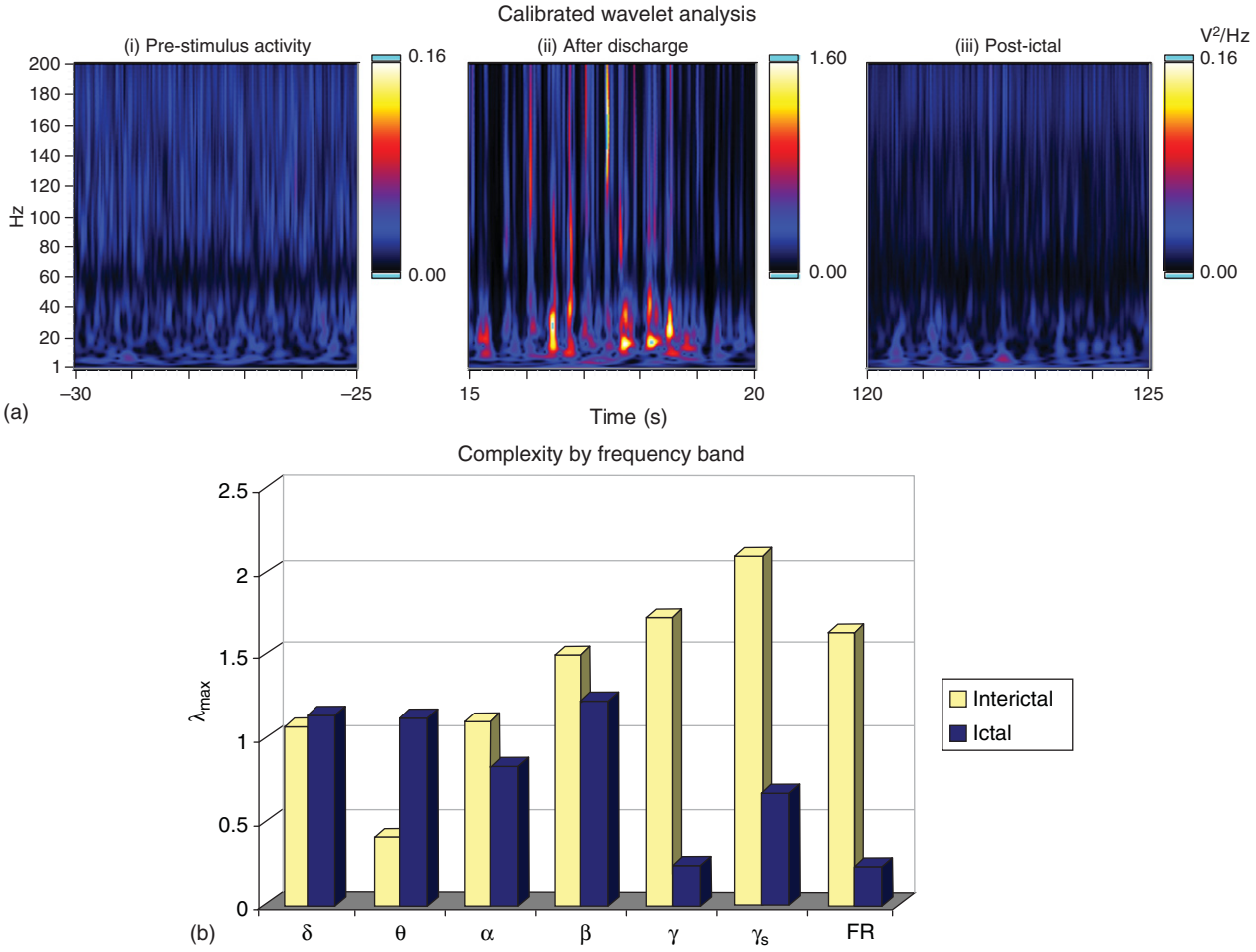


Figure 2 High frequency and complexity. (a) Changes in high-frequency events during kindled seizures. The figure shows the CAWT analysis for 5 s of (i) preictal, (ii) after discharge, and (iii) postictal hippocampal activity in kindled seizures. The kindling stimulus was applied to the perforant path and is indexed here as $t = 0$. This normalized amplitude wavelet analysis confirmed previously reported changes in power at lower frequencies but also demonstrated that changes extended into the high-frequency bands of 80–200 Hz. Note that the *Z* amplitudes are zoomed (10×) for the pre- and postictal events in order to show the activity in these periods. As such, the changes in activity are even more pronounced than the graphs otherwise indicate. The darker banding at 60 Hz is due to a notch filter. Adapted from Ohayon EL (2000) Connectionism and Wavelets in the Modeling and Analysis of Neural System Dynamics. M.Sc. Thesis., Ottawa: National Library of Canada, Ottawa. (b) Complexity measures across frequency bands. The maximum Lyapunov exponents (λ_{max}) were computed for each frequency band in hippocampal recordings in the Mg^{2+} model of epilepsy. A time series at each frequency band was constructed from the average wavelet coefficients in frequency. It is shown that the most significant decrease in complexities occurred in the higher frequency dynamics (>40 Hz). The frequency ranges shown are as follows: delta (δ, <4 Hz), theta (θ, 5–8 Hz), alpha (α, 8–15 Hz), beta (β, 15–40 Hz), gamma (γ; 40–100 Hz), super gamma (γ_s, 100–250 Hz), and fast ripples (FR, 250–400 Hz). Adapted from Chiu AWL, Jahromi SS, Khosravani H, Carlen PL, and Bardakjian BL (2006a) The effects of high-frequency oscillations in hippocampal electrical activities on the classification of epileptiform events using artificial neural networks. *Journal of Neural Engineering* 3: 9–20.

oscillations. We found that field and gap junctional coupling pathways exhibited significant stochastic resonance effects in the 4–8-Hz frequency range, but did not exhibit stochastic or coherence resonance phenomena at 0.5 Hz. This helped explain why reported in vitro seizure control strategies using pulse-trains could be effective at 0.5 Hz.

2. *The mode transitions from higher to lower complexity modes.* We were also interested in how the transitions might occur. We found spontaneous intermittent mode transitions, from higher complexity modes (possibly chaotic) to lower complexity (possibly rhythmic) modes, similar to those observed in in vitro hippocampal slice models under low-magnesium conditions. Mode transitions from high to low complexity were also shown to be associated with epileptic seizures in humans and onset of SLE in hippocampal in vitro slice models.

Synchrony, Complexity, and Intermittency in Network Models

In addition to the observations in the coupled oscillator models, the importance of intermittency for understanding autonomous transitions in epilepsy (and the potential therapies based on an understanding of intermittency) was explored in networks with random connectivity. When we tested networks with initial random connectivity and random activity, the vast majority of networks quickly stopped oscillating (**Figure 3(a)**) or settled to limit-cycles with periodic and fully synchronized activity (**Figure 3(b)**). These findings suggest that the inherent propensity of clusters and larger networks to enter into periodic synchronized activity – and, by extension, seizures – may be more prevalent than previously recognized.

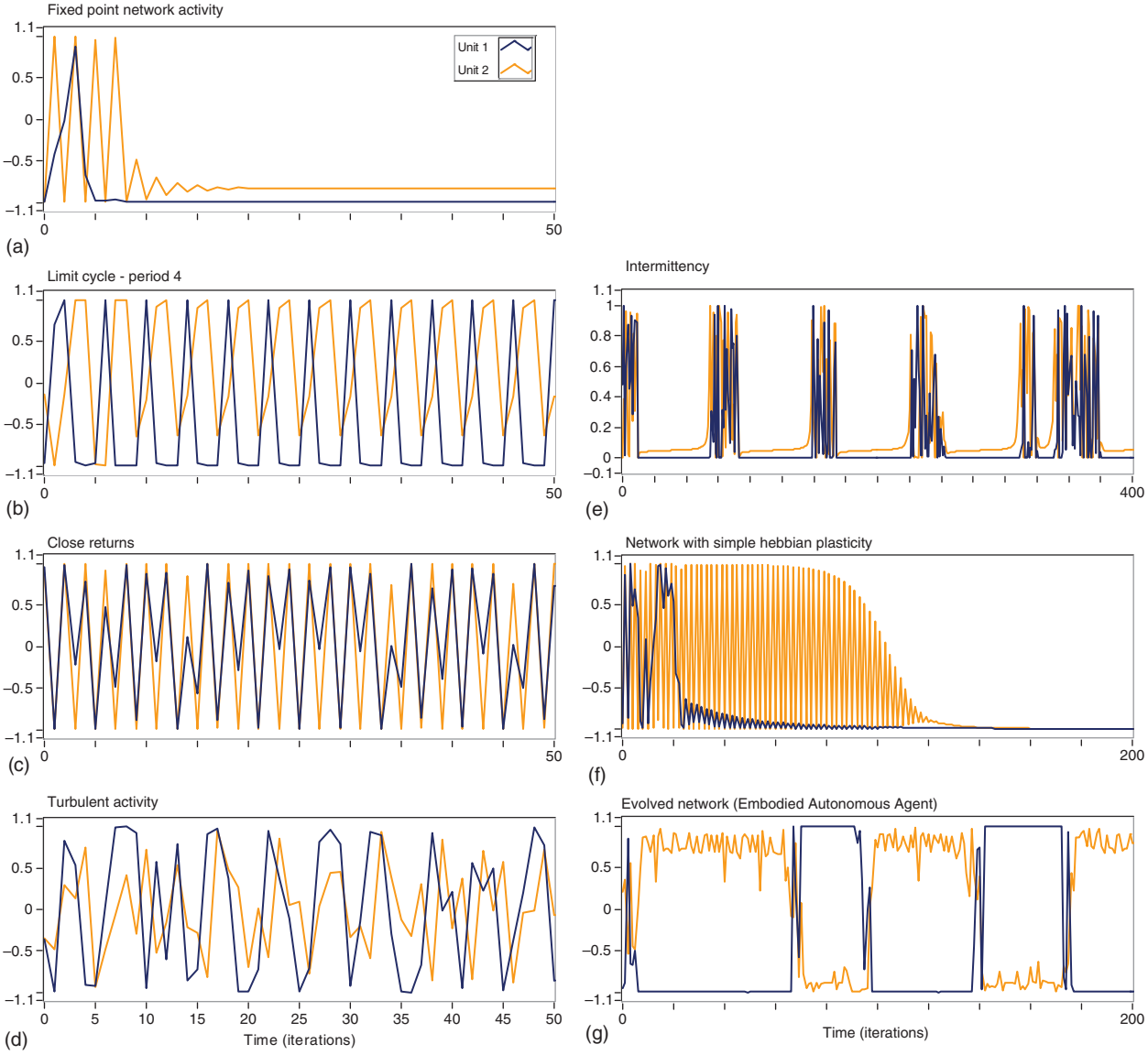


Figure 3 Activity dynamics in random and embodied neural network models. Most random networks settled to (a) fixed point and (b) limit-cycles characterized by periodic, synchronized activity (81% in the sigmoidal random networks). The remaining networks showed activities that were either (c) close to periodic but never precisely repetitive (13%) or were entirely (d) turbulent (6%). A subset of the nonperiodic networks showed (e) intermittent changes in phase reminiscent of transitions seen in epilepsy. The addition of simple Hebbian plasticity reduced complexity in random networks and quickly led to fixed points (f). On the other hand, embodied networks that were evolved to interact with the world showed (g) persistent nonperiodic activity. (Graphs show activity of 2 of 5 units; *y* axis is activity level normalized so that a value of 1 represents full excitation.) Reproduced from Ohayon EL, Kalitzin S, Suffczynski P, *et al*. (2004) Charting epilepsy by searching for intelligence in network space with the help of evolving autonomous agents. *Journal of Physiology-Paris* 98: 507–529.

However, these models have also demonstrated that complex oscillatory patterns can be an emergent property of network structure. For example, some networks were shown to generate complex oscillatory activity even if the constituent units were not themselves oscillators. In these cases, the nonperiodic activity showed either close to periodic but never precisely repetitive activity ('close returns') (**Figure 3(c)**) or highly turbulent activity (**Figure 3(d)**). By further examining these nonperiodic networks, we were able to demonstrate that in certain cases, the activity could switch autonomously between turbulent and quiet phases (**Figure 3(e)**). The change between the two phases of activity in these networks showed a distribution known to be characteristic of intermittency. Given that the units in all of these models were identical, the model demonstrates that intermittency can be the direct result of network structure and need not depend on changes to intrinsic cell properties. The autonomous switching in these models due to network structure thus demonstrates that some transitions into and out of seizures may be occurring in the absence of external perturbation or noise, and possibly without any change to neuronal properties. The continuous nature of these autonomous transitions also suggests that the very notion of a distinct epileptic 'state' may be questionable.

Although ongoing changes to the structure were not *necessary* for intermittency in these models, the activity was shown to exhibit changes in the statistics of transitions as a result of alterations to connectivity. In other words, changes in network structure could modulate the properties of the intermittency. As such, the model illustrates that intermittency mechanisms of transition are in fact compatible with the other mechanisms of seizure transitions enumerated by Lopes da Silva *et al.* (see Transitions, Intermittency, and Modulation in Background), and that the various mechanisms of transition are probably taking place concurrently in an interactive manner.

With regards to changes in connectivity, it is interesting to note that the addition of simple Hebbian plasticity drove even the most complex network activity to synchrony and eventually to the cessation of oscillations (**Figure 3(f)**). This finding suggests that it will be critical to identify the types of plasticity that generate brain structures that can maintain complex persistent activity and avoid seizures.

Interaction with the environment may be another factor important in avoiding periodic oscillations or fixed point activity. To demonstrate this point, the random networks were connected to external sensors and transducers (robot chassis). These embodied networks were then evolved over several generations, using an evolutionary algorithm that selected for continuous movement and the ability to avoid obstacles. As the behavior evolved, so did the dynamics of the activity, going from the largely fixed point and synchronized periodicity of the random networks to more complex multistable activity (**Figure 3(g)**). This demonstration is the first embodied modeling of epilepsy, illustrating the importance of the environment in producing structural changes that avoid seizures.

Future Directions

Technical Considerations

Our research highlights the need to consider multiple sources and scales when studying oscillations and transitions to seizure. Many challenges remain for the future. There are important questions of measurement and other technical issues. For example, the location and method of measurement, the chosen frequencies, and the use of linear versus nonlinear techniques are all ongoing debates in the field. Should we continue to rely primarily on electrical measures or is the better measure of epileptiform activity a reflection of something else, such as magnetic fields or chemical movements?

Autonomous Neurodynamics, Environment, and Embodiment

Our research demonstrates that the analysis must consider events in the higher frequencies, and forms of transition that go beyond simple attractors and parametric changes. In particular, the various intermittent forms of activity seen in the biological and model systems suggest a possible explanation for how certain transitions might occur autonomously, without a triggering stimulus. The research into autonomous neurodynamics is currently a major interest of our group, both in terms of understanding neural activity in epilepsy as well as how transitions in brain dynamics might occur in other cognitive conditions. As such, our research paradigms and models must also increasingly consider the role of bidirectional interactions of the embodied brain and the environment. Many laboratories are now uncovering the importance of enriched environment on development in general and susceptibility to seizures in particular. Our own research has shown that modeling of embodied networks connected to the environment can lead to increased complexity in oscillatory activity and to the escape from seizures. These connections – involving interaction within the environment, plasticity, and resultant oscillatory activity – need to be further explored. The attention to evolutionary sensorimotor modeling is also notable in that it moves away from modeling seizures as the primary goal and turns toward modeling the manner in which biological systems *avoid* pathological synchrony.

Relation between Network Structure and Oscillatory Activity

Another important frontier is the uncovering of the relation between network structure and the propagation of oscillatory activity. Netoff *et al.* and other groups have demonstrated that small world architectures, for example, can be an important factor in seizure dynamics. Our own work with random networks suggests that many seizure-like phenomena may arise even in the absence of complex architectures or plasticity. Current work is concentrating on modeling the propagation in spatial networks; results to date suggest that heterogeneity in structure (not just unit properties) may play a critical role in the form of oscillations and their propagation. These findings might help explain why the probability of seizures rises following cell damage or cell death in cases of trauma and aging. The most recent findings suggest that architecture may even override factors such as the balance between inhibitory and excitatory components.

Prediction of Seizure Activity

An artificial neural network possesses the ability to learn, generalize, and solve complex problems, and it is ideally suited for biological systems because of its inherent nonlinearity and its capacity to adapt its synaptic weights in response to changes in external environment. Our group's wavelet-based ANN design has been shown to be capable of classifying modes of brain activity and their transitions in an in vitro model of epilepsy from hippocampal extracellular recordings. Such a network was able to predict the onset of the ictal state within *minutes*. This delay provides a feasible time to facilitate appropriate modulatory actions to suppress the ictal state, avoid the 'locked-in' oscillatory low-complexity modes of a seizure, and increase system autonomy. This approach can lead to further success as the investigation is expanded to include both various signal features and the many new decision-making tools available to computer scientists (e.g., support vector machines).

Concluding Remarks

The brain is a complex, dynamic compilation of many interacting systems, which can adopt many oscillatory modes in space and time. To understand seizure modes, we need to consider the intrinsic properties of the multiple elements of the different systems, the network structure, connectivity, and the dynamics of interaction across spatiotemporal scales and with the external environment. This knowledge will provide us with the framework to develop therapeutic tools to measure, anticipate, and prevent (or eradicate) epileptic seizures.

See also: **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; Role of Gap Junctions in Epileptic Synchronizations; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures; Neuronal Networks Generating Pathological High Frequency Oscillations.

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Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures

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Introduction

Epileptiform discharges consist of the synchronized firing of principal neurons, usually in the form of bursts. The discharges, however, may repeat so as to form an oscillating activity pattern, at frequencies ranging from <1 Hz to over 20 Hz. In addition, neuronal network oscillations can occur prior to, during, and following the seizure proper, and there is a wide range of frequencies at which such oscillations can be recorded. Consideration of the oscillation mechanisms is important. An understanding of these mechanisms may allow prediction of an incipient seizure. And if there is a causal relationship between the events generating a pre-seizure oscillation and the events constituting the seizure proper, then that relationship might suggest a specific approach to therapy. Understanding the intricacies of this subject is not straightforward. The relevant oscillations involve a number of distinct mechanisms – synaptic and gap junctional – and the relation between different types of oscillation can be subtle. Furthermore, data are incomplete. We will present several examples illustrating some of the issues, and the interested reader can then delve further into the literature.

Background

In vitro models have been widely used to gain some understanding of the cellular mechanisms underlying epileptic activities in the intact brain. Until about 1999, most of the work with in vitro models employed experimental techniques that were designed to alter the balance between synaptic excitation and inhibition, in accord with what might be called the 'classical' idea of how epilepsy works. For example, synaptic inhibition might be reduced or suppressed completely with bath-applied GABA receptor blockers; recurrent excitation might be enhanced, by increasing NMDA receptor mediated currents in low $[Mg^{2+}]_o$ media (which also suppress synaptic inhibition); or axonal excitability might be enhanced and large depolarizing GABA currents produced with 4-aminopyridine. It is now apparent, however, that the generation of epileptiform activity involves more than a disturbed balance between synaptic excitation and inhibition. There are numerous phenomena associated with epileptiform discharge, including a variety of network oscillations, that can only be understood in terms of electrical coupling via gap junctions. The realization that gap junctions must play a critical role came largely from two experimental observations: first, that very fast oscillations (VFO) can occur without chemical synaptic transmission; and, second, that most experimental models of gamma and beta oscillations were dependent on gap junctions.

Methods

In our studies, we use both physiological experimentation and computer modeling. In vitro slice experiments have been carried out on hippocampus, entorhinal cortex, neocortex, and cerebellum, with tissue from rats, mice, or human patients (obtained, with proper consent, during neurosurgical procedures for medically intractable epilepsy). In parallel, we have generated detailed network models simulated on a supercomputer. In this latter work,

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we provide detail in the representation of individual neurons, in the number of neurons (thousands), and in the synaptic and gap junctional connectivity. These studies rely on a continual interplay between computer modeling and experimental discovery. The data from slice experiments are sometimes hypothesis-driven (including the testing of specific model predictions), but also sometimes serendipitous. The computer modeling attempts to replicate experimental observations, with economical assumptions, that sometimes lead to predictions. In addition, we draw on published in vivo data from patients and experimental animals, and collaborate with a molecular neurobiologist (Hannah Monyer), who is often able to engineer a genetically modified mouse that sheds light on oscillation mechanisms.

Recent Results

Figure 1 shows subdural recordings from an infant with intractable seizures of focal origin. Each seizure began with some slow waves, and then very fast oscillations (VFO, >70 Hz that appear fuzzy in Figure 1(a) but are seen clearly in Figure 1(b). The burst discharges in the seizure proper are of much higher amplitude than the VFO, and recur here at about 1 Hz. Interestingly, as Figure 1(c) shows, VFO are also superimposed on burst discharges. These patterns of pre-seizure VFO, and VFO superimposed on synchronized bursts, are both seen in in vitro and in vivo epilepsy models. The in vitro modeling shown in Figure 2 helps to sort out the respective mechanisms.

Figure 2-I (and also other data, see later) indicate that, under special 'epileptogenic' conditions such as disinhibition, neurons tend to fire bursts of action potentials that are synchronized on two different time scales. The first time scale is on the order of tens of ms, and synchronization on this time scale can be accounted for by the fact that principal neurons in cortical regions have recurrent excitatory (chemical synaptic) connections with one another, provided that at least some of these recurrent connections are sufficiently strong that a burst of action potentials in one pyramidal cell can induce a similar burst in a monosynaptically-connected second pyramidal cell. [Note: It is not clear if this situation holds in neocortex, and the situation becomes complicated when gap junctions are present; action potentials may be able to spread via the gap junctions as well as across chemical synapses.] The second time scale is much faster, as illustrated by the epileptiform burst shown in Figure 2-IB2; these bursts often have a superimposed VFO. The VFO is not reproduced by models of neuronal populations interconnected solely by chemical synapses. However, as shown in Figure 2-II, addition of electrical synapses (gap junctions) to the population model does generate the superimposed VFO. Presumably, a similar mechanism applies to the human case, as shown in Figure 1(c).

The gap junctions used in the model of Figure 2-II were located on the axons of pyramidal neurons. This choice was motivated by a body of physiological and dye-coupling evidence (see 'Further Reading'). More recently published ultrastructural data indicate that gap junctions can be found on hippocampal mossy fibers (axons of the excitatory dentate granule cells). The putative axonal location of gap junctions has critical physiological implications. At such a location, action potentials can, in principle, cross from axon to electrically coupled axon – that in turn permits the generation of very fast network oscillations amongst pyramidal and other principal neurons in a local population. Axonal coupling also allows the generation of other types of network oscillations, as further figures demonstrate.

Figure 3 illustrates data from a disinhibited neocortical slice, and from an extremely detailed thalamocortical model. The figure shows a pair of synchronized epileptiform bursts in succession. One might consider this pattern the in vitro correlate of an EEG polyspike. Each of the individual bursts resembles the epileptiform event shown in Figure 2-I, with the bursts of action potentials in individual cells riding on a large synaptic depolarization, along with some superimposed VFO. There are some differences with Figure 2-I, however. First, in the neocortex the epileptiform activity involves a number of distinct principal cell types simultaneously. Next, layer 4 spiny stellate cells fire continuously between the bursts, and this activity is coincident with the very fast oscillatory activity in the field potential recording (bottom trace). In simulations, one can show that the VFO are generated by gap junctional coupling, and that the VFO help to initiate the second burst. Indeed, under conditions of sufficient excitability, the pattern of activity observed in cortical neurons can continue in the form of a lengthy series of bursts at >10 Hz. Such activity resembles so-called 'fast runs' occurring in vivo, and consist of a series of epileptiform bursts at 10 Hz to about 25 Hz.

It is presumed that the VFO occurring before a seizure are generated by mechanisms similar to those of the VFO occurring on top of and between bursts. This presumption remains to be proved.

EEG oscillations at beta (10–30 Hz) and gamma (30–70 Hz) frequencies also are known to occur prior to seizures in some patients. It is therefore necessary to consider the cellular mechanisms of oscillations at these frequencies. In vitro studies and computer models provide access to this analysis, but the experimentalist must recognize the possibility that the EEG oscillations occurring in the clinical context may perhaps be generated differently.

Gamma oscillations arise in various physiological contexts, including brief sensory or electrical stimulation.

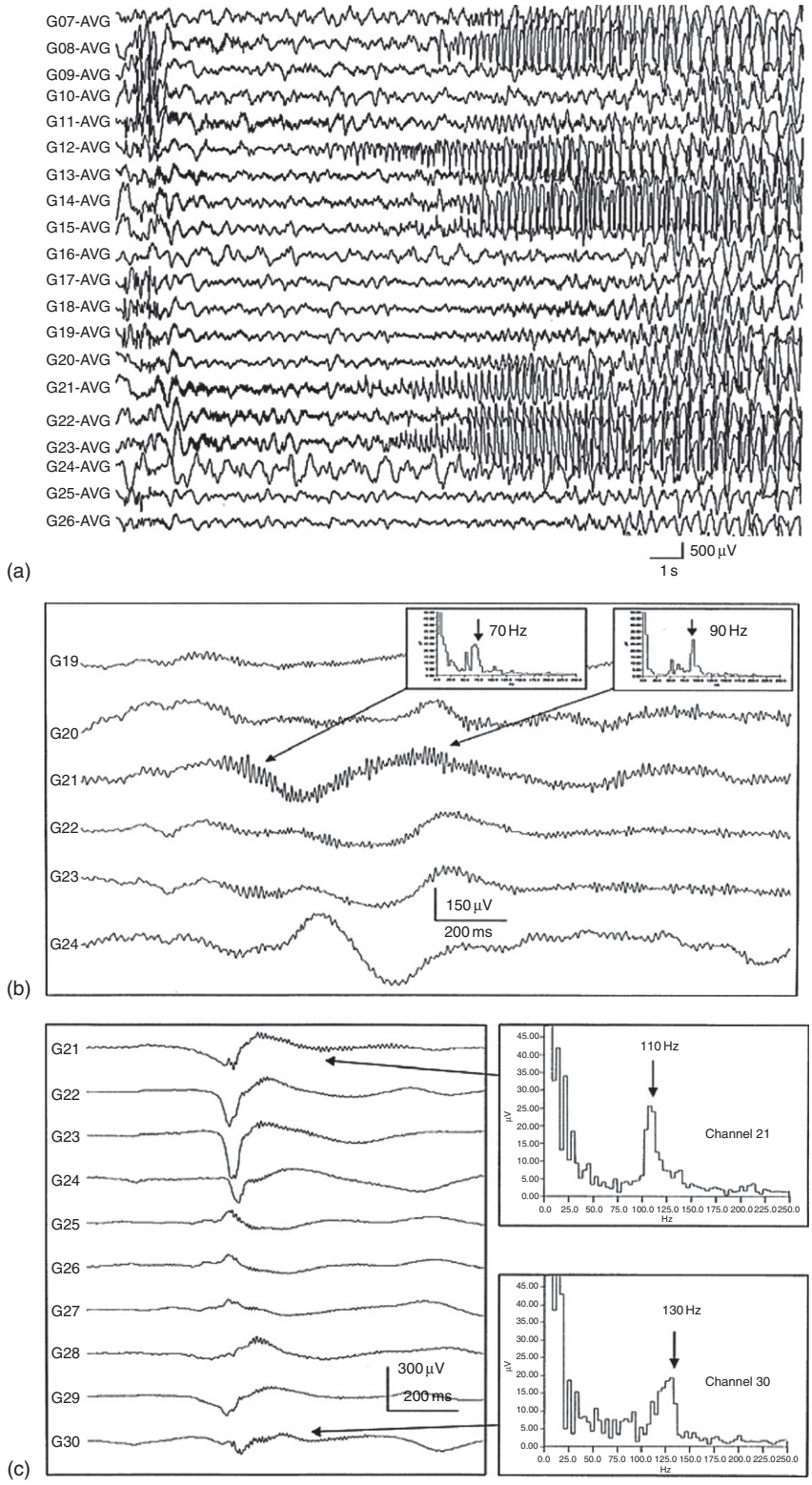


Figure 1 Very fast oscillations (VFO) recorded in subdural grid EEG signals, prior to and during seizures in a 13-month old child with a right frontal subcortical dysplasia. (a) widespread electrographic seizure activity is preceded by low-amplitude VFO in selected derivations, including G21-G23. (b) A different recording technique shows the pre-seizure VFO at 70–90 Hz. (c) 110–130 Hz low-amplitude EEG oscillations also occur during and immediately following burst complexes during the seizure itself. The seizure proper consists of EEG spikes and spike-wave complexes recurring at ~1 Hz. Adapted from Traub RD *et al*. (2001) A possible role for gap junctions in generation of very fast EEG oscillations preceding the onset of, and perhaps initiating, seizures. *Epilepsia* 42: 153–170.

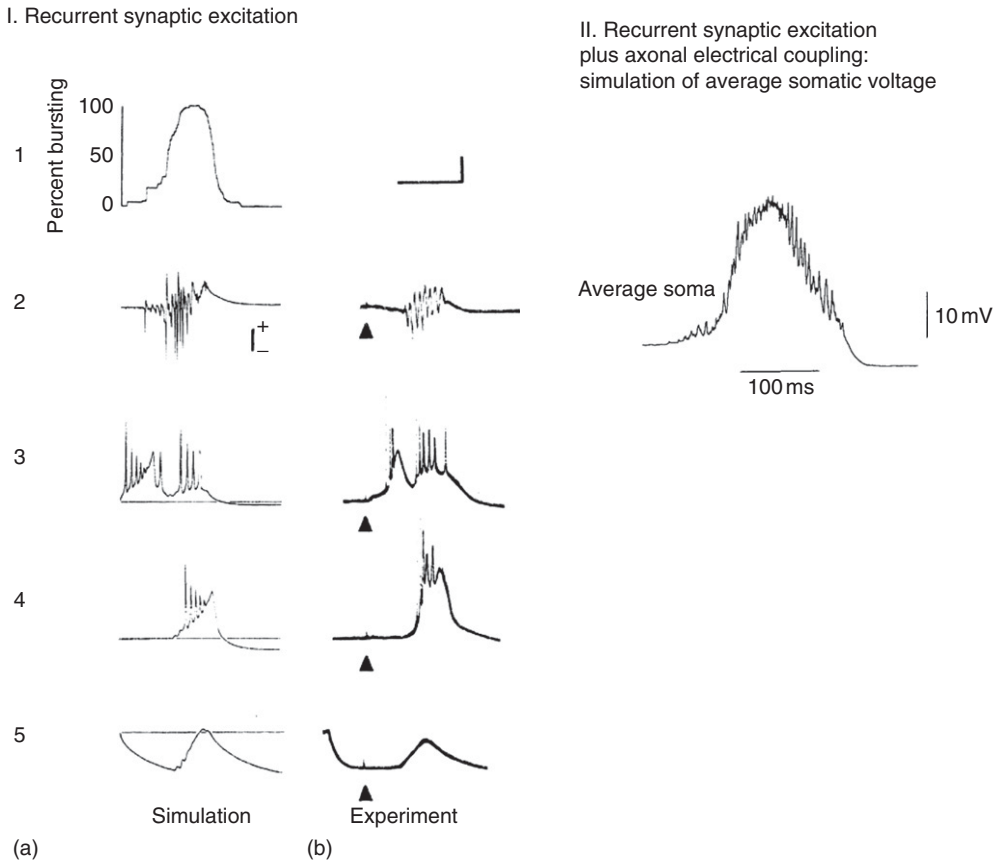


Figure 2 In the disinhibited hippocampal CA3 region in vitro, the primary mechanism of synchronization of bursting activity is by recurrent excitatory chemical synapses; electrical coupling between pyramidal cells may, however, be responsible for the VFO superimposed on an epileptiform burst. (I) Experiment (b) shows stratum pyramidale field potential (line 2) in a disinhibited hippocampal slice, CA3 region, following a small localized extracellular stimulus (arrowhead): note the latency of tens of ms prior to the epileptiform field potential, as well as the comb-shaped appearance of the latter, due to a superimposed VFO. Line 3 shows an intracellularly recorded burst in a pyramidal cell that happens to burst early on, and then generates a second burst. Line 4 shows a more typical pyramidal cell, that has negligible depolarization during the latent period, but then bursts during the field event. Line 5 shows the same cell as in line 4, hyperpolarized with current injection so as to prevent firing: this reveals the large EPSP during the field event, but no apparent activity during the latent period. A network model with 100 pyramidal neurons (a) produces exactly these features under conditions of (i) intrinsic bursting; (ii) sparse recurrent excitatory synaptic connections; the synaptic connections have the property that a burst in one cell can induce a burst in follower cells. The combination of sparseness with connection strength accounts for the growth of synchronization, the long latent period, and the absence of synaptic inputs in most cells during the latent period. However, this model does not generate a VFO superimposed on the epileptiform field potential. Scale bars: 50 ms (simulation), 60 ms (experiment); 4 mV (b2), 25 mV (a3, a4, a5), 20 mV (b3, b4, b5). (II) A simulation was performed according to the principles of I, but with more pyramidal neurons, and with axonal electrical coupling. The synchronized epileptiform burst now has a superimposed VFO. Data in I appeared originally in Traub and Wong (1982); data in II appeared originally in Traub *et al*. (1999). This figure reproduced from Traub *et al*. (2005c) Combined experimental/simulation studies of cellular and network mechanisms of epileptogenesis in vitro and in vivo. *Journal of Clinical Neurophysiology* 22: 330–342.

Probably the type of gamma oscillation most relevant to epileptogenesis is so-called 'persistent' gamma, discovered by Eberhard Buhl and collaborators. This type of gamma oscillation can be induced in entorhinal, neocortical, and even cerebellar slices by inclusion of an appropriate drug in the bath – for example, carbachol, kainate, or the metabotropic glutamate receptor agonist DHPG. Generation of persistent gamma oscillations involves an interplay between electrical coupling via gap junctions, and synaptic excitation and inhibition. Gap junctions between interneurons and between pyramidal neurons are involved, the former being modulatory and the latter critical. The most plausible mechanism is as follows: Electrical coupling between pyramidal cell axons (not too far from the soma) leads to high-frequency bursts of action potentials that cause barrages of rapid EPSPs in interneurons. The interneurons then fire almost synchronously and the resulting perisomatic inhibition onto pyramidal cells not only produces IPSPs in the pyramidal cell somata, but also terminates the pyramidal cell axonal

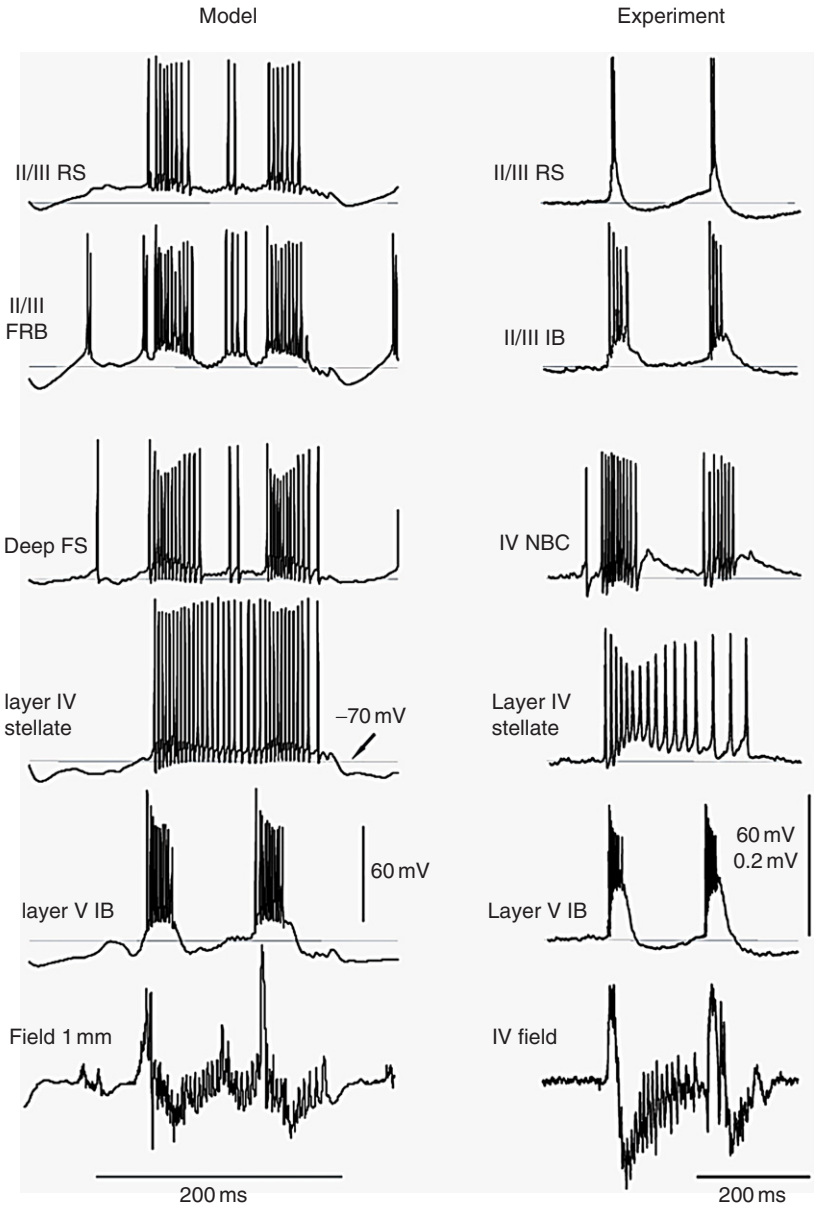


Figure 3 VFO during and after an epileptiform burst in neocortex occur in vitro, as well as in vivo. The VFO also contributes to the initiation of the second burst in this in vitro 'polyspike.' In similar model conditions, sustained ~10 bursts can occur, similar to 'fast runs' of epileptic bursts in vivo (not shown here). The model data are from a large neocortical network simulation, in which layer 4 spiny stellate cells were interconnected by voltage- and magnesium-independent NMDA receptors; in addition to conventional types of chemical synaptic interactions, there were axonal gap junctions between superficial pyramidal cells, spiny stellate cells, and layer 6 pyramidal cells. A synchronized epileptiform burst occurs, but note the prominent VFO in the field, as well as the prolonged firing of the layer 4 spiny stellate neuron. Experimental data show epileptiform activity in rat auditory cortex in vitro, bathed in kainate and picrotoxin. Note the similar very fast oscillatory activity in the field, as well as the prolonged depolarization in the layer 4 stellate neuron. Adapted from Traub *et al*. (2005a) Single-column thalamocortical network model exhibiting gamma oscillations, sleep spindles and epileptogenic bursts. *Journal of Neurophysiology* 93: 2194–2232.

firing. The inhibitory period – tens of ms – is the main determinant of the gamma oscillation period. This scheme accounts for pharmacological data – specifically, the sensitivity of persistent gamma to blockers of glutamate receptors, to blockers of $GABA_A$ receptors, and to blockers of gap junctions. It also explains the existence of a very high frequency peak in the power spectrum, a peak that is not a harmonic of the main gamma peak. Modeling further indicates that excessive recurrent excitation between pyramidal cells should disrupt gamma, while synaptic excitation of fast-spiking interneurons should stabilize the gamma. The data shown in **Figure 4** are

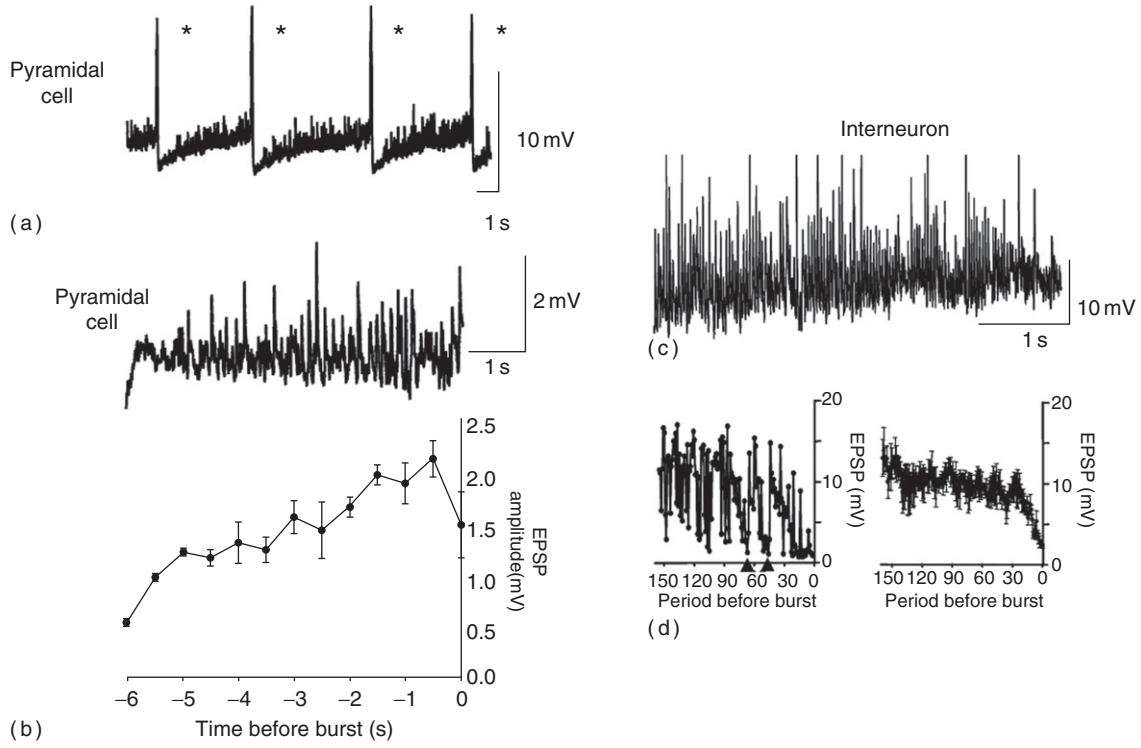


Figure 4 During between-burst gamma epochs induced by DHPG (a metabotropic glutamate receptor agonist), gamma-frequency EPSPs in pyramidal cells increase in amplitude, while EPSPs in interneurons diminish. (a) intracellular recording of repeating synchronized epileptiform bursts (*) in a CA3 pyramidal cell, with current injection to give mean resting membrane potential of −70 mV. (b) high-pass filtered data, showing EPSPs in the pyramidal cell. Graph below is pooled data (three interburst intervals from each of five pyramidal cells), showing increase in mean EPSP size during the interburst interval. (c) EPSPs in a fast-spiking s. pyramidale interneuron (injected with −0.2 nA current, to give a mean resting membrane potential of −70 mV), during the interburst interval. Action potentials truncated. (d) *left* shows EPSPs in the interneuron plotted as a function of gamma oscillation period, working backward from burst initiation. *Right* shows pooled data (mean ± standard error) from five burst intervals. Note the sudden decline in EPSP amplitude in the ~25 gamma periods (~750 ms) prior to the burst. Adapted from Traub *et al.* (2005b) Transient depression of excitatory synapses on interneurons contributes to epileptiform bursts intermixed with gamma oscillations in the mouse hippocampal slice. *Journal of Neurophysiology* 94: 1225–1235.

consistent with this prediction. These data also shed some light on how gamma oscillations might be relevant to epileptogenesis.

Figure 4 illustrates data from the CA3 region of a mouse hippocampal slice bathed in DHPG. In these conditions, epochs of several seconds of gamma oscillation alternate with brief epileptiform bursts (asterisks (*) in **Figure 4(a)**). Interestingly, short term plasticity occurs during the gamma intervals, consisting of gradual enhancement of EPSPs in pyramidal cells (**Figure 4(b)**), and collapse of EPSPs in interneurons (**Figure 4(c)**). As noted above, modeling suggests that these changes should destabilize the gamma, and that is what occurs. We do not understand, however, how the epileptiform burst resets the conditions so that a new gamma cycle can start. Intriguingly, it is a reasonable hypothesis that, during the gamma that can occur prior to a seizure in a patient, a similar type of short-term synaptic plasticity takes place, and that this plasticity leads to a switch to epileptiform discharge.

A robust in vitro model of beta oscillations occurs in intrinsically bursting (IB) cells in layer 5 of somatosensory cortex bathed in kainate (**Figure 5**). The in vivo correlate of this type of beta oscillation appears to occur during the immobile state of waiting for a sensory cue (as seen in rat, monkey and human). Currently, we can only speculate that pre-seizure beta oscillation is physiologically similar to this state-dependent beta; however, the tendency of seizures to begin, in certain seizure models where this feature has been tested, in layers 4 and 5 makes this a reasonable speculation. Additionally, kainate receptor-induced beta oscillations (as well as gamma and VFO) occur on upstates (i.e., during sustained – hundreds of ms – intracellular depolarizations of cortical neurons) during the slow (<1 Hz) oscillation of sleep; the upstates are the times when seizures are likely to be initiated.

Kainate-induced beta oscillation is quite remarkable. It depends on gap junctional coupling, but not on chemical synapses. One observable correlate of the electrical

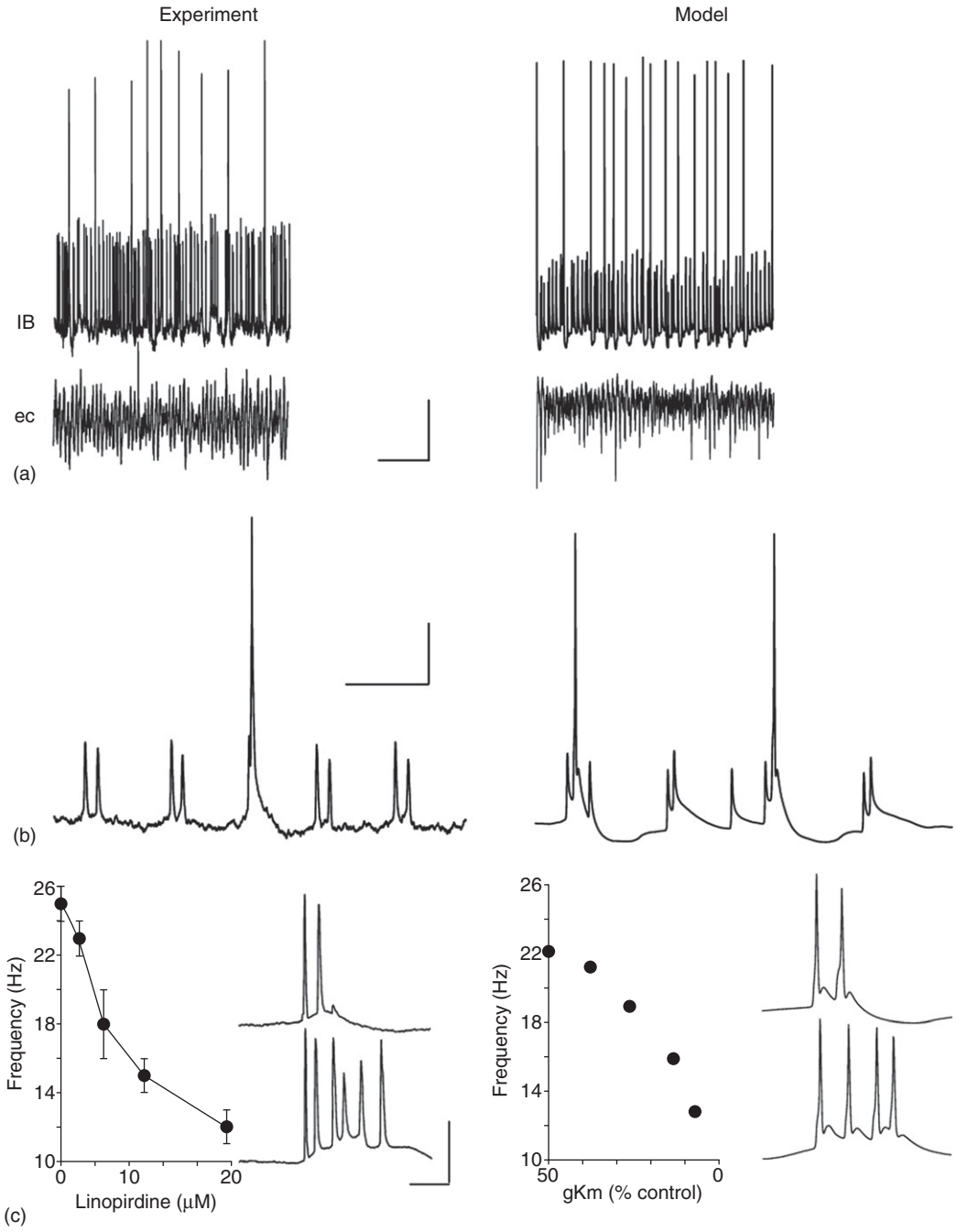


Figure 5 Kainate (400 nM) induces a beta2 (~25 Hz) population rhythm in layer 5 intrinsically bursting pyramidal neurons in vitro, with prominent spikelets. The rhythm period is determined by the M type of K^+ conductance, and the oscillation can be accounted for with a network model of pyramidal neurons electrically coupled via their axons. This type of oscillation may be related to beta-frequency activity prior to a seizure. (a) (left panel). 2.5 s example trace from an electrophysiologically and anatomically identified layer V intrinsically bursting (IB) cell reveals combinations of spikelets (fast prepotentials), single action potentials and action potential-bursts at beta2 frequencies, coherent with the local field (ec, extracellular). (Right panel) Field and tufted IB cell data are accurately predicted from antidromic spiking in a network model that includes 2000 IB pyramidal cells, electrically coupled through their axons, along with other model neurons. Scale bars 10 mV (experiment intracellular), 100 μV (experiment field), 15 mV (model intracellular), 500 ms. (b) Expanded timescale examples of layer V IB cell activity during field beta2 rhythms. Note the spikelets, that can occur in pairs, as well as the notch on the rising phase of the action potentials (arising from axonal spiking in the model, antidromically conducted). Scale bars (experiment) 10 mV, 15 mV (model), 10 ms. (c) Beta2 frequency is correlated with $g_{K(M)}$-mediated control of burst duration. Left panel shows mean peak frequency ($n = 5$) of layer V field potentials. The decrease in population frequency with M-current reduction (linopirdine 0–20 μM) was accompanied by increased IB cell burst duration and slowing of the population frequency. Right panel shows peak population frequency of model IB cells using different $g_{K(M)}$ from 50% to 5% of control. Scale bars (experiment) 30 mV, (model) 40 mV, 10 ms. From Roopun *et al*. (2006) A beta2-frequency (20–30 Hz) oscillation in non-synaptic networks of somatosensory cortex. *Proceedings of the National Academy of Sciences of the United States of America* 103: 15646–15650.

coupling is the frequent occurrence of spikelets, or fast prepotentials, readily observable in Figure 5(a) and 5(b). While the period of gamma oscillations is primarily determined by the time course of inhibitory synaptic conductances, the period of kainate-induced beta is primarily determined by an intrinsic membrane potassium current, the M current (Figure 5(c)).

To the best of our knowledge, there is no experimental model that sheds light on how a beta-frequency network oscillation might transform into a seizure. Modeling indicates that speeding up of M current kinetics could produce a transition from beta to VFO, but it is not known if that transition occurs physiologically (but see below for another alternative). It also remains to be determined how a run of VFO can be transformed into a seizure.

Future Directions

A current challenge for us is to obtain a more refined and quantitative understanding of VFO in the cortex. One new body of data is proving useful in this context. McCormick and colleagues have shown, using patch recordings of the axons of layer V pyramidal cells, that the axons contain a large transient K^+ conductance (a so-called D type conductance), blockade of which leads to axonal depolarization and hyperexcitability. As our published simulations to date had not incorporated such a conductance, we may have been simulating, in effect, over-excited axons. Preliminary simulations, in which a large transient K^+ conductance is included in axons, indicate that axonal gap junction conductance exhibits a threshold phenomenon; below this threshold, networks of layer V pyramids make beta oscillations, whereas above the threshold, there is VFO, with frequency depending on the gap junction conductance. If these preliminary data can be confirmed experimentally, it would provide an important link between beta oscillations and VFO. VFO also tends to occur in situations where chemical synaptic transmission is reduced or suppressed, and it will be necessary to determine how such a state might arise in vivo.

A final, and major, outstanding issue remains the ultrastructural demonstration of gap junctions between pyramidal cell axons, preferably using FRIL (freeze-fracture immunogold labelling). Knowing that axonal gap junctions exist on dentate granule cell axons (mossy fibers) is a start, but is not sufficient.

Acknowledgements

We thank Andrea Bibbig, Diego Contreras, Mark Cunningham, Fiona LeBeau, Steven Middleton, and Anita Roopun. This work is supported by the Wellcome Trust, MRC (U.K.), NIH/NINDS, and the Alexander von Humboldt Stiftung.

See also: **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; Role of Gap Junctions in Epileptic Synchronizations; **Oscillatory Activity**: Cellular and Network Oscillations in Epilepsy; High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures; Neuronal Networks Generating Pathological High Frequency Oscillations.

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High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures

J G R Jefferys and P Jiruska, University of Birmingham, Birmingham, UK

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Introduction

In the majority of cases of epilepsy, brain activity looks normal for most of the time. A fundamental question is how the transition is made from this relatively normal interictal activity to the hypersynchronous hyperactivity of an epileptic seizure. A related key question is whether the transition is sudden or a slow progressive process that starts significantly before the actual seizure onset. These questions are important for understanding the basic mechanisms of epilepsies. The answers have implications for treatments, both in finding novel and potentially more specific drug treatments, and also in developing better prediction or early detection, of seizures that can be used to trigger warnings and, potentially, responsive treatments such as therapeutic stimulation or focal drug application. In this article, we focus on the events which occur immediately at the onset of the seizure, and particularly on high-frequency activity (HFA).

Background

Transition to Seizure

Several mechanisms have been proposed to explain the transition to epileptic seizures. Modulation of glutamatergic synaptic transmission can cause changes in excitation and further promote seizure-like events in brain slices. Activation of presynaptic receptors can modify neuronal activity and has significant impact on seizure generation. Changes in interneuronal function have been implicated in transition to seizure, and may be more important than changes in excitatory transmission. Potential mechanisms involving interneurons include: decrease in probability of GABA release due to activation of presynaptic $GABA_B$ receptors; depletion of the available pool of GABA stores due to intense interneuronal activity; desensitization of GABA receptors; depolarizing block of interneurons; and depolarizing inhibitory synaptic potentials (IPSPs may become depolarizing instead of hyperpolarizing due to impaired Cl^- homeostasis and/or intracellular accumulation of Cl^- due to excessive neuronal activity).

All these changes occur at the cellular level. At the network level, inhibitory transmission controls not only the excitability of individual cells, but also the spread of epileptic activity across the neuronal network. Failure of GABA transmission may lead to disappearance of surround inhibition, resulting in a loss of ability to 'contain' the epileptic focus, and the subsequent spatial expansion of epileptic activity – which then may lead to transition to seizure, and further to the spread of epileptic activity to more distant regions. This type of expansion of the region of hyperexcitability may be essential to recruit a minimal population of neurons required to trigger seizure activity – the epileptic aggregate or critical mass.

High-Frequency Activity

HFA has been linked with the initiation of epileptic seizures since the early 1990s. Allen and Fisher simultaneously presented observations from human patients implanted with invasive electrodes (**Figure 1(a)**). They reported that seizures in some patients could start with low-amplitude HFA. Frequencies of HFA are typically >100 Hz, outside the range of frequencies recorded by most clinical recording systems. Preseizure HFA typically occurs in a small volume of tissue, which makes it useful in localizing the seizure onset zone, but complicates its detection because it is best detected with relatively small, preferably intracerebral, electrodes – and of course, those electrodes have to be in the right place. These early

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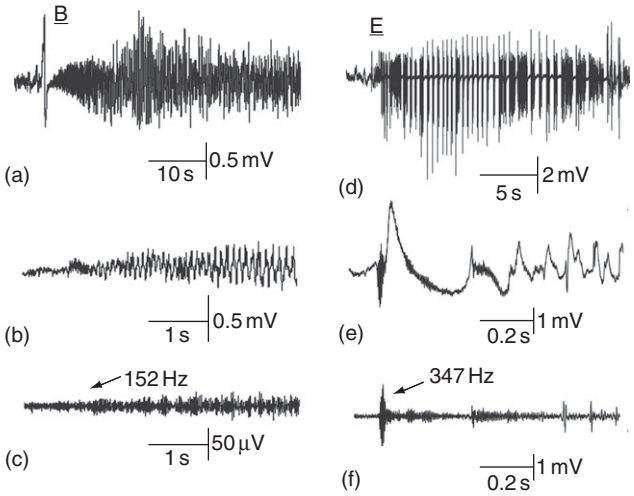


Figure 1 HFA at the onset of seizures. (a) Seizure recorded from epileptic hippocampus using depth electrodes in patient with mesial temporal lobe epilepsy. (b) Detail of seizure onset, characterized by a buildup of HFA, which is later superimposed on slower rhythmic waves. (c) Band pass filtered data (80–500 Hz) demonstrating HFA alone and its gradual buildup. (d) Seizure recorded from epileptic hippocampus in chronic animal model of mesial temporal lobe epilepsy. (e) Detail of seizure onset characterized by presence of high-amplitude HFA superimposed on sharp wave discharges. (f) Band pass filtered data. (Human data with permission from Dr P Marusic & Dr M Tomasek, 2nd Medical School, Prague, Czech Republic, in vivo data from the authors.)

clinical papers have led to a line of epilepsy research focusing on mechanisms of HFA, with the aim of gaining insights into ictogenesis and epileptogenesis.

Methodological Approaches

Experimental models of clinical conditions such as epilepsy exist to provide better control and reproducibility than is possible with clinical material. They also need to provide appropriate levels of simplification, to allow the development of insights into the fundamental mechanisms that can be used to formulate hypotheses for progressively more realistic, and complex, models.

Some of the more simplified models of epileptic seizures are those generated in brain slices in vitro, in the absence of any synaptic transmission. Decreasing extracellular calcium concentration to <0.2 mM effectively blocks synaptic transmission. Hippocampal slices perfused with low-Ca^{2+} artificial cerebrospinal fluid generate spontaneous, repeated seizure-like activity, with each episode lasting tens of seconds. This observation shows that nonsynaptic mechanisms are sufficient for the generation of prolonged seizure-like discharges. Similar observations have been described in the high-potassium model, in which synaptic activity is functional. In this latter case, seizures persist even when excitatory transmission is blocked. Methods for recording such activities in brain slice (in vitro) studies include: intra- and extracellular recording, usually with glass micropipettes; monitoring of extracellular ion concentrations with ion-sensitive electrodes; assessment of network activity with electrode arrays; and optical recording to measure the spread of activity across large areas of tissue.

While in vitro approaches with acute slices provide a useful simplification, chronic models of epilepsy provide closer parallels with the clinical situation. In such models, the tissue exhibits abnormalities of structure and function that lead to spontaneous seizures (or in the case of kindling, reduced seizure threshold). Tissue from these chronic models can be, and is, studied in slices in vitro. However, in situ analysis – in the intact animal – allows investigations of seizures in a 'complete' system, consisting of structures involved in the complex generation and modulation of seizure activity. The intact preparation is not constrained by problems of in vitro maintenance (e.g., proper oxygenation of the tissue), or the absence of inputs from and outputs to other brain regions, or by the complex changes in tissue milieu that are eliminated by brain slice recording. The main chronic models that have been used in the context of HFA studies to date are those in which a period of status epilepticus is induced (e.g., by intrahippocampal injection of kainic acid). After a latent period of a week or two, spontaneous seizures start and recur for the rest of the life of the animal. In some intact models, spontaneous epileptic activity (often spike and wave discharges) occurs in the absence of a prior stimulus or insult, presumably a product of gene abnormalities. The main recording methods used in intact animal preparations include monitoring of electrical activity (EEG and cellular discharge) with chronically implanted electrodes, often microwires. Some studies on anesthetized animals involve the introduction of glass microelectrodes and ion sensitive electrodes, as in slice experiments.

Detailed recordings from human patients are often made during clinical evaluation for potential surgery. Technical details for such recordings vary among centers, but typically include subdural strips and grids, and depth electrodes. The invasive electrodes usually have relatively large recording surfaces, but can include microwires (or microcontacts on larger electrodes) similar to those used in some animal studies; that type of electrode has proved particularly useful in the context of HFA analysis.

Important Results to Date

HFA in vivo

Bragin *et al.*, working in vivo on the kainate model of temporal lobe epilepsy, described the presence of HFA in the range 100–600 Hz. Similar observations were later described in human patients with epilepsy who were implanted with specially designed microelectrodes. These investigators identified two different subtypes of HFA which differed in morphology and spatial properties: ripples (80–160 Hz) and fast ripples (200–500 Hz). Fast ripples usually had a shorter duration, amplitudes reaching up to 1250 μV, and were spatially confined to the epileptic focus. Fast ripples could occur alone or superimposed on interictal spikes. The volume of tissue generating this epileptiform pattern was estimated at 1 mm^3. In the kainate model, the presence of fast ripples during the latent period was a marker for the subsequent development of spontaneous recurrent seizures. The most common seizure onset pattern was hypersynchronous spiking, typical for seizures originating in the hippocampus in both experimental animals and humans. In this pattern, the seizure usually starts with a discharge of the same morphology as interictal discharges, with HFA (typically high amplitude) superimposed on a slower component. The similarity of the interictal and ictal fast ripples suggests that they share common pathophysiological mechanisms.

In human TLE patients, Bragin *et al.* found that ripples and fast ripples could be present in the same recordings but that fast ripples were limited to epileptogenic areas. On the basis of these findings, the investigators proposed that fast ripples could be used as a surrogate marker for epileptogenic tissue. Whether this subclassification of HFA is useful remains a matter of debate in the literature.

Spontaneous spike-and-wave seizure activity in anesthetized cats provides another in vivo model, in neocortex, in which HFA occurs at the onset of seizures. In this model, HFA had even greater amplitudes than observed in the rat kainate model, reaching nearly 5 mV. Grenier *et al.* reported that HFA was present not only in the seizure onset zone but also in areas to which epileptic activity propagated; HFA was not spatially restricted and could be synchronized over distances of 9 mm. However, the HFA had its greatest amplitude in the seizure onset zone.

HFA in Brain Slices

HFA at the beginning of seizures has also been described in various in vitro models of epileptic seizures, including those in which synaptic activity is blocked. In the low-Ca model, low-amplitude HFA in the frequency range of 150–200 Hz occurs during several seconds before the seizure onset (**Figure 2**). This HFA pattern consists of repeated very small, negative, population spikes which have maximal amplitude in the pyramidal layer of CA1. The spikes are correlated with the firing of individual pyramidal cells, but each of these neurons fire at much lower rates (as one might expect for this type of neuron), typically at around 30 Hz – that is, once every several cycles of HFA. It appears that small groups of neurons fire synchronously, within 1–2 ms, and that the collective activity of multiple groups leads to the net HFA of 150–200 Hz. The full seizure-like event emerges as these small groups fuse into a single large synchronous population that discharges at a few tens of Hertz The fact

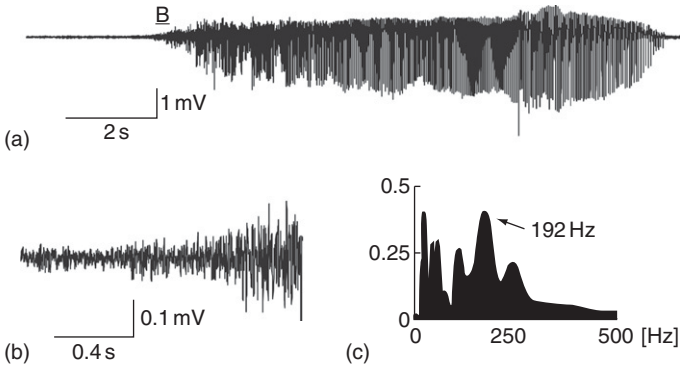


Figure 2 Low-calcium in vitro model of epileptic seizures. (a) Hippocampal slices perfused with artificial cerebrospinal fluid containing low levels of calcium generate spontaneous repeated seizure-like events. (b) Onset of the seizures is characterized by presence of HFA. (c) Corresponding power spectra demonstrating that HFA has peak at 192 Hz.

that HFA appears when chemical synapses cannot function normally suggests that nonsynaptic mechanisms must be responsible for synchronizing neuronal activity (see the following sections). Very similar HFA appears before seizurelike events in the high-K model, where although synaptic activity is preserved, there is some evidence that nonsynaptic mechanisms play a major role.

Cellular Mechanisms of Synchronization

Several theories exist for the cellular mechanisms of HFA. Under physiological conditions, HFA has been implicated in cognition and memory processing; in these cases. HFA is thought to result from interaction between fast spiking interneurons and pyramidal cells. Interneuronal activity generates inhibitory postsynaptic potentials on pyramidal neurons which restrict pyramidal cells to firing action potentials during 2–3 ms windows, resulting in ripple activity (**Figure 3(a)**). As outlined earlier, under pathological conditions, HFA may also represent synchronous action potential firing, probably due to pathologically interconnected hippocampal neurons. It is well known that one of the morphological changes in temporal lobe epilepsy is the sprouting of axons of principal neurons (mossy fibers, CA1 pyramidal cell axons), which potentially can then create autosynapses and synaptic contacts with adjacent neurons (**Figure 3(b)**). Activity in one neuron would be able to quickly evoke activity in synaptically coupled neurons and synchronize their action potential firing.

Work on the low-Ca model shows that nonsynaptic mechanisms are sufficient for HFA, although we cannot conclude that they are necessary. There is some evidence of electronic coupling between pyramidal cells by gap junctions, based on: (1) electrophysiological recordings of 'spikelets' (miniature action potentials that can be recorded intracellularly and believed to be generated sufficiently far from the recording site to attenuate them to a few mV); (2) pharmacological disruption of HFA (although the agents, such as octanol and carbenoxolone are nonspecific and far from ideal); and (3) rare cases of dye coupling. The synchronization of action potentials, on a millisecond time scale within the individual cycles of HFA, makes gap junction coupling at the soma or dendrites seem unlikely, given the membrane time constants of these structures are substantially slower; axons have

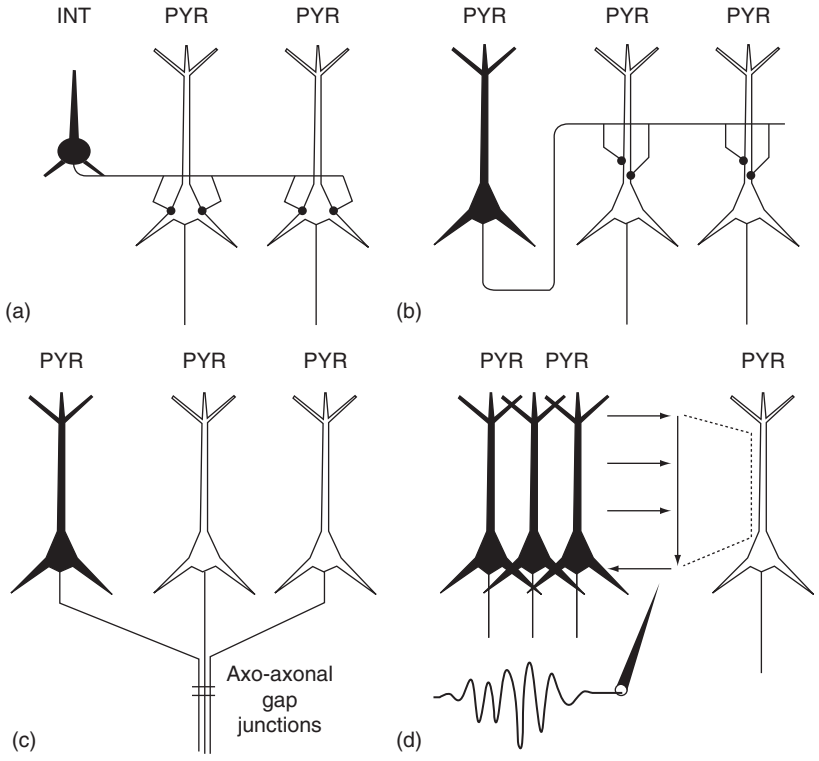


Figure 3 Mechanisms of HFA genesis. (a) Synchronous interneuronal input generates synchronous IPSPs and hence pyramidal cell firing, summation of which creates individual cycle of HFA. (b) Pathologically interconnected neuronal cluster. Physiological or abnormal axonal sprouting makes connection with adjacent neurons. There activation of one neuron effectively depolarizes synaptically coupled neurons into the action potential firing. (c) Axo-axonal gap-junction. Action potential generated in one neuron can electronically spread via gap-junction and depolarize coupled neurons and recruit them into synchronous activity. (d) Ephaptic interactions. Groups of neurons generate synchronous activity, which creates large field potentials in extracellular space. This large electric field can then depolarize adjacent cells and entrain them into synchronous activity.

faster time constants and provide a more plausible point of contact (**Figure 3(c)**). Computer simulations suggest that an axo-axonal syncytium could generate HFA. The other major class of fast nonsynaptic synchronization is ephaptic and field effect coupling (**Figure 3(d)**). Electric fields clearly can modulate neuronal excitability, with field strengths of $\sim$1 mV mm^{-1} being effective. The synchronization of individual HFA cycles is of a short range (a few hundred micrometers). Computer simulations suggest the maximum extracellular action potential of single neurons is at the axon hillock, and its effects on neighbouring neurons are fast and localized to within a few tens of micrometers. In the case of the HFA associated with spike-and-wave epilepsy in the cat, its large amplitude makes ephaptic coupling very likely indeed.

Challenges for the Future

The theories outlined in this article need experimental testing and translation to humans. The underlying cellular and network mechanisms remain a central challenge, particularly in terms of the behavior of individual neurons of various classes, their synchronization, and the role of HFA in transition to seizure. Examples where HFA precedes seizure onset by tens of seconds are particularly important and may provide novel targets for therapy.

The HFA that precedes seizures tends to be of a low amplitude and spatially localized to the seizure initiation zone. Under conventional clinical recording conditions, HFA can result in a flattening of the ECoG, or an electrodecremental pattern. The spatial restriction and small amplitude of HFA mean that it needs to be recorded with intracranial electrodes; scalp electrodes are too far from the highly localized sources of HFA (the large cortical area contributing to each electrode and the poor signal-to-noise ratio result in a loss of HFA). In contrast, HFA associated with evoked potentials can be recorded with surface electrodes, because of extensive averaging and the larger cortical volumes producing synchronous HFA.

Even with subdural electrodes or depth electrodes, the small size of the areas generating synchronous HFA can present problems. While conventional subdural and depth electrodes can record HFA, they do so less reliably and less well than microwires and other microelectrodes. Essentially, the larger the electrode, the greater the spatial averaging of the signal – which in turn will reduce signal-to-noise ratio, or create interference between multiple independent small HFA generators. Increasing knowledge about HFA and its association with epileptic foci and seizure genesis led to the development of novel 'hybrid' electrodes, which combine conventional macroelectrodes with microelectrodes. These electrodes have already been used in clinical practice with promising results.

Similar approaches are needed in in vivo studies of chronic animal models of epilepsy, both for comparison with clinical data (as part of the evaluation of those models), and for improving our understanding of mechanisms. In this experimental context, implanted microwire electrodes and/or multichannel probes (silicone probes, etc.) and perhaps tetrodes will provide the multichannel recordings that are needed to provide new insights into the temporal and spatial dynamics of the neuronal populations that generate HFA. The chronic animal models also are important in determining the applicability to intact functioning brain of mechanisms identified in vitro by intracellular studies (using sharp electrodes or patch-clamp with multichannel local field potential recordings).

Recordings of the kinds outlined here present challenges for analysis. Developments in time and frequency domain analyses have played a major role in recent advances in HFA research, particularly in dealing with spatial and temporal structure. Analyzing large numbers of channels of data remains a challenge in the investigations of HFA in epilepsy, and more generally in the analysis of neuronal networks.

Ultimately, the key question for us is whether HFA will provide useful leads into understanding or treating epilepsy and epileptic seizures. Improved seizure prediction, or at least early detection, would facilitate responsive treatments such as therapeutic stimulation or focal drug application. Understanding the process of transition to seizure may provide new, and hopefully more specific, therapeutic targets for novel drugs or stimulation protocols.

See also: **Models**: The Tetanus Toxin Model of Temporal Lobe Epilepsy; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; Role of Gap Junctions in Epileptic Synchronizations; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; Cellular and Network Oscillations in Epilepsy; Neuronal Networks Generating Pathological High Frequency Oscillations.

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Neuronal Networks Generating Pathological High Frequency Oscillations

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Introduction

Experimental animal models of epilepsy attempt to reproduce as many of the clinical and electrographic features of human epilepsy as possible in order to learn more about the neuronal mechanisms of epileptogenicity. In many of the chemically induced seizure models, focal application of chemical agents to the mammalian cortex produces acutely a seizure focus characterized by synchronous neuronal burst firing. Intracellular recordings within the focus reveal that a large percentage of neurons discharge bursts of action potentials that often are superimposed on a large amplitude depolarizing slow wave. This neuronal event, known as a 'paroxysmal depolarization shift,' occurs during the generation of epileptiform discharges and is believed to be the primary cellular mechanism contributing to the abnormal excitability and synchrony associated with epilepsy.

Unlike acute models, however, studies in patients (who require intracranial depth electrode recordings to locate brain areas where seizures begin) show that there is considerable variability in the number of neurons with abnormal firing properties in epileptogenic regions. Single neuron studies using chronic microelectrode recordings have not been able to provide consistent evidence for the extent of synchronous neuronal burst firing that is commonly observed in experimental seizure models. Yet, during some behavioral states such as sleep, it has been shown that greater burst firing and synchrony of discharges occur in brain areas ipsilateral to ictal onset (compared to remote contralateral sites). These changes in action potential firing and synchrony are due, in part, to changes in neuronal network oscillatory activity associated with particular states of vigilance.

The importance of network activity for information processing has been recognized for many years. However, the significance of pathological alterations in such networks has recently attracted considerable interest, because pathological network oscillations may be associated with abnormal synchronous discharges that contribute to epileptogenicity. Numerous studies have focused on fast network oscillations commonly referred to as 'high-frequency oscillations' (HFOs). Among the best studied physiological HFOs are Ripples. Ripples are brief, sinusoid-like oscillations, between 100 and 200 Hz, that occur within the hippocampus and entorhinal cortex of a number of mammalian species, including rats, mice, bats, cats, nonhuman primates, and humans. It is believed that the synchronous neuronal activity associated with Ripple oscillations may play a role in the consolidation of new memories. The pathological counterpart to Ripples are Fast Ripples (FRs). These pathological HFOs, typically between 200 and 600 Hz, are absent from normal limbic structures and occur primarily in areas of spontaneous seizure generation. FRs have been hypothesized to reflect the neuronal substrate of epileptogenicity.

Studies in animals with chronic limbic seizures and patients with temporal lobe epilepsy have identified a number of distinguishing features between Ripples and FRs with respect to their spatial distribution, spectral characteristics, and network properties, but the neuronal mechanisms supporting their generation are unclear. For several years our laboratory has used electrophysiological, histological, and neuroimaging techniques to study

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FR in order to better understand the role of pathological HFOs in the human epileptic brain and appropriate animal models. Our goal is to determine how functional and structural modifications in networks of neurons contribute to the generation of pathological HFOs and epileptogenicity. Studies that focus on pathological HFOs such as FR will provide insights that may lead to new strategies to treat, and possibly prevent, epilepsy.

Background

Studies investigating Ripple oscillations in rodents show that Ripples occur chiefly during periods of immobility or episodes of slow wave sleep (SWS). During theta-related states, such as during exploratory behaviors and rapid eye movement (REM) sleep, Ripples are largely absent. Ripples occur prominently in areas CA1 and CA3 of the non-primate hippocampus, and subicular and entorhinal cortices; there is little evidence for the presence of physiological Ripples within the dentate gyrus. One hypothesized role for Ripples is that they facilitate information transfer during sleep between hippocampal and cortical structures. This presumed role for Ripples is based on evidence that Ripples are temporally coupled with forebrain spindle oscillations that occur regularly during episodes of SWS. Coordinated discharges along hippocampal and neocortical pathways may be related to processes associated with memory consolidation when short-term hippocampal memory traces are transferred to long-term neocortical stores.

Much of what is known regarding the neuronal activity associated with Ripple oscillations derives from in vivo wide bandwidth recordings in rat hippocampus. In behaving rats, Ripples are often superimposed on large amplitude slow waves called sharp waves. During the large-scale neuronal activation associated with sharp waves, Ripples are typically largest in amplitude in the CA1 pyramidal cell layer. In vivo extracellular recordings during Ripples show that pyramidal cells tend to fire infrequently, but when they do fire their discharges are time-locked to the negative wave of the oscillation. In contrast, interneurons may fire during every cycle of the oscillation in a pattern that is consistently phase-aligned with respect to the Ripple. Intracellular recordings from pyramidal cells reveal the presence of a ripple-frequency potential that is smallest in amplitude at membrane potentials between -70 and -80 mV, and reverses in polarity relative to the extracellular Ripple at membrane potentials more negative than -80 mV. According to these data, it is likely that the fast intracellular potential is mediated by chloride ions due to the activation of $GABA_A$ receptors by GABAergic interneurons. While the mechanisms contributing to the synchrony of neuronal discharges during Ripples is not completely understood, one possibility is that phasic activation of interneurons by external input may produce rhythmic inhibitory post-synaptic potentials (IPSPs) that strongly regulate pyramidal cell discharges. This synchronous interaction between interneurons and pyramidal cells is thought to generate active inward and outward extracellular currents that contribute to the Ripple field potential.

Inhibitory interneurons can entrain discharging pyramidal neurons (or other interneurons) into a rhythmic pattern, but it is unclear whether interneuron-generated IPSPs facilitate the synchrony of discharges associated with Ripples. In vitro studies using hippocampal slices show that suppressing $GABA_A$ receptor activation does not abolish spontaneous or evoked Ripples in CA1. In contrast, application of agents such as octanol or carbenoxolone that interfere with the presumed electrical coupling between hippocampal neurons suppresses Ripples. These latter findings are consistent with the absence of Ripples in CA1 of rats under halothane anesthesia, and are predicted by computer models of Ripples generated from neuronal networks that include axon-to-axon gap junctions between pyramidal cells. These data indicate that inhibitory transmission may not be required for the generation of Ripples, but excitatory transmission mediated through chemical and possibly electrical synapses may contribute to the synchronous neuronal discharges associated with Ripples.

Ripples studied in the absence of inhibition, however, are often associated with epileptiform discharges, which raise questions about whether neuronal mechanisms generating Ripples in vitro are the same as those supporting physiological Ripples in vivo. Indeed, blocking $GABA_A$-mediated inhibition also produces Ripple-frequency oscillations in the isolated dentate gyrus, a region where Ripples are not found in the normal rat but occur in the seizure-generating dentate gyrus of epileptic rats. Evidence from the in vitro studies mentioned above show that these pathological HFOs in dentate gyrus reflect synchronously bursting granule cells that are dependent on AMPA/kainate-receptor mediated glutamatergic transmission.

In contrast to physiological Ripples, FRs are present only in spontaneously epileptic structures, and limited to regions in or adjacent to the epileptogenic lesion in epileptic rats. In epileptic patients, interictal rates of FR are significantly higher in seizure generating hippocampal areas compared to contralateral (nonseizure generating) sites. Simultaneous multiunit activity (MUA) and field potential recordings indicate that FRs reflect population spikes from bursting principal neurons. A number of studies, using acute models of epilepsy and hippocampal slices prepared from acute or chronic epileptic animals, show that blocking GABAergic transmission is an effective means of producing abnormal neuronal synchronization that contributes to the generation of population spike activity. In the systemic kainic acid (KA)

epileptic rat model, population spike bursts can be evoked in dentate gyrus showing extensive loss of parvalbumin interneurons; this observation indicates that even selective disruptions in inhibition may contribute to abnormal population synchrony. And in the penicillin model of epilepsy, population spike bursts, at a frequency greater than 200 Hz, occur in dentate gyrus and CA1. These data, too, indicate that the suppression of inhibition promotes abnormal neuronal synchrony, although it has not been determined how, and to what extent, local disturbances in inhibitory and excitatory networks contribute to the generation of pathological HFOs.

Methods

Epilepsy studies in our laboratory use chronic animal models of epilepsy that include the intrahippocampal KA rat, the systemic pilocarpine rat and mouse, and the self-sustained status epilepticus rat. Twenty-hour per day video and electrophysiological recordings of EEG, local field potentials, and single unit and MUA (using moveable and fixed microelectrodes) are regularly carried out in these animals with spontaneous epileptic seizures, as well as in treated animals that do not develop spontaneous seizures. Studies include analysis of ictal and interictal abnormalities.

Living brain slices are prepared from some epileptic animals at the end of chronic recordings for in vitro extracellular and intracellular recordings, to study the generation of local field potentials and single and MUA. Regularly, brain slices are fixed and stained following chronic in vivo recordings to locate the position of the microelectrodes in hippocampus and dentate gyrus. In addition, neo-Timm staining is used to evaluate the extent of mossy fiber reorganization that commonly occurs in dentate gyrus in animals that develop spontaneous limbic seizures.

A significant component of our research, that complements our investigations in experimental models, includes chronic microelectrode recordings in patients with medically intractable complex partial seizures that are suspected to arise from mesial temporal lobe limbic structures. Patients are candidates for epilepsy surgery, but require chronic intracranial depth electrode monitoring to locate the brain area where their seizures begin. During the 1–3 week period when patients are on the epilepsy telemetry ward for continuous video-EEG monitoring, we record from bundles of nine microwires (40 μm in diameter) that extend beyond the tip of each clinical depth electrode. These microwire bundles or 'microelectrodes' are regularly positioned within mesial temporal (amygdala, hippocampus, subiculum, entorhinal cortex, parahippocampal gyrus), extratemporal (orbital frontal, cingulate gyrus, supplementary and presupplementary motor), and other clinically relevant brain areas. Wide bandwidth EEG (1–6000 Hz) is recorded continuously during overnight polysomnography studies and analyzed for interictal and ictal abnormalities in relation to changes in states of vigilance (e.g., sleep and wakefulness), and in relation to epileptogenicity. Anatomical data obtained from resected epileptogenic tissue and MRI are correlated with EEG, local field potentials, single and MUA to correlate structural with functional abnormalities.

Results

Spatial Distribution of HFO Networks

Ripples occur simultaneously on both sides of the brain, with oscillations in either hemisphere leading the other. Ripples in epileptic rats, as well as in human patients with epilepsy, occur with equal probability in areas ipsilateral and contralateral to the hemisphere of seizure onset. Fast Ripples, in contrast, are strongly associated with sites ipsilateral to seizure onset. They occur simultaneously or separately between adjacent structures (e.g., dentate gyrus and entorhinal cortex) or at different recording sites within the same structure (separated by distances of 1 mm or more). These latter data highlight the spatial and occasional functional independence between networks generating interictal FR. In addition, these data and results from other FR studies indicate that individual FR generation sites are small and widely distributed throughout epileptogenic tissue.

Studies of hippocampal sharp wave-Ripple complexes indicate a unidirectional pattern of generation, beginning in hippocampal CA3, propagating to CA1 and subiculum, and then towards entorhinal cortex. Analysis of spontaneous FR show that there is a tendency for FR generated in entorhinal cortex to lead (by several milliseconds) FR in dentate gyrus. Yet, FR in dentate gyrus may precede those in entorhinal cortex. Thus, there appears to be no clear unidirectional propagation pattern for FR along the hippocampal trisynaptic circuitry, which suggests that the generation and synchronization of FR are not preferentially initiated from any one of potentially many distributed FR sites.

Structural and Functional Characteristics of Networks Supporting Pathological HFOs

Hippocampal neuron loss and synaptic reorganization are common pathological features found in KA-treated rats, as well as in patients with mesial temporal lobe epilepsy. In epileptic rats, FRs occur in areas of cell loss and intense mossy fiber sprouting. FR, however, can appear as early as 24-h following status epilepticus. The delay in the appearance of FR corresponds with excitotoxic cell loss

associated with status epilepticus; however, FRs clearly occur before the sprouting of new axon projections from surviving neurons in dentate gyrus and hippocampus. Once established, FR-generating sites are fixed spatially and surrounded by areas not generating FR. In patients with hippocampal sclerosis, reduced hippocampal volumes and neuron densities correlate with higher rates of FR and lower rates of physiological Ripples. Furthermore, neuron loss in CA1 most strongly predicts ratios of FR to Ripple rates. Based on these animal and patient data, it appears that morphological alterations linked with neuron loss promote the generation of FR but disrupt networks supporting Ripples.

In brain slices from epileptic rats, stimulation of perforant path fibers evokes FR-like responses – that is, burst of population spikes – in dentate gyrus; these bursts can be recorded within a radius of 3 mm from the KA lesion. FR-generating areas are estimated to be as small as 1 mm^3 (based on multisite recordings). Outside of these limited FR areas, stimulation evokes excitatory postsynaptic potentials that may or may not contain a single population spike. The volume of tissue supporting FR is determined, in part, by the functional circuitry maintained within and surrounding these pathological networks. Application of bicuculline to epileptic dentate gyrus slices enlarges the area from which FR can be evoked, and elicits spontaneous FR from the same sites where evoked FRs arise. In spite of the presence of intact inhibitory circuitry surrounding FR-generating areas, paired-pulse stimulation in FR networks generates augmented population spike responses at interstimulus intervals between 30 and 200 ms. Furthermore, test pulses at intervals between 100 and 500 ms can evoke enhanced FR-like responses. These data show that robust feedback inhibition is present surrounding FR generating areas, but suggest that local excitatory connections in FR areas are strong – and synchronous discharges may overcome feedback inhibition. Changes in inhibition can increase the size of individual FR-generating areas, and likely the synchrony between FR sites, that may lead to a seizure.

Sites Generating Physiological and Pathological HFOs

Voltage versus depth analysis of HFOs recorded from 16-contact electrodes that traverse CA1 and dentate gyrus of epileptic rats show that spontaneous Ripples are largest in amplitude on contacts positioned within stratum pyramidale of CA1 and absent from dentate gyrus (**Figure 1(a)**). FR, in contrast, occur in CA1 or dentate gyrus, with a maximum amplitude in CA1 pyramidal cell layer or dentate gyrus granular layer. CA1 Ripples reverse in polarity just below the pyramidal cell layer; the profile of CA1 FR is similar to the profile of Ripples, but the location of polarity reversal extends towards the hippocampal fissure (**Figure 1(b)**). This latter difference

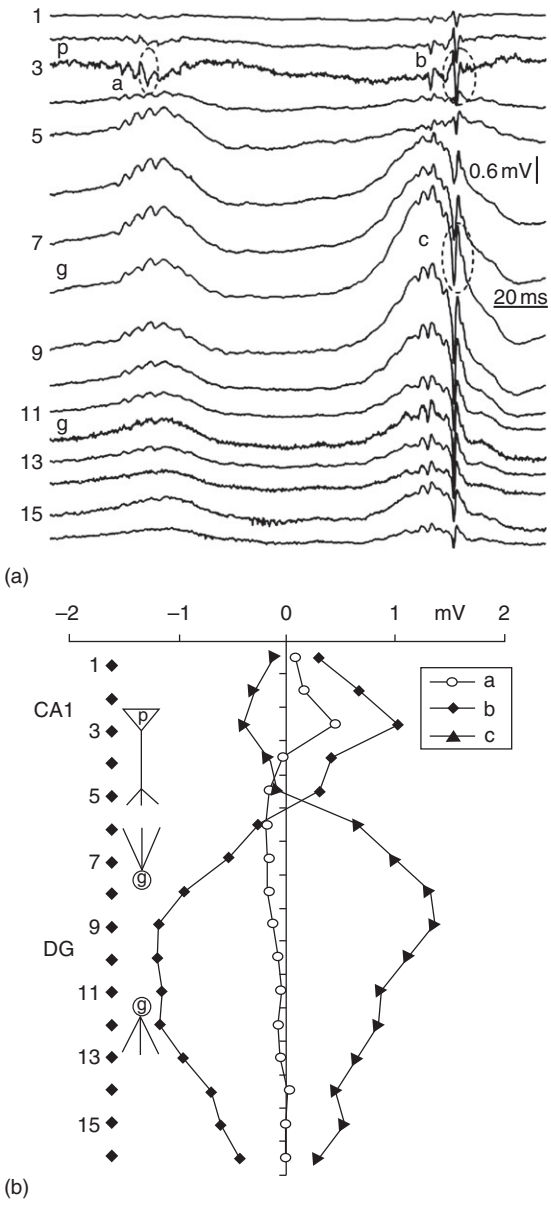


Figure 1 (a) Two-hundred millisecond continuous recording from a 16-contact recording electrode positioned in the hippocampus of a rat. Example of a spontaneous Ripple oscillation and associated positive sharp wave in CA1 ('a'), followed by a spontaneous HFO that occurs synchronously in CA1 ('b') and dentate gyrus ('c'). Dashed circles corresponding to a, b, and c indicate recording site of maximum amplitude. Approximate location of pyramidal cell and granule cell layers are denoted by 'p' and 'g', respectively. (b) Voltage depth profiles of HFOs a, b, and c from panel (a). Note that the maximum amplitude of the Ripple ('a') and pathological HFO ('b') occur within cellular layers, but the Ripple reverses in polarity just below the pyramidal cell layer, while the pathological HFO reverses near the hippocampal fissure. Reproduced from Bragin *et al*. (2007) *Epilepsia* 48(5): 35–40, with permission from Wiley-Blackwell.

may reflect back propagation of the population spike along dendrites and/or activation of a greater number of neurons associated with pathological HFOs. Ripples in CA1 are lower in amplitude compared to FR, and appear

as smooth sinusoid-like oscillations. FR recorded in either CA1 or dentate gyrus appear as bursts of population spikes, with intraburst population spike frequencies between 80 and 600 Hz (Figure 2). Outside these cellular layers, FR amplitude sharply decreases and the shape changes from sharp, irregular population spikes to a smoother oscillation.

In entorhinal cortex ipsilateral to the seizure onset region in epileptic rats, FR recorded simultaneously from sites 1.5 mm apart often do not reverse in polarity. These data suggest that FR networks in entorhinal cortex may be larger than those in dentate gyrus. Using microelectrodes with spaced tips oriented perpendicular to cortical laminae, we have shown in patients that FR are maximum in amplitude and reverse in polarity in middle layers of entorhinal cortex. Stimulating the afferents to entorhinal cortex reveals that these middle layers correspond with locations in which the evoked population response is largest in amplitude. In contrast, Ripples recorded from these same sites more often do not reverse in polarity across entorhinal cortex laminae. These data indicate that FR-generating areas in cellular layers are likely to be much smaller than networks supporting Ripples.

Neuronal Correlates of Ripples and FR

Similar to hippocampal Ripples, FR are most likely to occur during SWS, both in animals and patients with epilepsy. Unlike Ripples, FR occurrence remains elevated during REM sleep, and equivalent to rates during wakefulness. In these mesial temporal limbic areas, analysis of single neurons shows distinct changes in neuronal firing that correspond with occurrences of Ripples and FR. Single unit firing rates, burst firing, and synchrony of action potential discharges between pairs of neurons are higher during SWS compared to REM sleep. However, during REM sleep, neurons ipsilateral to seizure-generating areas have significantly higher firing and burst rates, and greater synchrony of discharges compared to neurons in contralateral sites. These data demonstrate that enhanced neuronal excitability and synchrony of discharges are associated with Ripple- and FR-generating areas. Yet, the neurochemical systems that govern REM sleep and suppress neuronal excitability (and large scale thalamo-cortical rhythms) have less influence on abnormal neuronal synchrony and local FR oscillations in primary epileptogenic brain areas.

Analysis of the relationship between neuronal discharges and occurrences of Ripples and FR in entorhinal cortex of epileptic patients reveals that MUA increases markedly during Ripples and FR, but the increase during Ripples is half that seen during FR. Analysis of single neurons indicates that the increase in MUA observed during Ripples is due, in part, to an increase in pyramidal cell discharge, because presumed pyramidal cells show maximum firing rates that coincide with the highest amplitude of the Ripple. Putative interneurons also increase their firing, but maximum firing rates occur before the Ripple oscillation. These data suggest that interneuron discharges may inhibit pyramidal firing at

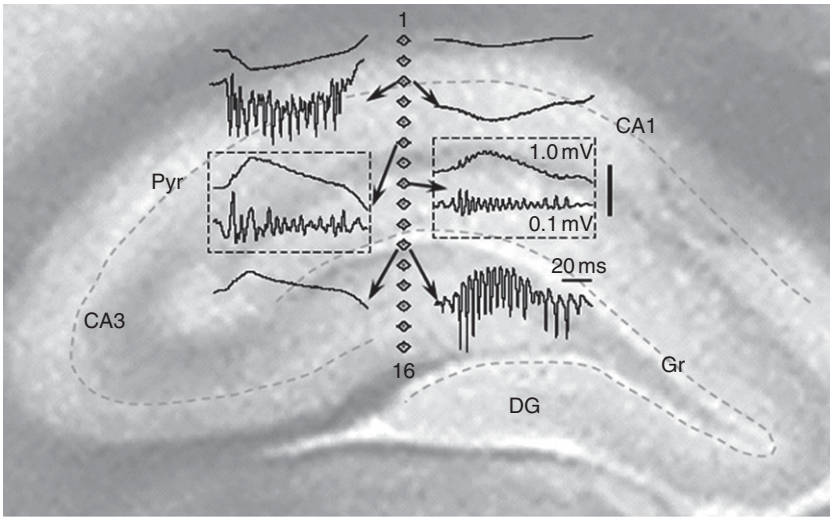


Figure 2 Two examples of pathological HFOs recorded from the hippocampus of a rat using a 16-contact recording electrode with intercontact spacing of 100 μm. The HFO on the left has a maximum amplitude in the pyramidal cell layer of CA1, while the other on the right has a maximum amplitude within the granule cell layer of dentate gyrus. Both HFOs reflect a burst of population spikes. Traces inside the dashed boxes correspond in time to the same bursts of population spikes, but at sites ~300 μm from the cellular layers. The bottom trace in each box is amplified 10 times and bandpass filtered 80–600 Hz. Reproduced from Bragin *et al*. (2007) *Epilepsia* 48(5): 35–40, with permission from Wiley-Blackwell.

the beginning of the local field oscillation, but pyramidal cells are 'released' to fire during the Ripple oscillation. Phase analysis between unit discharges and each Ripple cycle reveals that pyramidal cells fire preferentially during the negative phase, while interneuron discharges lag by approximately 0.5 ms. This coordinated pattern of firing during Ripples includes precisely timed discharges from interneurons that may regulate pyramidal cell discharges and control activation of postsynaptic targets.

Currently, there are no patient data regarding cell-specific discharges contributing to FR, but limited in vivo evidence from epileptic KA-treated rats shows that pyramidal cells fire during the negative phase of the extracellular FR oscillation. However, cellular recording has revealed no consistent firing pattern relationship between interneurons and FR, and recordings fail to show that either interneurons or pyramidal cells fire at FR frequencies. Thus, it is likely that FR emerge from synchronized action potential discharges from groups of neurons that include principal neurons primarily, but probably interneurons as well. How abnormal synchrony is generated in vivo during FR is unknown, but in vitro studies show that normal rat hippocampal slices bathed in a high potassium medium generate spontaneous FR-like field bursts in CA3. This hippocampal region contains a high density of recurrent excitatory connections between pyramidal cells. Chemical agents that block ionotropic glutamatergic signaling reduce synchronous discharges between pyramidal cells and attenuate FR bursts. Manipulations that increase the fidelity of pyramidal cell discharges increase FR amplitude, whereas disrupting intrinsic action potential generating mechanisms decreases FR amplitude. These data indicate that excitatory synaptic transmission may synchronize discharges between pyramidal cells that contribute to FR.

Future Goals

A challenge in studying neuronal networks supporting pathological HFOs is to reliably capture pathological HFOs at the source of their generation. FR-generating sites are small and distributed across seizure-generating brain areas, which make these pathological networks difficult to target in animals, and particularly in patients with epilepsy in whom the primary epileptogenic area is often not limited to a single structure. However, because higher rates of FR correlate with areas of greater atrophy and cell loss, MRI could help to identify atrophic hippocampal areas that may then be used to guide the placement of chronic microelectrodes in suspected FR-generating areas. Also, recent patient studies using conventional subdural or depth electrodes positioned in neocortical areas have found HFOs that are presumed to be pathological. These data suggest that the neuronal generators of neocortical HFOs may be larger than those in mesial temporal limbic areas, and therefore may be captured using noninvasive techniques – for example, magnetoencephalography (MEG).

Our past studies using chronic microelectrode recordings in animals and patients with epilepsy emphasized significant differences in spatial distribution and spectral frequencies between Ripples and FR. Our more recent analysis suggests that HFO shape is an important feature that may be useful to distinguish physiological and pathological HFOs (that is, pathological HFOs with Ripple frequencies). In addition, HFO shape may be especially important for studies using conventional depth electrodes in patients with epilepsy because large diameter electrodes are more likely to record HFOs from sites that are distant from the source of generation. Studies are needed to analyze and compare the spatial, temporal, and waveform electrophysiological features of HFOs recorded simultaneously on microelectrodes and clinical depth electrodes.

In order to identify neuronal mechanisms underlying pathological HFOs, additional electrophysiological studies are needed that specifically characterize neuronal cell types and their discharges during FR (e.g., in animal studies describing physiologically identified principal cells and interneurons during endogenous network oscillations). It is essential to determine how local excitatory circuits and inhibitory interneurons contribute to generation of pathological FR. A powerful approach, using juxtacellular labeling and immunohistochemical identification following in vivo recordings, will provide important morphological and neurochemical information about the roles of pyramidal cells, granule cells, and various classes of interneurons in generation of pathological HFOs.

Studies of HFOs suggest that pathological HFOs have tremendous potential as a surrogate marker of epileptogenicity, which may provide valuable localizing information to identify areas for surgical resection. Furthermore, the presence of FR in primary epileptogenic areas may be an indication of pharmacologically resistant epilepsy, and assist in determining effective treatment. In addition, pathological HFO may be used to screen for potential anti-epileptic compounds. Finally, there is evidence that FR are involved in epileptogenesis, and studies are needed to investigate pathological HFOs in animal models and, if possible, in patients who are at risk for developing epilepsy.

See also: **GABA**: Synchrony Through GABAergic Inhibition; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; Role of Gap Junctions in Epileptic Synchronizations; **Oscillatory Activity**: Cellular Mechanisms of Network Oscillations

Occurring Prior to, and during, Seizures; Cellular and Network Oscillations in Epilepsy; High-Frequency Weakly-Synchronized Activity at the Onset Focal Seizures.

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Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations

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Introduction

Over a century ago, on the basis of clinical investigations, Gower suggested that 'seizures beget seizures.' Implicit in that notion is that seizures lead to long-lasting alterations in the mode of operation of neurons and networks, and that these alterations in turn induce the chronic epileptic state – i.e., a permanent reduced threshold for seizure generation. How this transformation occurs – from a naïve network to one that generates seizures – is a basic question in neurobiology, since it serves as a template for how episodes of hyperactivity may profoundly and often irreversibly alter the molecular and cellular properties of neurons. The transformation is of obvious major interest in relation to the epilepsies; its elucidation should lead to improved understanding of epileptogenesis, including the unique features of epileptic tissue, the mechanisms that lead to the transformation, and their relation to neuronal plasticity. Further, understanding these transformative mechanisms should also provide insight into treatments that might prevent epileptogenesis (antiepileptic drugs) and drugs that only block seizures (anticonvulsive agents).

Studies relying on experimental adult animals have provided a solid rationale for the concept that seizures beget seizures. Indeed, recurring seizures such as those produced by kainic acid lead to long-lasting alterations in the circuit, including cell loss and the formation of novel aberrant synapses that are now known to play a central role in the generation of seizures by the reorganized (now 'epileptic') network. The inaugurating status epilepticus induced by kainic acid injection is followed by a latent phase, during which overt behavioral (or electrographic) seizures are not readily generated; it is only a few weeks later that a chronic epileptic state, with spontaneous seizures and a reduced threshold for the generation of additional seizures, is apparent. During this latent phase, a plethora of genes often expressed in the developing – but not the naïve adult – brain are reexpressed and lead to the sprouting of fibres and the formation of novel aberrant synapses that will directly contribute to the generation of

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Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations

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Studies relying on experimental adult animals have provided a solid rationale for the concept that seizures beget seizures. Indeed, recurring seizures such as those produced by kainic acid lead to long-lasting alterations in the circuit, including cell loss and the formation of novel aberrant synapses that are now known to play a central role in the generation of seizures by the reorganized (now 'epileptic') network. The inaugurating status epilepticus induced by kainic acid injection is followed by a latent phase, during which overt behavioral (or electrographic) seizures are not readily generated; it is only a few weeks later that a chronic epileptic state, with spontaneous seizures and a reduced threshold for the generation of additional seizures, is apparent. During this latent phase, a plethora of genes often expressed in the developing – but not the naïve adult – brain are reexpressed and lead to the sprouting of fibres and the formation of novel aberrant synapses that will directly contribute to the generation of

further seizures. This 'reactive plasticity' is instrumental in the pathogenesis of many forms of epilepsy. A concept similar to the 'seizures beget seizures' hypothesis is intrinsic to the 'kindling' process, in which recurrent electrical brain stimulation that initially generates only a brief response eventually triggers (after many stimuli) a generalized convulsive seizure. Here, also, reactive plasticity may occur and contribute to the deleterious sequelae.

Instrumental to our understanding of these processes are parallel observations made by researchers on the mechanisms of synaptic plasticity. These studies have focused on how short episodes of augmented activity leads to persistent alterations of synaptic efficacy. These studies on long-term potentiation and depression (LTP and LTD) have unravelled important basic mechanisms of synaptic plasticity, including the short lasting rise of intracellular calcium (mediated by activation of NMDA receptors and/or voltage-gated calcium currents) that triggers a cascade of events that induces a postsynaptic insertion of novel AMPA receptors. A similar rational applied to epileptic activities has led to the idea that hyperactivity first leads to increased synaptic efficacy followed by long-lasting synaptic rearrangements. Seizures are now known to trigger LTP processes triggered by NMDA receptors and associated with a more efficient glutamatergic responses. 'Epileptic' LTP (or other forms of pathological LTP) differs, however, from the memory-related LTP; in classical memory-linked LTP, NMDA receptors are required for the induction of the process but are not modified during its expression. In contrast, in epileptic LTP, NMDA receptor-mediated signals are persistently augmented, and this persistent change is an important factor in the resulting functional pathology. Nevertheless, these classical LTP-related observations provided a framework studying the relation between hyperactivity and its long-lasting consequences. They also suggest a link between physiological and pathological alterations of synaptic plasticity and their consequences.

Yet, another major player must be considered in studying the transformation of naïve tissue to the epileptic state. GABAergic neurons and synapses control the activity of neural networks and play a central role in the generation of behaviorally-relevant oscillations and pathological patterns. They also provide a source of inhibition that prevents the generation of hyperactivity. Extensive investigations have shown that GABAergic inhibitory postsynaptic currents (IPSCs) are obliterated quite rapidly by episodes of hyperactivity. Subsequent studies showed that some GABAergic neurons are also vulnerable to seizures; subtypes of interneurons, such as the somtostatin OLM interneurons, readily degenerate after seizures. Also, recordings made weeks after the insult show a permanent reduction of the inhibitory drive onto dendrites of pyramidal neurons, suggesting a permanent loss of inhibitory drive; no reactive plasticity or sprouting of GABAergic axons appear to compensate for the loss. Recent studies have suggested that a shift of polarity in the action of GABA (from hyperpolarizing to depolarizing) plays a central role in epilepsy. Recordings made in chronic human epileptic foci showed a persistent accumulation of intracellular chloride in neurons, leading to excitatory effects of GABAergic synapses. Since GABAergic synapses are also excitatory in immature neurons (for the same reason), this observation has suggested that 'epileptogenesis recapitulates ontogenesis.'

In this review, we shall briefly deal with the long-lasting consequences of seizures in the developing brain. The developing brain is known to have a high incidence of seizures, and exhibits significant (and long-term) seizure consequences. Recurring seizures in newborns may lead to life-long deleterious sequelae, suggesting that seizures at that stage of development may transform naïve networks into networks that generate seizures. Paradoxically, in spite of their low threshold for seizure generation, immature neurons are more resistant to insults than adult cells. For example, immature neurons are not readily killed by anoxic episodes or recurrent seizures. Thus, in the very young postnatal rat, seizures generated by kainic acid activate limbic networks but do not lead to cell loss in the hippocampus; however, by the 2nd postnatal week this resistance to injury disappears. Therefore, the long-lasting consequences of seizures in the very young brain may well be due to mechanism other than cell loss and reactive plasticity. One plausible mechanism is that seizures alter developmental programs in a time- and developmental stage-dependent manner, thereby leading to persistent maladaptive construction of networks (including malformations) and synapses. These networks, in turn, are responsible for the long-term seizure sequelae. Hence, it remains important to test whether seizures, even those not associated with structural damage, beget seizures in the developing brain at an early developmental stage.

Background

Activity Dependent Alterations of $[Cl^-]_i$

The regulation of $[Cl^-]_i$ by neuronal activity has been studied extensively by Poo and colleagues, using various preparations to determine how altering neuronal activity modifies the chloride gradient and thus the actions of GABA. In an earlier paper, they showed a progressive reduction of $[Cl^-]_i$ in neuronal cultures, which is associated with an expression of the chloride transporter KCC2. This shift was prevented by applications of a GABA receptor antagonist, suggesting that GABA itself contributes to the reduction of $[Cl^-]_i$. These investigators then showed that postsynaptic action potential generation shifted the reversal potential of GABA-induced Cl^- currents (E_{Cl^-}) toward positive levels in a duration- and frequency-dependent manner. This activity-dependent shift is regulated by cytosolic Ca^{2+} elevation and mimicked by releasing Ca^{2+}

from internal stores. This activity-dependent effect plays an important role in seizure generation, inasmuch as it is quite persistent. Indeed, in earlier studies in the adult hippocampus in vivo, investigators showed that high frequency stimulation produced long-lasting increases in the efficacy of glutamatergic synaptic currents and an elimination of GABAergic synaptic currents. This opposite action of electrical activity on excitatory and inhibitory currents can lead to higher excitability.

Alterations of $[Cl^-]_i$ also Regulate Construction of Cortical Networks

GABA receptors are present well before synapses are formed, and their activation can modulate early cellular function. Indeed, the ongoing release of GABA exerts an important tonic and phasic action on neuronal migration in neurons that bear no operative synapses. In addition, alterations of $[Cl^-]_i$ can affect the construction of networks. This point was directly demonstrated using a transfection technique to express KCC2 (the late-appearing chloride co-transporter) in immature neurons; such expression led to the excitatory-to-inhibitory shift of the actions of GABA. This shift also led to an unexpected and dramatic increase in the density of GABAergic synapses and of miniature GABA currents, indicating that more functional synapses are established when the chloride shift occurs. In subsequent experiments, Poo and colleagues used an in utero electroporation technique to introduce KCC2 into a subpopulation of rat progenitor cells. This introduction did not alter radial migration to layer II/III of the somatosensory cortex, but did impair the morphological maturation of neurons. These results are consistent with earlier studies that suggested a trophic action of excitatory GABA on neurons, and illustrate how early actions of excitatory GABA (and the underlying chloride gradient) act to modulate the construction of networks. Alterations in the polarity of GABA action would be expected to have long-lasting consequences on excitability. For example, drugs that alter GABA function at an early stage of brain development may also affect cortical networks and lead to malformations; such results have been shown in recent studies devoted to test the effects of in utero drugs on the formation of heterotopic masses. It is also likely that early seizures, and other episodes of enhanced activity, lead to long-lasting sequelae mediated by these mechanisms.

Seizures Beget Seizures in vitro in the Hippocampus

To determine if seizures produce long-lasting alterations of network activity, we developed an intact in vitro hippocampi preparation in which the two intact hippocampi from immature rodent were dissected together with their commissural connections. This preparation is placed in a chamber with three independent compartments (one compartment for each hippocampus and one for the connecting commissures). Since the compartment are isolated from each other, we can independently perfuse the two hippocampi and the connections with different liquids. Using this preparation, we generated seizures in one hippocampus (with kainate or bicuculline) while recording from fields and single neuron activity in both the stimulated and naïve hippocampus. The connections between hippocampi can be reversibly interrupted – e.g., after the propagation of a single or multiple seizures – by applications of TTX. We found that the propagation of single seizure did not lead to long-lasting consequences; ongoing spontaneous and evoked activity was restored to baseline levels after the seizure event. In contrast, after recurring seizures, the naïve hippocampus – i.e., the hippocampus that had not received a convulsive agent but was subjected to recurring propagated seizures – became epileptic: it generated spontaneous long-lasting ictal episodes, and electrical stimuli elicited all-or-none epileptiform events. This transformation was long-lasting; slices prepared from the epileptic hippocampus still generated epileptiform activities 48 h later. We concluded that recurring seizures do transform a naïve hippocampus to one that generates seizure activity.

Interestingly, blocking NMDA receptors in the recipient naïve hippocampus did not block the generation of propagated seizures, but did prevented the transformation of the naïve hippocampus into an independently epileptic seizure-generating site, suggesting that NMDA receptors are required for this transformation. This observation is consistent with the involvement of alterations of synaptic efficacy in this process, analogos to those changes seen in LTP (and other paradigms of synaptic plasticity) that also necessitate the activation of NMDA receptors. Somewhat more unexpectedly, blocking GABA receptors also prevented the transformation. Therefore, the activation of GABA *and* NMDA receptors is instrumental for seizures to beget seizures.

Persistent Increase of $[Cl^-]_i$ in the Mirror Focus

A major role for GABA and chloride was suggested by the observations that in the epileptogenic 'mirror focus' (as described above), GABA again excites neurons. Recurring seizures appear to down-regulate the mechanisms that control chloride extrusion, thus leading to an accumulation of intracellular chloride. Extensive investigations now suggest that the chloride co-transport extruder KCC2 is vulnerable to insults, and is reduced by lesions and traumatic injuries. Other observations also suggest that recurring activation of neurons favors an accumulation of chloride and a shift of the actions of GABA to more excitatory actions. This observation on the plasticity of $[Cl^-]_i$ suggests that this mechanism plays a central role in long-lasting effects of

neuronal activity on the excitatory/inhibitory actions of GABA (also see below). The importance of these observations is also reflected in the excitatory actions of GABA consequent to the high $[Cl^-]_i$ reported in human epileptic neurons recorded in vitro following surgery.

Methods

Intact neonatal hippocampi are dissected and placed in a triple chamber. Both neuronal and population activities are recorded, and a mathematical analysis of the activities is carried out to describe the power spectra of the observed oscillations.

Recent Results

High Frequency Oscillations are Required for Seizures to Beget Seizures

Why do recurring seizures lead to long-lasting alterations and transform a naïve network into one that generates seizure activity? Why are both GABA and NMDA receptors required for seizures to beget seizures? The development of our triple chamber, and our confirmation of the formation of an epileptogenic mirror focus in that preparation, gave us a unique opportunity to test the mechanisms. Toward this goal, we applied drugs separately to the generating hippocampus and the transformed one, and compared the properties of seizure activity that effectively begat seizures to properties of seizures that did not cause this transformation. We found that only seizures that included high frequency oscillations (HFOs, above 40 Hz) lead to long-lasting consequences. Seizures with only lower frequency components fail to so initiate the transformation; after a large number of recurring seizures, the naive hippocampus will not generate spontaneous or evoked seizures (when it is disconnected from the treated hippocampus).

How are HFOs generated? When GABA and/or NMDA receptors are blocked, the seizures fail to generate HFOs – and loose their long-term consequences. In other words, the activation of these receptors enables the generation of HFOs, that in turn trigger the events that lead to the formation of an epileptogenic mirror focus. It is likely that seizures activate GABA PSCs, leading to the activation of NMDA receptor channels (by the removal of the voltage-dependent Mg^{++} block), thereby enabling both the generation of HFOs and long-lasting potentiation of synaptic currents. The parallel loss of regulation of chloride removal, by an activity-dependent inactivation of KCC2, becomes progressively irreversible (by an as yet unknown mechanism), leading to a positive feedback loop (i.e., more excitatory actions of GABA, more seizures, more intracellular chloride, etc.). To summarize, the induction of the transformation is due to HFOs generated by the activation of GABA and NMDA receptors; the expression of the mirror focus is in part due to a permanent accumulation of chloride and excitatory actions of GABA. The clinical implications of these observations may be quite important, both in terms of diagnosing deleterious seizures that require aggressive treatments and also in differentiating the inhibitory and the apparently pro-epileptic actions of GABA (since the activation of GABA receptors is needed for seizures to beget seizures).

In preliminary experiments, we've recently made single GABA channel recordings (cell attached configuration) from neurons in the mirror focus. We found significant alterations of $[Cl^-]_i$ associated with the expected changes of E_{GABA}. These observations provide direct evidence that there is, in the mirror focus, an increased intracellular chloride concentration.

Future Goals

Observations from our experimental model clearly suggest that seizures do beget seizures, and that GABA-chloride plasticity plays a central role in long-term consequences of seizures and other insults. A deficit in the regulation of chloride during episodes of hyperactivity is central to this effect, in that neurons loose their capacity to respond adequately to hyperactivity and even to physiologically relevant episodes of enhanced activity that can be triggered under these conditions. Future experiments should focus on confirming these observations in vivo, using appropriate recording techniques (and in particular, with single GABA channels recordings that prevent intracellular dialysis of chloride and provide an adequate measure of chloride shifts). Also crucial will be the determination of the dynamic of chloride shifts; methods used at present are less than adequate considering that they rely on invasive whole cell recordings. Most important and difficult will be the determination of the type of mechanisms that can transform a transient shift of chloride to a quasi-permanent one: At what point is a neuron unable to return to its normal baseline?. How is that shift controlled? Answers to these questions will, in turn, give important insights into the mechanisms that underlie the transformation of a transiently seizing network to one that is genuinely epileptic. The use of the triple chamber in vitro, combined with powerful imaging techniques, will provide a unique opportunity to investigate the relation between HFOs and epileptogenicity. Single GABA channel recordings in the epileptic tissue will provide a unique opportunity to investigate how the epileptic networks and GABA signalling behave and respond to various stimuli, including behaviorally-relevant oscillations.

Parallel studies on human brain slices, removed after surgical interventions, will enable investigators to test the relevance of the proposed mechanisms. While it is already apparent that GABA excites epileptic neurons, it is still unknown how many neurons, which ones, and in relation to what patterns of activity (e.g., HFOs?). The use of in vivo preparations that enable experimenters to perfuse the cortex and hippocampus while recording from identified neurons will provide opportunities to test in situ the propagation of seizures in animals that been made epileptic by treatment with convulsive agents. Finally, the impact of fast imaging techniques in determining the mechanisms of epileptogenicity should not be underestimated. The generators of various patterns of epileptiform activities are now being unravelled in brain slices, using fast two photon imaging. The identification of the generators of spontaneous seizures will be of the utmost importance, as this determination will provide an approach to some of the most difficult questions in that field: What are the relations between physiologically- and behaviorally-relevant patterns of activity and seizures? Are they produced by the same generators? What are the neuronal properties of these generators, and how do they shift from one pattern to another?

See also: **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; $GABA_A$ Receptor-Mediated Mechanisms and Epileptiform Synchronization; Plasticity of GABAergic Systems during Epileptogenesis; Synchrony Through GABAergic Inhibition; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; **Transporters**: Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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PEDIATRIC EPILEPSY

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Age-Specific Responses to Antiepileptic Drugs

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Introduction

When treating infants and children with epilepsy, two things become obvious. First, seizures are prevalent at these very young ages of life and second, the seizures and epileptic syndromes occurring in the pediatric population have a wide variability in semiology and evolution, as well as in their response to therapies, which distinguish them from those in adults. These unique features of the pediatric epilepsy patient are due, in part, to the fact that the developing brain first encounters stressors (and other conditions) that can trigger epileptic events – at a developmental stage when its defense mechanisms have not fully matured. For example, certain seizure-controlling networks may not have been fully formed, and/or there is a relative abundance of excitatory/synchronizing mechanisms with respect to inhibitory control mechanisms. Furthermore, paradoxical "excitatory" effects may be triggered from classical inhibitory systems, as is the case in $GABA_A$ receptor signaling. In fact, the maturation of the mechanisms that control excitability is a dynamic and asymmetric process across brain regions and cell types, presenting interneuronal communication patterns that are more complex and diverse, when compared not only to adults but also to any given developmental stage.

In this article, we briefly introduce the factors responsible for the age-specific effects of antiepileptic drugs (AEDs) and then focus on $GABA_A$ signaling, describing how its role in seizure control and brain development changes with age. We also present recent advances that demonstrate how $GABA_A$ signaling is influenced by intrinsic and epigenetic factors, which may therefore modify the effects of AEDs on the brain.

Background

Age-Specific Effects of AEDs: Treating a Moving Target

Given the often stormy course of many neonatal and childhood seizures, the first priority of the pediatric epileptologist is to find the most effective anticonvulsant

therapy. The armamentarium of available AEDs is similar across ages, and, AEDs that are effective in adults can also be useful in the pediatric population. However, unlike the adult epileptologist who typically treats temporal lobe or extratemporal focal-onset epilepsies, pediatric epileptologists encounter a richer spectrum of epileptic seizures and syndromes that may differ in their responsiveness to AEDs. This variability in responsiveness reflects, at least in part, the many different pathophysiological processes underlying early life seizures and epilepsies – mechanisms that are distinct from those seen in adults. Such is the case, for instance, with epilepsies of genetic, metabolic, or febrile etiology that tend to appear in young patients. Of equal importance is the fact that the developing brain operates differently than the adult. As a result, seizure semiology may differ widely with age. A dramatic example of this feature is seen in infantile spasms, a classical seizure type of infancy that requires its own specialized types of therapies (e.g., adrenocorticotropin hormone (ACTH)). Alternatively, the effects of drugs may differ (between adult and pediatric case) because the targeted neurotransmitter systems may have different signaling consequences in immature versus more mature neurons, or their effects may be less pronounced in a specific age group due to differences in the expression of related receptors (Table 1). The effects of AEDs may be further diversified by age-specific differences in the organization of the brain, and its still developing interactions with other systems (e.g., the endocrine or immune systems) and with the environment.

Age not only matters when considering AED efficacy, but also influences the effects of AEDs on brain development and function. The age-specific vulnerability of the patient who is still developing has best been exemplified by drug-induced neurodegenerative changes. Ikonomidou's group first observed increased apoptosis in multiple areas of the brain in seizure-naïve neonatal rats, a result that was not nearly as dramatic as in more mature animals. Existing evidence supports the view that AEDs may interfere with higher cognitive functions, even during early postnatal life. Comparison of clinical studies across ages and populations is hampered by the fact that etiology and frequency/severity of epilepsy may independently affect the cognitive function. There are limited studies that specifically address the neurodevelopmental impact of AEDs on immature animals with seizures. Existing studies suggest that at least certain AEDs may not have significant adverse effects in animals that had experienced prior seizures. Obviously, this issue needs to be explored further.

Developmental Changes in $GABA_A$ Signaling

GABA has a central role in epilepsy research, as it is the classical inhibitory neurotransmitter in the mature brain and a favorite target of many of the available AEDs. GABAergic interneurons control the activity of regions involved in seizure generation, such as the hippocampus, but are also major components of subcortical nuclei involved in the control of seizure propagation, such as

Table 1 Age-specific differences in selected neurotransmitter systems

Factor	*Immature brain*	*Mature brain*	*Potential significance*
NMDA receptors			
– binding studies	Peak at ~PN5	Intermediate	– Higher excitability of immature brain – Different sensitivity to selective inhibitors
– subunit expression	NR2B vs NR2A High	NR2B vs NR2A: Low	Shorter NMDA-postsynaptic currents
AMPA receptors			
– binding studies	Peak at PN10	Intermediate	– Higher excitability of immature brain
– subunit expression	GluR2B expression: Low	GluR2B expression: High	– Increased calcium permeability in immature neurons – Different sensitivity to selective inhibitors
Kainate receptors			
– binding studies	Low	High	Different sensitivity to selective inhibitors
Glutamate uptake	Low	High	Higher excitability of immature brain
GABAA receptors			
– signaling effects	Depolarizing	Hyperpolarizing	– More effective GABAA inhibition in mature neurons – Age-specific translational effects
– subunit expression	α2/α3 vs α1: high	α2/α3 vs α1: low	Higher affinity for benzodiazepines in mature brain
GABAB receptors			
– binding studies	High	Low	Higher sensitivity to $GABA_B$-acting drugs
GABA reuptake			
– GAT expression	Higher GAT-3	Higher GAT-1 (peak at PN10–30)	Different sensitivity to specific GAT inhibitors

Simplified summary of the known age differences in neurotransmitter systems. It should be noted that cell type, region, and sex specific differences have been reported.
GAT is the GABA transporter.

the substantia nigra pars reticulata (SNR). The role of the SNR and the age- and sex-specific changes in the function of its GABA-sensitive seizure controlling networks is the focus of another article in this volume (see Scantlebury *et al.*). Here, we will rather address how the age-related changes in $GABA_A$ receptor signaling – from depolarization to hyperpolarization – may impact both the effects of $GABA_A$ergic drugs in seizure control and also their effects on brain development.

$GABA_A$ receptors are pentameric ligand-gated Cl^- channels that can assume both excitatory and inhibitory functions, depending on the existing Cl^- gradient across the plasma membrane. They play central roles in brain development and neuronal communication. Very immature neurons maintain high intracellular Cl^- concentrations due to overabundance of inward Cl^- transporters, such as the sodium potassium chloride cotransporter NKCC1 (Figure 1). Consequently, the opening of $GABA_A$ receptors leads to depolarization, activation of L-type voltage-sensitive calcium channels (L-VSCCs), and activation of calcium-sensitive processes that are important for neuronal differentiation. The depolarizing effects of GABA are important for a number of processes, including neuronal proliferation, migration, synaptogenesis, transcriptional regulation, and neuronal electrical activity. During development, there is a gradual shift in the balance of cation chloride cotransporters (CCCs) that transport Cl^- through the cellular membrane, and this shift ultimately decreases intracellular Cl^- to favor hyperpolarizing $GABA_A$ responses in mature neurons. These changes occur via a gradual decline in Cl^- importers (like the sodium potassium chloride cotransporter NKCC1) and a concomitant increase in the neuronal-specific potassium chloride cotransporter KCC2. These changes have been

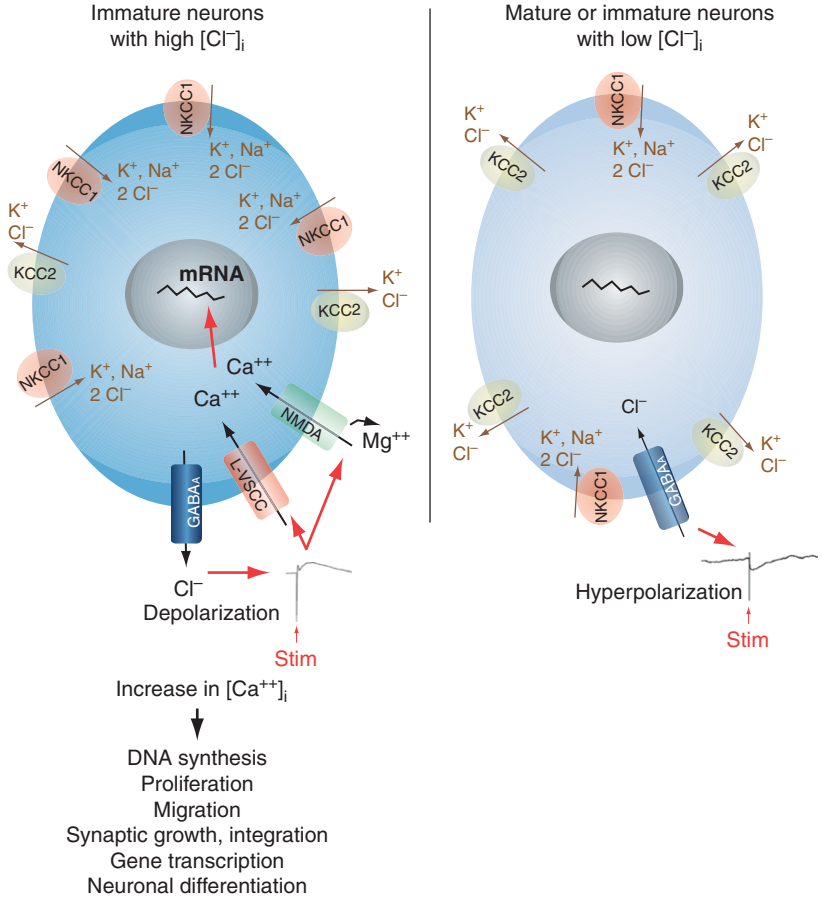


Figure 1 Developmental changes in chloride cotransporters and $GABA_A$ signaling. In immature neurons (left panel) there is a high $[Cl^-]_i$, due to a relative abundance of NKCC1 over KCC2. As a result, activation of $GABA_A$ receptors leads to depolarization, activation of L-VSCCs and NMDA receptors (via release of Mg^{2+} block) and increase in intracellular calcium. This promotes many calcium-sensitive signaling cascades that are involved in brain development. During development, there is a gradual increase in KCC2 and decrease in NKCC1 which decreases $[Cl^-]_i$ and therefore facilitates the appearance of hyperpolarizing actions of $GABA_A$ receptors (right panel). In this scenario, $GABA_A$ activation cannot influence directly the calcium-sensitive processes. Modified from Galanopoulou AS (in press) GABAA receptors in normal development and seizures: Friends or foes? *Current Neuropharmacology*, with permission from Bentham Science Publishers.

shown, with antisense knockout animals or in functional inhibition experiments, to trigger the functional switch of $GABA_A$ signaling from depolarizing to hyperpolarizing. This functional switch occurs at specific time points during development, and in a cell type and sex-specific manner.

Several studies have indicated that the presence of depolarizing $GABA_A$ signaling may increase the excitability of very immature neurons and, under certain conditions, trigger ictal epileptiform events. Untimely re-appearance of depolarizing $GABA_A$ signaling or shift in the balance of CCCs to favor depolarizing GABA has been described in human tissues from temporal lobectomies of adults with temporal lobe epilepsy, as well as in tissue from pediatric epilepsy surgeries for cortical dysplasias. Paradoxically, drugs that normally trigger epileptiform activities in naïve adult neurons, like the $GABA_A$ receptor antagonists bicuculline or picrotoxin, can suppress epileptiform discharges in certain neurons with depolarizing $GABA_A$ signaling. Consistent with these results, functional inhibition of NKCC1 with bumetanide has been shown to suppress neonatal seizures in vivo in the kainic acid model. However, in in vitro studies, bumetanide suppressed ictal epileptiform activities only in the high potassium model, but not in the low magnesium or kainic acid model. These results suggest that age, as well as the type of seizures or the timing of bumetanide administration, may be an important factor in determining the bumetanide effects on seizure control. It is, however, difficult at the moment to speculate on the efficacy of such treatments in vivo, in humans, as the NKCC1-selective inhibition by bumetanide occurs only at very low concentrations of the drug.

In human patients, it is not yet known when the $GABA_A$ switch occurs. In the only available human postmortem study of patients dying from nonneurological causes, the levels of KCC2 and NKCC1 were compared and a transition from a NKCC1-dominant state to a KCC2-dominant state was reported to occur during the perinatal period, suggesting that this period may also correspond to the time of the $GABA_A$ switch. Occasional reports of paradoxical exacerbation of seizures by $GABA_A$-acting AEDs in premature, low-birth weight infants do exist. It is tempting to speculate that this may indeed be due to excitatory effects of $GABA_A$ signaling. These reports also raise the possibility that, in premature babies and possibly in a subpopulation of older patients with epilepsy, one of the reasons for medical refractoriness of their seizures to $GABA_A$ergic AEDs is the presence of depolarizing $GABA_A$ signaling.

In our laboratory, we have been particularly interested in the effects of $GABA_A$-acting AEDs on brain development, when given in seizure-naïve rats or in rats that had prior seizures. As discussed above, a number of studies have clearly demonstrated that the developmental changes in $GABA_A$ signaling lead to age-specific effects of $GABA_A$ergic AEDs on processes involved in neuronal differentiation. Recent advances have indicated that chronological age does not always correspond to biological age. Endogenous factors like sex, as well as epigenetic influences like seizures, stress, or drugs, may change the ontogeny of signaling pathways, thus altering the developmental stage of the brain. To understand better how to translate the experimental literature with respect to effects of AEDs on brain development, it is therefore imperative to dissect these mechanisms.

Methods

Our studies have been carried out in Sprague–Dawley rats (Taconic Farms, NY). Developmental comparisons of mRNA or protein expression levels are done using in situ hybridization and immunochemistry experiments, respectively. The experiments described below have focused on the SNR, known for its significant role in seizure control, and the hippocampus, well-known for its involvement in the networks that generate seizures, as well as its vulnerability in epileptic patients with memory deficits. Functional characterization of $GABA_A$ signaling is done with gramicidin-perforated patch clamp recordings, a method that preserves the Cl^- gradient and can therefore determine the direction of $GABA_A$ergic responses. In these experiments, slice recordings were carried out in the presence of glutamatergic inhibitors (D(−)-2-amino-5-phosphonopentanoic acid (AP5) 50 μM and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) 10 μM). Under these conditions, stimulation elicited only $GABA_A$ergic postsynaptic responses. $GABA_A$ergic responses were therefore assessed either by electrical stimulation or by bath application of $GABA_A$ergic agonists such as muscimol (10 μM exposure for 3 min). To assess whether such $GABA_A$ergic agonists can increase intracellular calcium, we used fura-2 AM imaging, in the presence of AP5 and CNQX.

Seizure induction was done with intraperitoneal kainic acid (KA) injections. We induced three episodes of status epilepticus (SE), hereafter referred to as 3KA-SE. In prior studies from other laboratories, brief recurrent seizures or a single episode of SE could impair performance on behavioral tests, but did not suffice to induce neurodegeneration or epilepsy. We wanted to maximize the seizure load (using three episodes of neonatal KA-SE) to test the effects of this level of activation on $GABA_A$ signaling, brain injury, and seizure outcome. Neurodegeneration was assessed by FluoroJade B staining. In vivo electroencephalographic (EEG) and video monitoring was carried out in selected pups either during KA-SE or at 2–3 months of age to detect seizures.

Recent Results

Sex Differences in the Age-Specific Effects of $GABA_A$ergic Drugs, in Seizure-Naïve Rats

Prior studies had indicated that several structures involved in seizure networks, like the SNR and hippocampus, are sexually dimorphic. Given the known role of depolarizing $GABA_A$ signaling in brain development, we wanted to determine whether sex differences existed in $GABA_A$ signaling – and if so, to elucidate their importance for the sexual differentiation of the SNR. Using KCC2-specific in situ hybridization, and subsequently KCC2 and NKCC1-specific immunochemistry, we confirmed that the expression of KCC2 mRNA and immunoreactivity (-ir) increased, whereas NKCC1-ir decreased (as described in the hippocampus and cortex) between the second and fourth postnatal week (Figure 2). As anticipated by these changes, synaptic stimulation of GABAergic SNR neurons, in the presence of glutamatergic inhibitors, elicited depolarizing $GABA_A$ergic responses in male rats prior to PN16 and hyperpolarizing responses after PN17.

Interestingly, the timeline of these changes was accelerated in females. KCC2 mRNA expression was higher in female than in age-matched male rat SNR GABAergic neurons at PN15, PN30, and PN60. Furthermore, the $GABA_A$ switch occurred at a younger age (PN10) in female rat SNR. The pharmacologic effects of muscimol also differed in male and female rat SNR GABAergic neurons, when assessed at PN15. In males, muscimol triggered depolarizations, increases in intracellular calcium, and the phosphorylated form of cAMP responsive element binding protein (pCREB-ir) (which regulates calcium-sensitive gene transcription). In contrast, in females, muscimol caused hyperpolarizations without influencing calcium or pCREB-ir (Figure 3). Independent studies, both from our own laboratory and from others, have shown similar sex differences in CCC expression or the direction of $GABA_A$ergic signaling in the CA1 pyramidal region of hippocampus and in hypothalamus of developing rats. However, the timing of these events follows region-specific patterns.

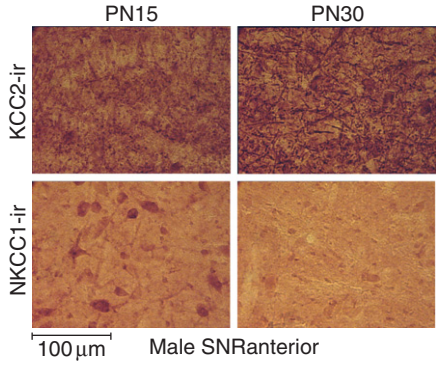


Figure 2 Age-specific changes in KCC2-ir and NKCC1-ir in male rat SNR.Using specific immunochemistries on PN15 and PN30 male SNR sagittal sections, KCC2-ir was detected in both somata and processes (upper panels). A gradual increase in KCC2-ir was noted, which was more evident in the neuronal processes at PN30. In contrast, NKCC1-ir declined with age (lower panels). Photographs were obtained with 400× magnification.

In vivo experiments aiming to evaluate whether the age-specific effects of a systemically administered $GABA_A$ergic drug may be due to the presence of depolarizing versus hyperpolarizing $GABA_A$ signaling have several limitations, if done only in young versus older animals. First, the biology and structure of the brain, and other interacting systems, such as the endocrine and immune systems, are completely different across ages. It is, therefore, difficult to dissociate their impact from the individual impact of the specific drug. Second, the epigenetic influences of the life experiences of a young versus older animal may also interfere. Third, the metabolism and bioavailability of the drug may be different. In our experiments, we took advantage of the sex differences in the direction of $GABA_A$ergic signaling in the neonatal hippocampus; specifically, we tested whether the existence of depolarizing versus hyperpolarizing $GABA_A$ergic responses modifies the effects of $GABA_A$-acting drugs or seizures on the developing hippocampus.

One of the calcium-regulated genes that is transcriptionally controlled by $GABA_A$ signaling is KCC2. GABA can control the timing of the $GABA_A$ switch via altering the expression of KCC2. In our studies, muscimol increased KCC2 mRNA in neurons with depolarizing $GABA_A$ signaling (i.e., immature neurons), but decreased it in neurons with hyperpolarizing signaling (i.e., mature neurons). This demonstration suggests the possibility of age- and possibly sex-specific translational effects of $GABA_A$ergic AEDs. Of interest, it has been shown that prenatal exposure to benzodiazepines leads to sex-specific differences in susceptibility to pentylenetetrazole-induced seizures in adult rats. However, the molecular pathways involved in these late effects have not yet been defined.

The different patterns of $GABA_A$ergic signaling also modify the effects of other pathways that are important in neuronal differentiation or survival. For instance, 17β-estradiol-mediated downregulation of KCC2 mRNA occurs only in neurons in which depolarizing $GABA_A$ signaling can activate L-VSCCs (Figure 3). Such interactions may further amplify the diversity of neurotrophic or translational effects of $GABA_A$ergic drugs, if given at different developmental stages. Interestingly, the neurodegeneration induced by AEDs, including effects of $GABA_A$ergic drugs, is maximal during the first 1–2 postnatal weeks, which correlates with the presence of depolarizing $GABA_A$ signaling in most studied brain regions. It is tempting to speculate that there is a link between the

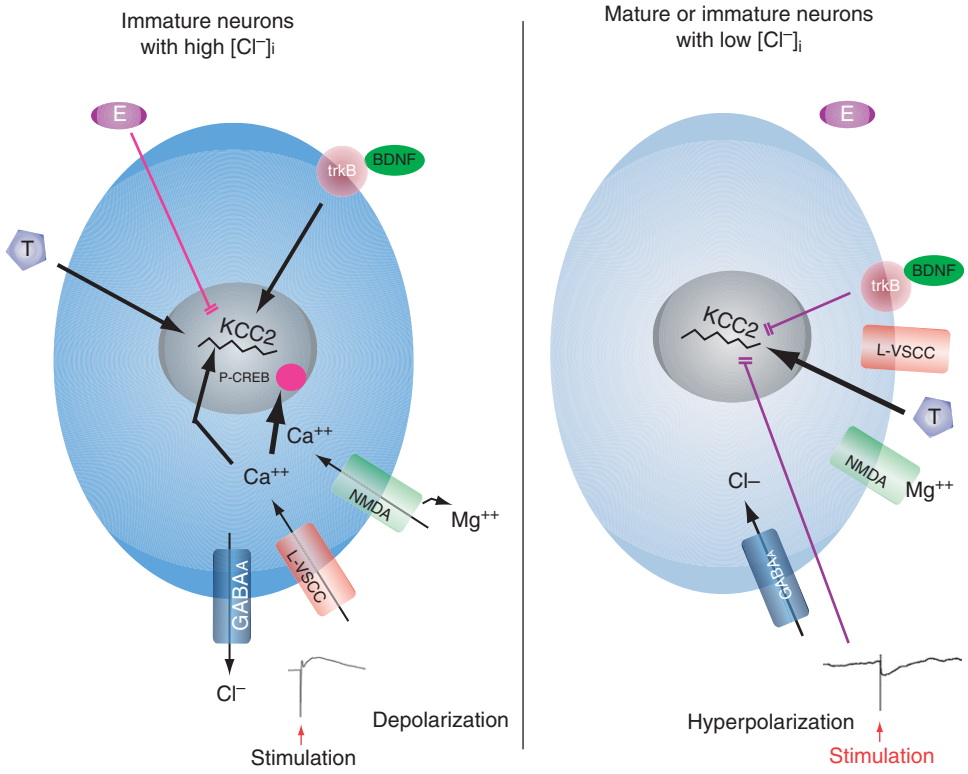


Figure 3 Different modes of regulatory control of KCC2 mRNA and pCREB-ir operate in neurons with depolarizing (left panel) or hyperpolarizing (right panel) $GABA_A$ signaling. *Left panel:* In immature neurons with high intracellular Cl^- ($[Cl^-]_i$), $GABA_A$ receptor activation causes depolarization, increases intracellular calcium and pCREB-ir, via activation of L-VSCCs and/or NMDA receptors. 17β-estradiol (E) decreases KCC2 mRNA; this effect is not seen in the presence of L-VSCC or $GABA_A$ receptor inhibitors. Brain derived neurotrophic factor (BDNF) increases KCC2 mRNA. Testosterone (T) and its androgenic derivatives also increase KCC2 mRNA. *Right panel:* In mature or immature neurons with low $[Cl^-]_i$, $GABA_A$ receptor activation causes hyperpolarization without altering calcium or pCREB-ir. In contrast, it decreases KCC2 mRNA. BDNF decreases whereas estradiol has no effect on KCC2 mRNA. Testosterone and its androgenic derivatives increase KCC2 mRNA regardless of the mode of $GABA_A$ signaling. These data are compilation of our own findings as well as data from the literature (referenced in Further Reading list). Modified from Galanopoulou AS (2008b) GABAA receptors in normal development and seizures: Friends or foes? *Current Neuropharmacology*, with permission from Bentham Science Publishers.

two processes (depolarizing $GABA_A$ signaling and neurodegeneration), but there is no direct proof yet. It is important to remember that these events have been described in seizure-naïve rats. How relevant are they for patients who need to take $GABA_A$-acting AEDs for seizure control (or for other neurological reasons)? This question has been a central focus of research in our laboratory; as described below, there are fundamental differences in $GABA_A$ signaling in the brain of rats with prior seizures or stress, which may help determine the effects of AEDs.

Seizures and Stress Can Modify the Biological Age: Lessons from $GABA_A$ Receptors

Numerous studies have shown that the outcomes after early life seizures differ from seizures first appearing in older animals. The immature brain is more susceptible to seizures. However, the degree of associated brain injury, cognitive deficit, and probability to develop later epilepsy increase with the age of first seizure onset. Many of the previously stated age-specific differences in the structure and physiology of the brain and its interactions with other systems, have been implicated. In our laboratory, we have been particularly interested in deciphering how $GABA_A$ signaling interferes with the late effects of early life seizures for two reasons – first, because of the importance of GABA in brain development and second, because most of the currently available antiepileptic drugs administered in neonates and infants act on $GABA_A$ receptors.

We have used the 3KA-SE model in rats, inducing three episodes of SE, at PN4, PN5, and PN6. In terms of brain development, this age corresponds roughly to a preterm human neonate. 3KA-SE did not induce detectable neurodegeneration as assessed at PN7, and mortality associated with this procedure was very low. However, 3KA-SE at PN4–6 switched the direction of $GABA_A$ signaling during the second week of life. Specifically, in males, it accelerated the functional maturation of $GABA_A$ signaling, causing precocious appearance of hyperpolarizing $GABA_A$ signaling. This influence, however,

can deprive the hippocampal CA1 pyramidal neurons of the neurotrophic influence of depolarizing GABA, and could thus contribute to long-term functional deficits. In contrast, in females, it produced a transient appearance of depolarizing $GABA_A$ signaling between PN8–13 (Figure 4). This influence may reactivate dormant calcium-sensitive processes, which in turn may cause phenotypic changes in the 3KA-SE females. Interestingly, the aberrant 3KA-SE induced switch in $GABA_A$ signaling did not occur in rats that had been treated with bicuculline at the time of KA-SE (i.e., given 10 min prior and 6 h after each KA injection; Figure 4), suggesting that $GABA_A$ receptors are mediators of the effects of 3KA-SE.

Comparison of our findings with published reports on the effects of seizures on $GABA_A$ signaling reveals that the female type of response to seizures resembles that of adults (work by Rivera *et al.*). Kindling in adult rats shifts CCC expression to favor a NKCC1-dominant state. Similarly, studies of human patients with temporal lobectomies have indicated either depolarizing $GABA_A$ signaling in a significant portion of subicular neurons or a shift to favor a NKCC1-dominant state. The only other in vitro developmental study was done on neonatal hippocampal neurons with hyperpolarizing $GABA_A$ signaling at the time of seizure induction; these experiments showed a similar switch to depolarizing $GABA_A$ signaling that drove spontaneous seizures. To determine whether the direction of $GABA_A$ signaling, rather than chronological age, is indeed a key factor in determining the effects of seizures on $GABA_A$ signaling, we examined the effects of 3KA-SE on rats that had been treated with bicuculline at the time of KA-SE (i.e., given 10 min prior and 6 h after

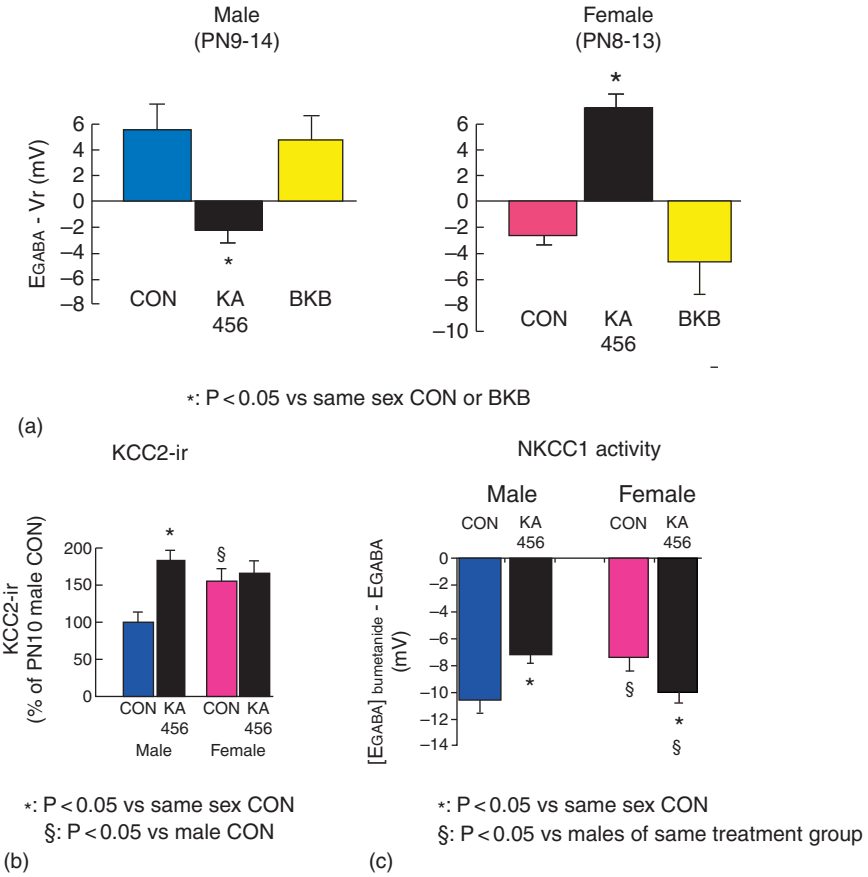


Figure 4 3KA-SE alters CCC expression and $GABA_A$ signaling in rat CA1 pyramidal neurons, via pathways that involve $GABA_A$ receptors. Using gramicidin perforated patch clamp, we assessed the direction of $GABA_A$ signaling in control (CON) pups or pups subjected to 3KA-SE at PN4–6 (KA456). E_{GABA} is the reversal potential for $GABA_A$ responses, in the presence of AP5 and CNQX. Vr is the resting membrane potential. (a) 3KA-SE at PN4–6 (KA456) switch the direction of $GABA_A$ signaling to hyperpolarizing in male PN9–14 and depolarizing in female PN8–13 rat CA1 pyramidal neurons. Such effects do not occur in rats that were injected with bicuculline at the time of 3KA-SE (BKB). (b) 3KA-SE at PN4–6 increases KCC2-ir in male PN10 CA1 pyramidal neurons but has no effect in females. (c) The activity of NKCC1-like Cl^- cotransporters was assessed with the bumetanide inhibition assay. Low doses of bumetanide (20 μM for 10 min) selectively inhibit NKCC1-like activity, shifting E_{GABA} to more negative values. Male PN9–14 KA456 rat CA1 pyramidal neurons had less bumetanide-sensitive NKCC1-like activity. In female PN9–14 KA456 rats, bumetanide-sensitive NKCC1-like activity was stronger than in CON. Modified from Galanopoulou AS (2008a) Dissociated gender-specific effects of recurrent seizures on GABA signaling in CA1 pyramidal neurons: role of GABAA receptors. *Journal of Neuroscience* 28(7): 1557–1567, with permission from Journal of Neuroscience.

each KA injection; BKB group in Figure 4). Indeed, in BKB rats, $GABA_A$ signaling remained similar to control (CON) after the three episodes of SE at PN4–6, suggesting that $GABA_A$ receptors are mediators of the effects of 3KA-SE. Further studies are under way in our laboratory to further delineate the exact molecular pathways involved in, or interacting with, $GABA_A$ receptors that contribute to these effects. Other signaling pathways that seem to be important for the cell type specific patterns of regulation of KCC2 include brain derived neurotrophic factor (BDNF), which is known to be released with seizure activity (Figure 3).

To dissociate the effects of 3KA-SE from those of maternal separation, we subjected pups to maternal separation for equivalent periods of time at PN4–6 (SS456 group). In males, SS456 pups also exhibited accelerated appearance of hyperpolarizing $GABA_A$ signaling, although its effects on CCCs were quantitatively different than seen in 3KA-SE animals. Maternal separation has more robust effects on decreasing bumetanide-sensitive NKCC1-like activity. In females, maternal separation caused a shift in E_{GABA} to more hyperpolarizing values, due to a decrease in bumetanide-sensitive NKCC1-like activity.

Considering the short lifespan and the rapid rate of rat brain development, this week-long disruption of processes that depend on $GABA_A$ pathways could contribute to long-term functional deficits. We were particularly interested in testing whether such changes may lead to late epilepsy. We performed long-term EEG monitoring in 2–3 months old (i.e., adult) female KA456 rats, using hippocampal electrodes. No spontaneous seizures were detected (Figure 5). If indeed the persistence of depolarizing $GABA_A$ signaling may prove important for epileptogenesis, it is possible that the molecular mechanisms involved in the resistance of the immature brain to sustain depolarizing $GABA_A$ signaling after seizures may protect it from subsequent epileptogenesis.

These findings raise a few important points. First, they reiterate the validity of the classical concept that the effects of seizures are different in immature and mature animals, partially due to the immaturity of the existing signaling pathways. Second, the data indicate that changes within the immature brain are not linear, but specific to discrete time windows that may reflect stages of the newborn period, infancy, and childhood. Certain patterns appear only during specific times, and these time points may differ as a function of sex. Third, $GABA_A$ signaling may be an important mediator of these age- and sex-specific effects. Consequently, the commonly used antiepileptic drugs that act upon $GABA_A$ receptors may also interfere with and/or modify these processes. These findings also emphasize that when age is considered, chronology does not always equate with biology. Intrinsic factors like sex, or epigenetic influences like seizures or stress, may alter the course of developmental processes. When administering AEDs, it is therefore important to consider not only age (in broad terms, child vs. adult) as a factor, but also the developmental stage of the targeted brain structures and whether these structures are sexually dimorphic, to improve efficacy and minimize side effects.

Future Goals

GABA is not the only neuroactive factor that shows age-specific roles important to brain functions. However, it provides a window through which to study developmental rules and principles, which may also be operable in other signaling cascades. We need a better understanding of the diversity that exists across ages in these molecular

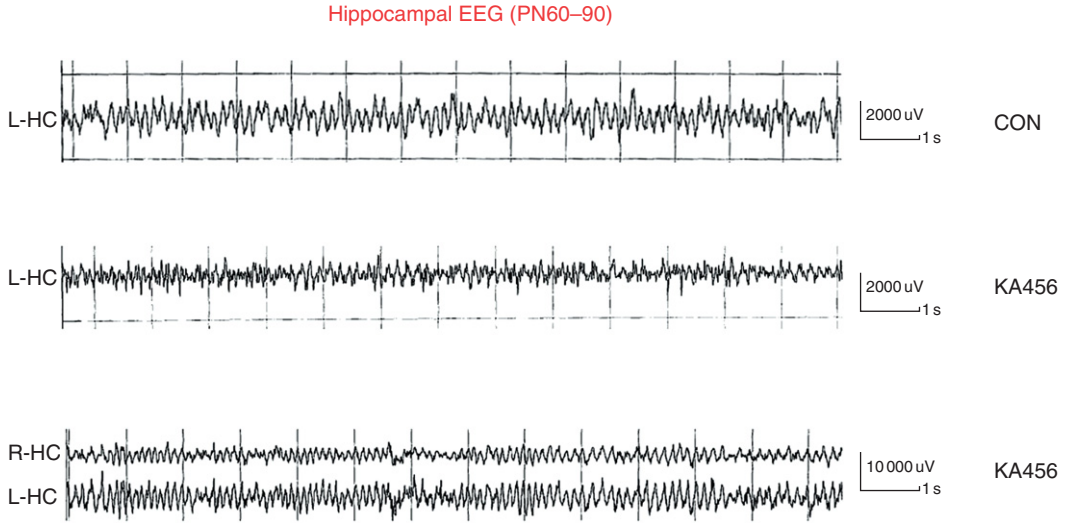


Figure 5 Hippocampal EEG recordings from 2.5 month old female control (CON) and KA456 rats do not reveal any spontaneous seizure activity. EEG recordings were done either unilaterally or bilaterally (R-HC: right hippocampus; L-HC: left hippocampus).

mechanisms to be able to make better predictions about their consequences. However, an important challenge in such efforts will be to translate such findings to the clinical arena. Although experimental studies have offered insights into the mechanisms of AED actions that could not have been obtained from human studies, it is important to remember that there are significant biological and functional differences among species. These differences may influence our ability to study specific outcomes in experimental animals or extend the animal findings into humans. Also, if the net effect of a signaling pathway in vivo is indeed a conglomerate of biological factors and pathways (i.e., age, sex, developmental stage, and epigenetic influences like seizures or stress), we will need better strategies to predict the type of response we expect in vivo. Useful tools may include imaging studies, electrophysiological tests or other specialized functional tests.

Although abundant evidence exists to support the central role of GABA in brain development, neuronal communication, and plasticity, it is important to provide more direct links between the seizure-induced disruption of $GABA_A$ signaling and long-term outcomes. Specifically, it is important to define better the functional and cognitive deficits resulting from disrupted $GABA_A$-related neuronal differentiation, to be in a better position to design specific therapies aimed at curing these deficits without side effects.

A key question is to understand the role of depolarizing $GABA_A$ signaling in epileptogenesis. Such signaling is obviously not sufficient for epileptogenesis, as it is present in normally developing animals. What are the pathological conditions under which seizures can induce the permanent appearance of depolarizing $GABA_A$ signaling? When this state occurs, can it result in epilepsy? Or, given the neurotrophic actions of depolarizing GABA, is it possible that the untimely switch of GABA to depolarization is an adaptive, regenerative process aimed at healing the brain? If so, how can we distinguish these regenerative processes from the epileptogenic processes so that we can create therapeutic interventions that specifically stop epilepsy without interfering with the healing process? Knowledge of the underlying molecular and electrophysiological mechanisms involved will be essential in our quest to understand the significance of these events and to design specific antiepileptogenic therapies without halting restorative processes.

Acknowledgements

The authors would like to acknowledge that funding for this work has been obtained by NIH NINDS research grants NS20253, NS45243, NS62947, NS58303, and grants from the Rett Syndrome Research Foundation, International Rett Syndrome Foundation, People Against Childhood Epilepsy, and the Heffer Family Foundation. Solomon L. Moshé is the recipient of a Martin A. and Emily L. Fisher fellowship in Neurology and Pediatrics.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy; **Hormones and Gender**: Sex Differences in Seizure Sensitivity; **Models**: Model Characterization in Relationship to Human Disorders; **Networks**: The Substantia Nigra in the Control of Seizures; **Pediatric Epilepsy**: Developmental Aspects of Seizures; Epileptogenesis in Childhood; Quantitative and Qualitative Changes of Action of Antiepileptic Drugs in Developing Experimental Animals; **Transporters**: Neuronal Cl Transport: Setting the Stage for Successful Inhibition; Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

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Animal Models of Catastrophic Epilepsies of Childhood

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Introduction

Catastrophic epilepsies are defined as age-specific epilepsy syndromes, occurring in infants and young children, in which severe seizures become refractory to medical treatment. Seizures in catastrophic epilepsies are accompanied by plateau or regression in developmental milestones (encephalopathy). Examples include relatively common syndromes such as infantile spasms (West syndrome) and Lennox-Gastaut syndrome, and rare epilepsies such as early infantile epileptic encephalopathy with suppression burst (Ohtahara syndrome), severe myoclonic epilepsy of infancy (Dravet syndrome), and epilepsy with myoclonic-astatic seizures (Doose syndrome). These syndromes are caused by a wide variety of brain insults or congenital or genetic causes. Infantile spasms, the most frequent type, have an incidence of ~1 case per 3200 live births, comprising about 9% of childhood epilepsies. Catastrophic epilepsy syndromes wreak havoc on the lives of children, families, and society, as the seizures tend to become refractory over time and are associated with major cognitive impairment that lasts a lifetime. Better seizure control often translates into preservation of intellectual and behavioral function in affected children. Current therapies for these syndromes are serendipitous or inadequate, both in terms of seizure control and prevention of cognitive deficits.

The main impetus for the development of an animal model is to understand the mechanisms and so create more appropriate and specific therapies. So far, attempts to replicate the features of the human catastrophic epilepsies in animal models have been disappointing. Inherent interspecies differences between brains of experimental animals (usually rodents) and humans contribute to the lack of a model. Nevertheless, to truly understand the neurobiological substrates of these catastrophic epilepsy syndromes, animal models must be designed to allow experimental manipulations of seizure frequency, type, and duration, as well as analysis of brain structure and function and behavioral consequences. Furthermore, experimental treatments can be tested on animal models prior to their introduction in humans.

Criteria for an ideal animal model of infantile spasms are listed in Table 1. So far, all animal models of infantile spasms have failed to mimic at least some of those criteria. Although it is not essential that an animal model fulfils each criterion to yield insights about developmental epilepsy, the most informative model will closely mimic the clinical situation. For example, responsiveness to adrencorticotrophic hormone (ACTH) does not necessarily make or break a model, but, as we discuss in this article, it enhances the model's credibility.

A model of infantile spasms that meets some of the criteria was developed in the laboratory of Dr. Tallie Baram. Since infantile spasms have multiple etiologies, Baram and colleagues proposed that infantile spasms occur because of a nonspecific brain insult during a developmental window during which the brain is particularly susceptible to pathological stresses. They hypothesized that stress may increase endogenous corticotrophin-releasing hormone (CRH) levels in seizure-prone areas of the developing brain, leading to neuronal damage, axonal reorganization, long-term cognitive defects, and possibly age-related seizures such as infantile spasms. Rat pups injected with minute doses of CRH intracerebroventricularly (i.c.v.) develop severe seizures, while mature rats develop CRH-induced seizures only at much higher doses, if at all. However, developmental seizures induced by i.c.v. injection of CRH are limbic in nature; they begin in the amygdala and spread to the hippocampus. The seizures in this model are not spasms, but rather consist of face and forelimb clonus, mimicking limbic convulsions. The electroencephalographic (EEG) abnormalities in CRH-induced seizures do not precisely reproduce those in children with infantile spasms. Furthermore, CRH-induced seizures are not responsive to ACTH, though ACTH does reduce CRH gene expression in certain neuronal populations.

Another attempt to model infantile spasms has involved intraperitoneal (i.p.) injection of *N*-methyl-D-aspartic acid (NMDA) in infant rats, which causes a clinical seizure described as emprosthotonus or hyperflexion. Emprosthotonus consists of whole-body tonic flexion with loss of the righting reflex and no clonic component. Although the EEG findings are variable, emprosthotonic

Table 1 Ideal criteria for an animal model of infantile spasms

Unprovoked epileptic spasms or myoclonic seizures during early postnatal development
Interictal EEG abnormalities (hypsarrhythmia)
Ictal EEG correlate (electrodecremental response)
Response to relevant treatment (e.g., ACTH)
Behavioral or cognitive sequelae

seizures are often accompanied by a reduction of EEG amplitude, resembling the electrodecremental response in the human EEG. Interictal hypsarrhythmia (as seen in human EEGs) is lacking, although large-amplitude non-synchronous waves frequently occur interictally, which may represent rat 'hypsarrhythmia'; these high-amplitude waves are never seen during routine EEG recordings. In this model, no spontaneous seizures have been recorded and the seizures are not responsive to ACTH.

Background

Intraperitoneal injection of NMDA induces hyperflexion seizures only in developing rats, with a seizure semiology similar to infantile spasms. These spasm-like whole-body flexions last for several seconds and may repeat in clusters. The ictal EEG shows depression similar to the electrodecremental EEG in infantile spasms. Additionally, interictal EEG often shows chaotic asynchronous high-voltage waves somewhat resembling hypsarrhythmia; this EEG pattern is definitely not normal at this stage of brain maturation in rodents. Learning and memory deficits occur in adulthood in rats having experienced NMDA spasms on postnatal day 15, and the rats have an increased susceptibility to generalized seizures induced by the GABA antagonist pentylenetetrazole in adulthood. However, this model has been limited by the lack of responsiveness to ACTH and other drugs used to treat infantile spasms such as vigabatrin. Clinical studies have demonstrated that patients with infantile spasms have decreased ACTH and cortisol levels in their cerebrospinal fluid. This finding, along with a unique and very specific therapeutic response of infantile spasms to ACTH, indicates that the control mechanisms of their hypothalamus–pituitary–adrenal (HPA) axis are impaired. Experimental studies using prenatal exposure to synthetic corticosteroids, especially to betamethasone, have demonstrated that this prenatal insult also decreases ACTH and cortisol levels. Furthermore, expression of glucocorticoid receptors is decreased in animals prenatally exposed to synthetic corticosteroids. Thus, this experimental approach mimics HPA impairments in patients with infantile spasms.

Methods

The current model involves a '2-hit' approach. Infant rats are exposed prenatally to betamethasone and then are treated postnatally with systemic NMDA. This approach is based on preliminary evidence showing that betamethasone impairs the HPA control systems, which may also have relevance in human infantile spasms. Prenatal betamethasone does not produce any macroscopic cerebral abnormalities, likening this model to one of idiopathic or cryptogenic infantile spasms.

On gestational day 15 (G15), pregnant rats are injected with betamethasone (two doses of 0.4 mg kg^{-1}, i.p., 10 h apart) resulting in the exposure of embryos to betamethasone. Then, on postnatal day 15, the offspring of rats exposed prenatally receive doses of NMDA at 7.5, 15, or 30 mg kg^{-1}, i.p. Pilot experiments showed that this NMDA dose range elicited hyperflexion seizures in more than 80% of rat pups with a greater than 80% survival rate. ACTH treatment was initiated with both the full length ACTH molecule (39 amino acids) as well as a 24-amino acid fragment ($ACTH_{1–24}$). The EEG was recorded in neocortex or hippocampus. Later, c-fos expression was evaluated at different times after spasm onset using immunohistochemistry, and [^{14}C]2-deoxyglucose imaging was used to identify brain structures activated by the flexion seizures.

Results

Several previous studies have shown that NMDA induces hyperflexion spasms in rats. NMDA-injected rats develop severe flexion seizures (emprosthotonus) in spasms lasting up to tens of seconds, with multiple recurrences; the seizures are intermixed with interictal abnormal running or walking behaviors (**Figure 1(a)**). These flexion seizures are insensitive to ACTH. In rats that received betamethasone prenatally, the semiology of NMDA-induced clinical signs was generally similar to that of prenatally naïve rats (i.e., no betamethasone exposure). However, rats having received prenatal betamethasone had significantly decreased latency to onset of tail-twisting and flexion spasms following postnatal NMDA challenge. Thus, these seizure signs occurred earlier in betamethasone-exposed rats than in untreated controls.

Electrographic recordings showed that before NMDA administration, P15 rats exposed prenatally to betamethasone showed no EEG abnormalities. After NMDA injection, two principal patterns were found during flexion spasms in betamethasone-exposed pups. The first was a high-amplitude discharge followed by attenuation of the signal (**Figure 1(b)**). The second pattern was a high-amplitude discharge followed by rhythmic afterdischarge (**Figure 1(c)**). Treatment with ACTH (0.1 mg kg^{-1}, i.p.) significantly delayed the occurrence of all NMDA-induced signs elicited in betamethasone-exposed rats (**Figure 1(d)**). Specifically, ACTH lengthened the latency to onset of tail-twisting, arching, and flexion spasms compared with saline-exposed control rats without ACTH.

Compared with rats treated with NMDA alone, betamethasone-exposed, NMDA-treated rats had markedly increased c-fos immunoexpression in limbic areas, hypothalamus, and brainstem. In the limbic system, c-fos expression was found in the medial amygdala nuclei and in the lateral septum, but not in the dorsal hippocampus. In the hypothalamus, c-fos expression was found in the

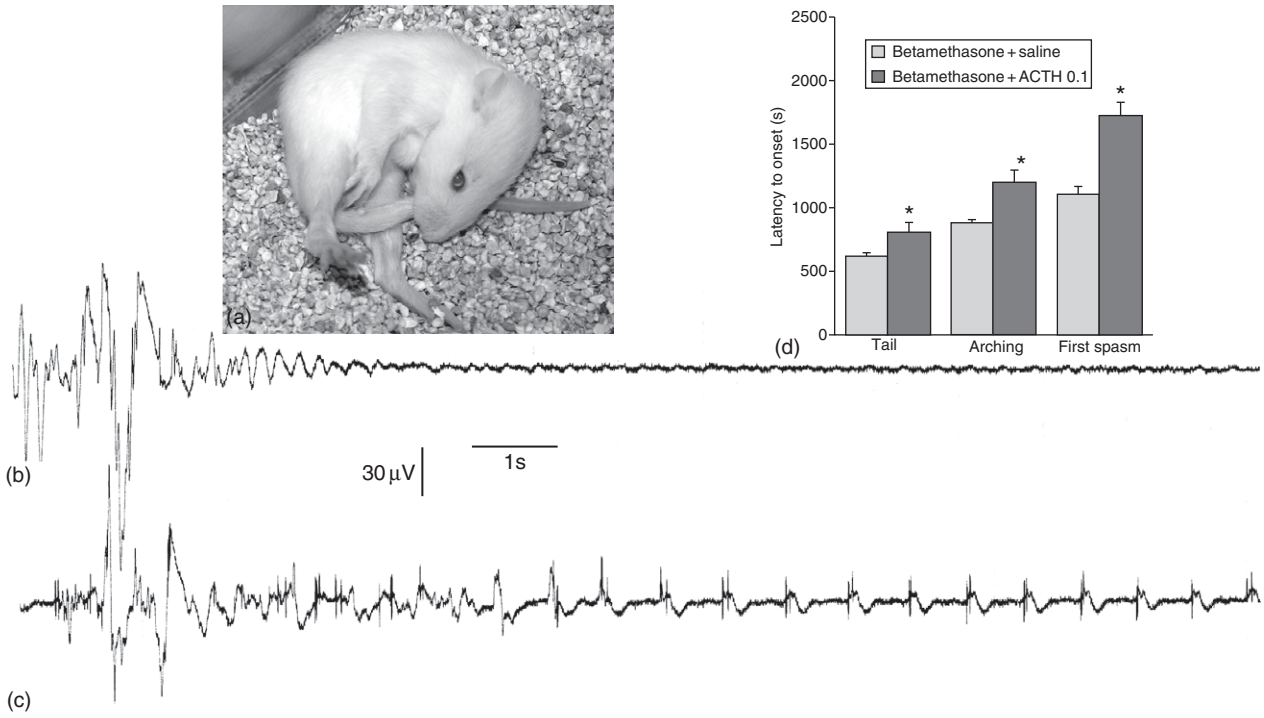


Figure 1 NMDA-induced flexion spasms in a 2-hit model of infantile spasms (1st hit: prenatal betamethasone exposure; 2nd hit: postnatal NMDA-induced seizures). (a) Photograph of postnatal day 15 rat exhibiting a flexion seizure (emprosthotonus). The rat was exposed prenatally to betamethasone and later treated on postnatal day 15 with NMDA (15 mg kg^{-1}, i.p.). The hyperflexion seizure involves the head, limbs, spine, and tail. This behavior is associated with EEG electrodecrement (see (b) and (c)). (b) EEG correlate (cortical recording) of NMDA-induced flexion spasm in 15-day-old rat. Large-amplitude sharp, rhythmic activity is followed by voltage attenuation. (c) Another example of an EEG correlate of flexion spasm consisting of a large-amplitude sharp waves followed by voltage decrement and rhythmic after discharges. (d) Latency to onset (in seconds) of NMDA-induced behaviors in 15-day-old rats prenatally exposed to betamethasone and postnatally treated either with saline or ACTH (0.1 mg kg^{-1}, i.p., in saline) 30 min prior to NMDA challenge (15 mg kg^{-1}, i.p.). Administration of ACTH increased latency to onset of NMDA-induced behaviors, including flexion spasms, consistent with an anticonvulsant effect of ACTH in this model. (a), (b), and (c) are reproduced from Velíšek L, Jehle K, Asche S, and Velíšková J (2007) Model of infantile spasms induced by *N*-methyl-D-aspartic acid in prenatally impaired brain. *Annals of Neurology* 61: 109–119.

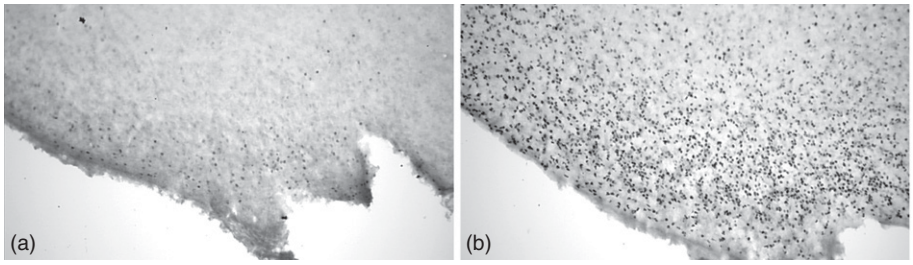


Figure 2 c-fos expression in the arcuate nucleus of the hypothalamus. (a) Section from a rat exposed to betamethasone prenatally and saline postantally. Few c-fos immunopositive cells are seen. (b) Section from a rat exposed to betamethasone prenatally, 1 h after the onset of NMDA-induced spasms. The arcuate nucleus is filled with c-fos immunopositive cells indicating activation of this hypothalamic structure.

supraoptic nucleus, arcuate nucleus, medial tuberal nucleus, and dorsomedial nucleus (**Figure 2**). Throughout the brainstem, nuclei were tagged with c-fos, including the ventral tegmental area, pedunculopontine tegmental nucleus, lateral parabrachial nucleus, dorsal nucleus of the lateral lemniscus, nuclei raphe, and the solitary tract nucleus. Increases in 2-deoxyglucose (2-DG) uptake in the limbic system were found in the lateral septum and significant decreases in the dorsal hippocampus. In the hypothalamus, increases in 2-DG uptake closely mimicked c-fos findings (**Figure 3**). In the brainstem, pedunculopontine tegmental nucleus was activated. These combined c-fos and 2-DG findings suggest the activation of these nuclei by NMDA seizures in betamethasone-treated rats.

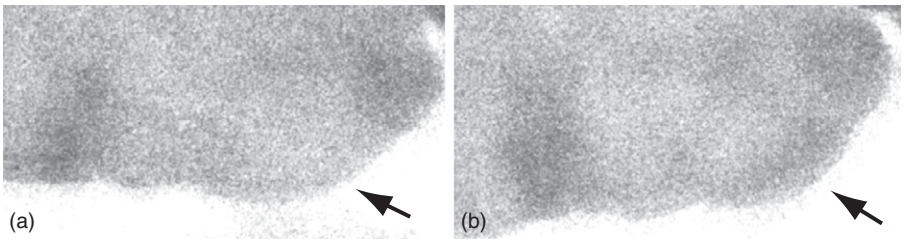


Figure 3 [^{14}C]2-deoxyglucose (2DG) uptake in the arcuate nucleus of the hypothalamus. (a) Section from a control rat injected with betamethasone prenatally and saline postantally. The arrow marks arcuate nucleus of the hypothalamus. (b) Section from a rat exposed to betamethasone prenatally, 2 min after the onset of NMDA-induced spasms. The arrow indicates a significant increase in 2DG uptake in the arcuate nucleus, consistent with an increase in synaptic activity.

The significant contribution of this 2-hit approach to modeling infantile spasms is the therapeutic response to ACTH, which has not been achieved in other models of infantile spasms or in other models of developmental seizures. This model has the potential to screen for novel therapies of infantile spasms.

Future Goals

Creation of an animal model of infantile spasms (the prototype catastrophic epilepsy of childhood) has been a daunting problem for investigators. Infantile spasms have numerous causes, including metabolic disorders, genetic mutations, malformations of cortical development, tuberous sclerosis, congenital or postnatal infections, strokes, and other causes. Therefore, it has been difficult to sort out essential elements of etiology from factors sufficient to provoke infantile spasms.

The studies introduced here aim to develop an alternative animal model of infantile spasms that may be of use in studying mechanisms of this disorder, and eventually in leading to therapeutic interventions. We show that prenatal administration of betamethasone, a corticosteroid that theoretically mimics a stress response, subsequently reduced the latency to postnatal NMDA seizures. That is, in rats prenatally exposed to betamethasone, NMDA at 2 weeks of age elicits flexion spasms resembling infantile spasms. In contrast to NMDA spasms elicited in naïve postnatal rats, spasms in prenatally betamethasone-exposed rats have a delayed onset when the animals are treated with ACTH. Although this ACTH protocol does not eliminate the spasm seizures, the sensitivity of the spasms to ACTH treatment constitutes an important step in the development of a clinically relevant model of infantile spasms. In this 2-hit model, the first hit – the prenatal stress caused by betamethasone – might somehow sensitize the brain to a later insult (NMDA seizures), resulting in enhanced seizure effects manifest by EEG findings reminiscent of infantile spasms. An important limitation of this method is the lack of spontaneous seizures. Given the need for double pharmacological stressors in this 2-hit protocol, perhaps this model should be considered a form of symptomatic infantile spasms, even though no gross structural abnormalities have been documented. However, it is possible that more detailed investigations will reveal changes at the cellular level that may mediate effects of prenatal betamethasone exposure on postnatal seizure susceptibility.

Numerous future studies are needed to complete the model development and validate its relationship to human infantile spasms. Most importantly, prenatal corticosteroids have been shown to alter many aspects of brain development, including subsequent behavioral and cognitive function. These effects must be more closely documented, as must the role of NMDA seizures in the manifestations of stress response. Since ACTH is given only immediately before NMDA, the time course of ACTH effects must be expanded. If cognitive sequelae can be determined in this model, the effect of ACTH on these cognitive changes can be studied. In human infantile spasms, ACTH is not given immediately before the spasms occur (as in this model). Rather, patients receive daily ACTH injections over several weeks once infantile spasms are diagnosed. In such ACTH-treated babies, the time course of ACTH effectiveness is usually on the order of several days (median, 2 days). Consideration of this time course is also important when testing other drugs. Within the infantile spasms spectrum, some drugs seem to be etiology specific. While ACTH seems to be more effective in idiopathic infantile spasms, vigabatrin, for example, is particularly beneficial in children with infantile spasms associated with tuberous sclerosis. The model proposed here might have value in the search for new therapies of infantile spasms. For example, drugs such as melanocortin receptor antagonists could be tested using this model. The interplay between intrinsic physiologic aspects of brain hyperexcitability (as a function of age) must be studied within the context of alterations induced by both of the stress-invoking agents discussed in this article.

Until recently, researchers have been somewhat pessimistic with regard to creating a useful animal model for the catastrophic epilepsies such as infantile spasms.

However, this pessimism is now changing to a tempered optimism. Within the next several years, this model – and hopefully others – will allow researchers to identify underlying mechanisms. Development of new therapeutic interventions based on those mechanisms can then be tested, to alleviate the consequences of the devastating catastrophic epilepsies of childhood.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Development/ Malformations**: Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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Animal Models of Infant-Onset Epileptic Encephalopathy

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Introduction

Epileptic encephalopathies are age-dependent clinical conditions characterized by severe epilepsy and cognitive disabilities. The interictal and ictal EEG abnormalities are typically so repetitive and frequent that it looks as if patients are in continuous electrical status epilepticus. The repetitive EEG abnormalities and clinical seizures are thought to directly contribute to poor cognitive development and mental retardation in patients with an epileptic encephalopathy. In infants, the best known syndromes are early epileptic encephalopathy with EEG suppression-burst (Ohtahara syndrome), neonatal myoclonic encephalopathy, and infantile epileptic encephalopathy (infantile spasms, West syndrome). Of these, infantile spasms are the most common epilepsy syndrome in the first year of life. This article will focus on infantile spasms and West syndrome as the prototypic infant-onset epileptic encephalopathy which should be modeled in animals, discuss the conceptual framework of current hypotheses attempting

However, this pessimism is now changing to a tempered optimism. Within the next several years, this model – and hopefully others – will allow researchers to identify underlying mechanisms. Development of new therapeutic interventions based on those mechanisms can then be tested, to alleviate the consequences of the devastating catastrophic epilepsies of childhood.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Development/ Malformations**: Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Ketogenic Diet**: Animal Models of the Ketogenic Diet and Other Dietary Therapies; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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Animal Models of Infant-Onset Epileptic Encephalopathy

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Introduction

Epileptic encephalopathies are age-dependent clinical conditions characterized by severe epilepsy and cognitive disabilities. The interictal and ictal EEG abnormalities are typically so repetitive and frequent that it looks as if patients are in continuous electrical status epilepticus. The repetitive EEG abnormalities and clinical seizures are thought to directly contribute to poor cognitive development and mental retardation in patients with an epileptic encephalopathy. In infants, the best known syndromes are early epileptic encephalopathy with EEG suppression-burst (Ohtahara syndrome), neonatal myoclonic encephalopathy, and infantile epileptic encephalopathy (infantile spasms, West syndrome). Of these, infantile spasms are the most common epilepsy syndrome in the first year of life. This article will focus on infantile spasms and West syndrome as the prototypic infant-onset epileptic encephalopathy which should be modeled in animals, discuss the conceptual framework of current hypotheses attempting

to explain the pathogenesis of this disorder, describe the pros and cons of currently available animal models that purport to reproduce this condition, and discuss future directions and goals for clinical and research investigations in this field.

Background

Human Characteristics of West Syndrome/ Infantile Spasms

Infantile spasms are a unique seizure type that occurs mostly during infancy and early childhood. In 1841, William West, a physician, first described the condition in his 4-month-old son. West syndrome consists of the triad of infantile spasms, severe cognitive dysfunction, and an interictal EEG pattern termed hypsarrhythmia. Thus, infantile spasms are the behavioral motor seizures that comprise one clinical component of West syndrome. The incidence of infantile spasms is ~1:3000 live births; the seizures generally begin in the first year of life (85% of cases), peak incidence is between 3 and 7 months of age, and familial occurrence is rare. The behavioral epileptic attacks are brief events, usually consisting of bilateral symmetric contractions of the muscles involving the extremities, neck, and truck (motor spasms). Other associated ictal behaviors may include attenuated responsiveness, eye deviation, respiratory pauses, and autonomic changes. Spasms typically occur in clusters lasting several minutes, appear frequently at sleep–wake transitions, and sometimes after a partial seizure. More than half of patients with infantile spasms exhibit major deficits on neurological examination. A normal developmental outcome is expected in less than 20% of children who have infantile spasms, and children with a history of spasms make up ~10% of all cases of childhood onset mental retardation. Shorter seizure duration is associated with a better long-term cognitive outcome in patients with symptomatic infantile spasms. Around 50% of babies with infantile spasms will eventually develop other therapy-resistant seizure types/ syndromes, such as the Lennox–Gastaut syndrome. Autism spectrum disorder is another common finding in children who have had infantile spasms.

The interictal EEG is almost always abnormal in children with infantile spasms. The most characteristic pattern consists of a disorganized high voltage background of slow waves intermixed with multifocal spikes termed hypsarrhythmia (**Figure 1(a)**). The ictal EEG pattern usually consists of an electrodecremental response associated with the motor spasms (**Figure 1(b)**). Spontaneous remission occurs in 25% of patients with infantile spasms within 12 months of onset. Infantile spasms are usually refractory to traditional antiepilepsy drugs. Medical therapy specific for patients with infantile spasms includes adrenocorticotropic hormone (ACTH) and corticosteroids (steroids), which are effective in half to three-quarter of patients, and vigabatrin, which is especially effective in patients with tuberous sclerosis complex (TSC) and infantile spasms. All of these medical treatments for infantile spasms are potentially toxic or are associated with significant side effects. Interestingly, ACTH is not a successful treatment for other forms of intractable generalized childhood-onset seizures. Thus, response to ACTH is considered one of the traits of patients with infantile spasms. About 5–30% of children with infantile spasms die within 12 months of treatment; 50% of the deaths are related to the medical treatment, and mortality is greater in infants with symptomatic causes. There are long-term negative consequences to those who had infantile spasms. A recent study reported that children who had a history of infantile spasms demonstrated microencephaly on long-term follow-up MRI scans.

There are a large number of etiologies associated with infantile spasms. Nearly 80% of cases of infantile spasms are symptomatic (genetic or structural etiology) and 20% are cryptogenic (no known etiology). There are over 200 identified symptomatic etiologies associated with infantile spasms, including at least 100 genetic conditions, such as Down syndrome, TSC, other gene mutations, and abnormalities of biochemical pathways such as pyridoxine deficiency, amino acid and, neuropeptide abnormalities. Other symptomatic etiologies include cortical and subcortical insults or injuries, such as from trauma, intraventricular hemorrhage, infections, toxins, hypoxia/ischemia, neoplasms, and malformations of cortical development (such as lissencephaly and cortical dysplasia). The most common etiology is hypoxic–ischemic encephalopathy, which is found in 25% of all cases of infantile spasms. Hence, an interesting clinical aspect of infantile spasms is that the time of the provoking etiology can be associated with a range of developmental periods, from intrauterine, prenatal, perinatal to immediately postnatal time points. Despite the temporal variability of the apparent precipitating cause, infantile spasms occur mostly in the first year of life. In addition, even though there are no consistent neuropathologic features found in all patients with infantile spasms, the seizures consists of such a well recognized clinical disorder that it is presumed that there must be a common developmental mechanism or pathway that produces the seizures and the associated encephalopathy.

The Ideal Animal Model of Infantile Spasms

Despite being a fairly rare clinical condition, developing animal models of infantile spasms and West syndrome has been identified as an important goal for research, since current therapies for this disorder are inadequate and prognosis is poor. This priority was established by a panel of experts invited to a workshop on Models of Pediatric Epilepsies sponsored by NINDS (National

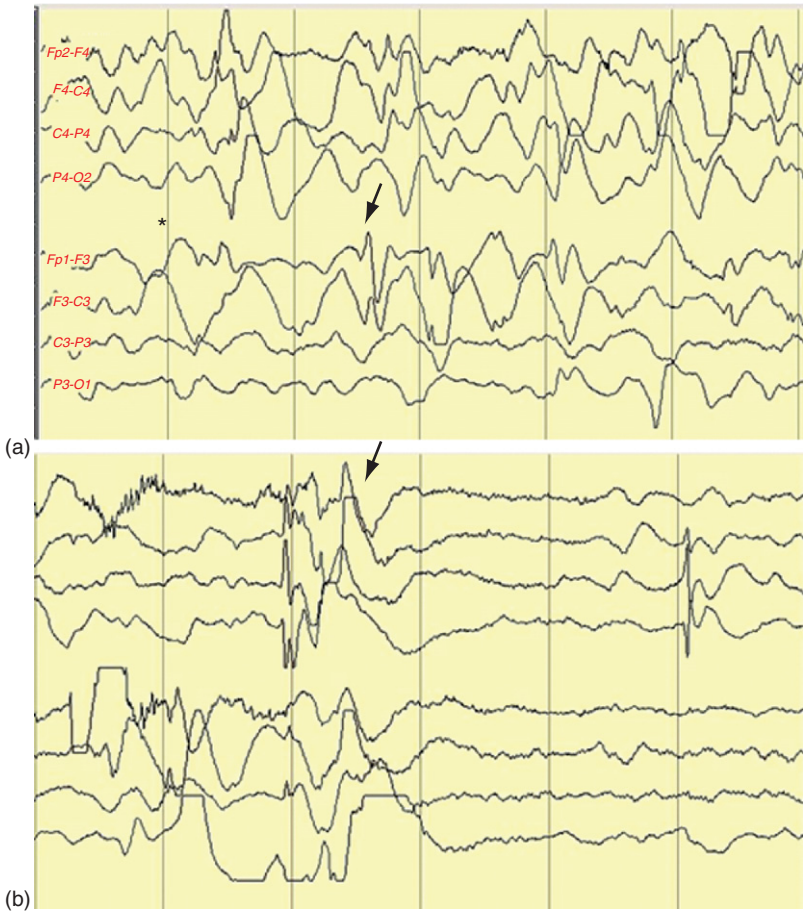


Figure 1 EEG examples of interictal hypsarrhythmia (a) and ictal electrodecremental response (b) in a child with behavioral infant spasms. (a) Interictal EEG shows periods of chaotic high-voltage slowing (asterisk) intermixed with high voltage spike discharges (arrow). (b) Ictal electrodecremental EEG response (arrow) associated with a motor spasm. Notice the lower voltage activity after the spasm. Montage for (a) is the same for (b). Low-frequency filter at 1 Hz, high-frequency filter at 70 Hz, sensitivity is 15 $\mu V\,mm^{-1}$, and each vertical line represents 1 s.

Table 1 Criteria for an animal model of infantile spasms

1. Unprovoked seizures with behavioral characteristics of spasms or myoclonic seizures that occur early in postnatal brain development
2. Ictal EEG correlates of seizure events that consist of electrodecremental response
3. Interictal EEG consisting of hypsarrhythmia or similar pattern
4. Model should respond to ACTH and/or vigabatrin
5. There should be evidence that untreated animals show signs of cognitive and behavioral sequelae from the seizures
6. Initial spasms seizures should eventually transition into generalized motor seizures
7. Not every animal with an etiology should develop characteristics of infantile spasms
8. There should be spontaneous remission of infantile spasm-like events with developmental maturation
9. Animals with infantile spasm-like events should demonstrate some mortality secondary to the seizures

Note: Criteria 1–5 were from the NIH workshop. Criteria 6–9 were added as secondary criteria.

Institutes of Health), the American Epilepsy Society, and the International League Against Epilepsy in May 2004. The goal of developing animal models of infantile spasms was to provide an experimental resource that could be used to develop novel treatments and understand basic mechanisms that produce spasms in the developing brain. The experts at the workshop also developed criteria for an ideal model of infantile spasms and West syndrome (Table 1; points 1–5). Additional secondary criteria are included in this table, based on the clinical features of children with infantile spasms. We will use these criteria to assess currently available animal models of infantile spasms.

Concepts of Pathogenesis

Over the past 40 years, several hypotheses have been proposed to explain the unique clinical characteristics of

infantile spasms, including the age-dependent onset, motor spasms, and a diffuse generalized EEG pattern. While none of these hypotheses have been proven or universally accepted, it is relevant to understand these ideas as they relate to development of different animal models proposed to reproduce infantile spasms.

Brainstem dysfunction

Based on the typical absence of focal clinical signs, the symmetric motor seizures, and diffuse EEG findings, a brainstem origin of infantile spasms has long been suspected. Autopsy studies have found brainstem pathology in individuals with a history of infantile spasms, clinical spasms have been noted in cases of hydranencephaly (no cerebral cortex), and infants with spasms have been found to have abnormal sleep patterns – all consistent with potential brainstem dysfunction. A common theme for several proposals is the notion that there are abnormalities in brainstem excitatory and inhibitory projection systems. One early hypothesis proposed that there was decreased output from excitatory cholinergic fibers arising from neurons of the pontine region, compared with inhibitory noradrenergic and serotonergic output from neurons of the locus ceruleus and dorsal raphe. In this scenario, the clinical spasms result from interference of the descending motor pathways that controls spinal cord reflex activity. Separately, the ascending tracts from the pons projecting to the cerebral cortex would produce EEG hypsarrhythmia. No animal models have been successfully developed based on this idea, however. Furthermore, agents that regulate serotonin or norepinephrine have been ineffective in clinical trials of children with infantile spasms.

Another hypothesis suggests that there are abnormalities of the serotonin–kynurenine pathways. In this scenario, there is an unknown mechanism that causes a metabolic shift in tryptophan metabolism that produces kynurenine or quinolinic acid, rather than serotonin. Quinolinic acid is a neurotoxin that can activate NMDA receptors, which in turn could produce seizures and infantile spasms by exciting neurons in the brainstem and limbic structures. The idea that dysfunction of brainstem neurotransmitter systems, especially those involving serotonin pathways, might be the common pathway in the generation of infantile spasms is an interesting concept. However, despite nearly 40 years of effort, this hypothesis is yet to be validated in clinical and basic science experiments.

Abnormal cortical–subcortical interactions

Many cases of infantile spasms involve patients with known cortical lesions identified by neuroimaging (MRI and FDG–PET), without evidence of brainstem dysfunction. Surgical removal of the cortical lesion stops infantile spasms in these patients, supporting the concept that there was a cortical origin to the seizures. These findings have led to the hypothesis that abnormal cortical dysfunction could be reflected at an early developmental age by subcortical structures that are still maturing. In other words, the primary problem is in the cerebral cortex (rather than the brainstem or other subcortical structures) that at young maturational stages projects abnormal activity onto the brainstem, basal ganglia, and other subcortical structures (including the serotonergic raphe nuclei), that then triggers the clinical spasms. Raphe–striatal pathway activation is suggested to produce the hypsarrhythmic EEG pattern over the cerebral cortex, and the downstream brainstem pathways have been postulated to generate the motor spasms (similar to the brainstem hypothesis). Support for this idea has emerged mainly from FDG–PET studies in children with active infantile spasm that show hypometabolism in regions of cortical dysplasia and hypermetabolism in the lenticular nuclei. Again an interesting hypothesis, this mechanism would only explain infantile spasms in those infants with known cortical lesions, and ignores other etiologies that might affect the brainstem or multiple brain systems (e.g., from genetic defects). This idea would also not explain infantile spasms in those with cryptogenic etiologies.

Hypothalamic-pituitary-adrenal axis dysfunction

As children with infantile spasms often respond fairly rapidly and robustly to treatment with ACTH and corticosteroids (spasms often completely stop within days), the hypothalamic-pituitary-adrenal axis has been suggested to play a role in the pathogenesis of this disorder. Activation of components of the brain stress response is hypothesized to promote neuronal hyperexcitability and provoke hypsarrhythmia and motor spasms. This idea has been supported by clinical studies documenting reduced CSF levels of ACTH and cortisol in children with infantile spasms. One group has hypothesized that the basic abnormality is excess intrinsic brain production of corticotrophin-releasing hormone (CRH), during stress events in utero or early postnatal life. ACTH treatment is proposed to downregulate CRH synthesis through activation of melanocortin receptors. CRH is known to be released from hippocampal, amygdala, and brainstem neurons, and exerts neuronal hyperexcitability partly by reducing after-hyperpolarizations (acting via a G-protein-coupled receptor). Thus, investigators have hypothesized that ACTH stops infantile spasms not by acting as an anticonvulsant, but by reducing expression and secretion of endogenous CRH in limbic structures and the brainstem. Animal models have been developed based on this idea that will be discussed later (see CRH).

Interneuronopathy

Another recent concept has emerged, based on findings of patients with gene defects that alter development of GABAergic interneurons. Gene defects in aristales related homeobox (ARX) and cyclin-dependent kinase-like 5 (CDKL5) have been found to be associated with

cryptogenic infantile spasms in a small group of patients with West syndrome. ARX is expressed at high levels in the neocortical ventricular and germinal zone of the ganglionic eminence, where these proteins are involved in the development and migration of GABAergic interneurons. Mice deficient in ARX have abnormal differentiation and migration of interneurons into the cerebral cortex and can have seizures – although it is not clear if those seizures are similar to infantile spasms. The precise function of CDKL5 is currently unknown, but a high percentage of patients with this mutation develop infantile spasms. Thus, in the first year of life, disturbances of GABA-mediated neurotransmission, due to a reduced number of GABAergic interneurons or alterations in GABA receptors, may be another mechanism that produces infantile spasms. However, animal models with these gene defects have yet to be validated to show behavioral and EEG signs of infantile spasm-like events.

Immune system dysfunction

Several investigators have proposed that defects of the immune system, with inflammation of brain structures, can result in infantile spasms. This idea has been supported by clinical studies showing antibodies to brain tissue in the sera, increased numbers of activated B and T cells, and abnormal human leukocyte antigen in patients with infantile spasms. While such findings indicate that the immune system is altered in some children with infantile spasms, these findings could be the result of ongoing seizure activity and brain damage, and not be the cause of spasms. Also, this concept does not explain the unique age-dependent characteristics of infantile spasms, the EEG findings, and the responsiveness to ACTH. Additional experimental and clinical studies showing a casual relationship between brain inflammation and infantile spasms are needed to further validate this hypothesis.

Developmental desynchronization

A relatively new hypothesis has been proposed that attempts to integrate the many diverse findings and ideas regarding cortical and brainstem dysfunction associated with age-dependent infantile spasms. The general idea is that infantile spasms are a developmentally unique manifestation, not of lesions in particular brain locations or defects in specific biochemical pathways, but of a temporal disturbance and desynchronization of one or more developmental brain processes that are active in the first year of life. In other words, the common denominator is disturbed in early developmental brain maturation – which could be caused by multiple factors. This conceptual approach would help to explain why infantile spasms are associated with so many different anatomical and biochemical abnormalities.

The hypothesis goes on to postulate that the main abnormalities are those that affect genes and pathways governing ontogenesis and transcriptional factors, or those resulting from direct environmental injuries or malformation of cortical development (including tumors) in the first year of life. According to this hypothesis, at least one developmental brain process lags behind others, resulting in abnormal integrated brain functions (desynchronization). If the imbalance among brain functions is sufficiently severe, then infantile spasms would occur. This concept helps explain the fact that in individuals with similar etiologies, only a sub-group of children develop infantile spasms; that no single pathologic factor has been associated with spasms; and that the response to treatment is often inconsistent and unpredictable. If this hypothesis is correct, the basic pathophysiologic substrate for infantile spasms would involve brain systems/processes that are developmentally active and regulated during the first year of life, such as synaptogenesis, myelination, glutamate, and GABA-mediated neurotransmission, development of serotonin, and acetylcholine pathways, and disorders of neuronal migration and differentiation.

This hypothesis is particularly attractive in that it may unite other ideas into a final common concept to understand the pathogenesis of infantile spasms. However, it should be clear from the earlier discussion that no single hypothesis is currently universally accepted to explain all aspects of the clinical features of infantile spasms and their EEG correlates. Therefore, it is anticipated that other ideas and concepts will likely be proposed to explain the pathogenesis of infantile spasms.

Methods and Results

Animal Models of Infantile Spasms

This section will describe the animal models currently purported to reproduce infantile spasms. We will compare and contrast the animal models with the ideal criteria shown in **Table 1**. Our purpose is to illustrate the variability of mechanisms that produce spasm-like events, and to consider how well these models fit with the human epilepsy syndrome. The models are presented in chronological order of publication.

Corticotrophin-releasing hormone

Model description

Baram and colleagues developed this model which was first published in 1991. This model helped develop the hypothalamic-pituitary-adrenal hypothesis of the pathogenesis of infantile spasms (see above). Small amounts of CRH are injected into the ventricle of 5–10-day-old rats. Within minutes, this produces acute behavioral seizures consisting of chewing, tonic–clonic activity of the limbs, and 'swimming' seizures lasting several hours. EEG correlates of the seizures are inconsistent, and when present consist of increased beta activity and semirhythmic sharp

waves (not hypsarrhythmia). The rat brains show long-term signs of hippocampal and limbic neuronal injury and synaptic reorganization. Treatment with ACTH did not alter the induction of acute seizures from CRH. Adult animals that received CRH as infants have not been reported to have chronic seizures, but do display some problems in memory tasks.

Pros and cons

This was the first model purported to test a mechanism that produces infantile spasms in humans, examining abnormal stress during early brain development. There is a developmental element to the model, inasmuch as the dose of CRH necessary to induce seizures in immature animals is less than that required in adult rats, and cause more severe seizures.

There are a number of problems with this model when compared against the ideal criteria for an animal model of infantile spasms (Table 2). The seizures require an agent (chemical convulsant) to induce the events, and are thus provoked (not spontaneous). The resulting ictal behaviors and ictal EEG are not typical of infantile spasms, but show more limbic brain stimulation and the interictal EEG does not show hypsarrhythmia. The seizures do not respond to ACTH and it does not appear that the acute seizures lead to chronic ones. It is also not clear what percent of animals given CRH have acute seizures. Thus, while CRH tests one hypothesis of the pathogenesis of infantile spasms, the resulting model lacks many of the clinical characteristics associated with human spasms and West syndrome.

NMDA/steroids

Model description

This model was developed in two stages by Velisek and colleagues. The model tests two components of pathogenic mechanisms involving disturbance of brainstem, and cortical excitation and inhibition, with prenatal hormonal stress. The first model, published in 1999, involved intraperitoneal injections of *N*-methyl-D-aspartate (NMDA) in 12–18-day-old rat pups. Behavioral seizures acutely developed consisting of hyperflexion emprosthotinic-like events that are prolonged (i.e., not brief) and occurred in clusters. The seizures were accompanied by electrodecremental cortical EEG responses. Interictal EEG consisted of large chaotic waves resembling hypsarrhythmia. Adult rats had impaired cognition after NMDA-induced flexion seizures as infants. However, ACTH and glucocorticoids were ineffective as acute treatments with NMDA injections in infant pups. In 2007, the authors published a revised version of their model, in which pregnant rats were given betamethasone (a steroid) as a form of prenatal developmental stress. The pups from these dams were then injected with NMDA on day 15 of life. Prenatal glucocorticoid exposure increased the sensitivity to NMDA-induced flexion seizures, and treatment with ACTH delayed the onset of the provoked NMDA-induced seizures. The authors concluded that ACTH responsiveness further validated NMDA injection as an animal model of infantile spasms.

Pros and cons

This animal model has more features of human infantile spasms (Table 2). The flexion spasms have similarities with motor spasms in human infants, and the acute interictal and ictal EEG patterns are like those expected in babies with infantile spasms. Also, there appears to be long-term cognitive sequelae in adult rats when they experience NMDA-induced seizures at P15. However, the model also has limitations and has not been completely validated against the ideal criteria for infantile spasms.

Table 2 Comparison of current animal models of infantile spasms

Model	*Seizure behavior*	*EEG*	*Response to ACTH*	*Cognitive sequela*	*Chronic seizures*	*Spontaneous remission*
CRH	No spasms. Acute limbic and tonic seizures. P 5–10	Inconsistent No hypsarrhythmia	No acute response	Yes; memory	None reported	N/A
NMDA/ Steroids	Flexion spasms (emprosthotonus) lasting several seconds P 12–18	Hypsarrhythmia-like Ictal electro-decrement	Partial response	Yes	Unknown	Unknown
TTX	Brief extension and flexion spasms P 20–40	Hypsarrhythmia-like Ictal electro-decrement	Unknown	Unknown	Unknown	Unknown

N/A: Not applicable.

The acute seizures are provoked, and the spasm-like events are more prolonged than the typical motor spasm seen in infants. Also, it is not clear if the animals show acute and chronic damage to the cortex, limbic system, or brainstem, and how that pathology reflects what we know in human patients with infantile spasms. Finally, we do not know if there are long-term intractable seizures in adult rats exposed to NMDA as pups. Thus, further studies are necessary for this animal model of infantile spasms.

Tetrodotoxin

Model description

This model was developed by Swann, Frost, and Hrachovy, and was first published in 2007. The hypothesis of developmental desynchronization is based in part on this model. The model consists of a chronic infusion of the sodium channel blocker tetrodotoxin (TTX) into the hippocampus or neocortex of 10–12-day-old rat pups. Within two weeks, unprovoked behavioral seizures begin, consisting of brief 1–2 s extensor or flexor spasms of the head and upper trunk, and many of the spasms were noted to occur in animals awakening from sleep. Similar to humans, infantile spasm-like seizures developed in about 30% of the animals treated with TTX. Ictal EEG showed voltage attenuation and electrodecrement with superimposed fast activity. Interictal EEG of most of those rats that also had behavioral spasms showed a high-voltage chaotic background resembling hypsarrhythmia. As this model was only recently developed, we do not know if the spasms respond to ACTH, if there are long-term chronic seizures, if there are subsequent cognitive deficits in adult rats that had seizures as pups, or if the spasms show spontaneous remission.

Pros and cons

Of all the animal models developed to date, this one has more of the features fulfilling the ideal criteria for a model of infantile spasms than the others. Unlike the prior two models, the seizures are unprovoked, and the events have behavioral and EEG features that are reasonably similar to human infantile spasms. However, this model also has its limitations. The onset of the unprovoked seizures, 2 weeks after TTX infusion, occurs when the rats are at a minimum of 20 days of age or older. P20 rats are more adolescent-like than infant-like, and thus considerably older than what would be expected if the model was to reflect infant-onset spasms. However, because it is so new, there are as yet few studies to attempt to validate this model – for example, to determine if the spontaneous spasm-like seizures respond to ACTH, if the animals have acute and chronic cortical or brainstem pathology, if there are long-term cognitive deficits, if these animals go on to have spontaneous seizures as adults or if there is spontaneous remission of the seizures. Thus, more studies are necessary before this model is fully characterized.

Summary and Future Directions/ Challenges

As discussed earlier, there is no single unifying hypothesis that explains the pathogenesis of infantile spasms in humans – and probably as a consequence, there is no single animal model that fulfills all the ideal criteria of this condition (**Tables 1** and **2**). Animal models have been developed based only on a few of the proposed hypotheses to explain the pathogenesis of infantile spasms. We lack animal models that effectively test brainstem abnormalities of excitatory and inhibitory axon systems, dysfunction of interneurons, or inflammation as possible causes of infantile spasms. Thus, there is considerable need to develop more and better animal models of infantile spasms based on existing pathogenic concepts. For infantile spasm research, having more than one model may be necessary. As there are multiple known etiologies, having several models that reproduce behavioral motor spasms and the EEG findings would be helpful in exploring common mechanisms, and to develop new treatments. Furthermore, we should not assume that all reasonable hypotheses regarding pathogenesis of infantile spasms have been developed; fresh ideas and concepts are certainly needed.

Any theory regarding the pathogenesis of infantile spasms has to account for the unique developmental features of this seizure disorder. Spasms should occur spontaneously during infancy with multiple genetic and acquired etiologies. The seizures should have characteristic behavioral motor spasms and EEG correlates of hypsarrhythmia. The events should be associated with severe long-term cognitive deficits, and the seizures should respond in some animals to treatment not by antiepilepsy drugs but by a hormone, ACTH. If possible, the model should also display some of the secondary features of infantile spasms, such as the variable response to treatment, and high mortality associated with the seizures. Hence, this field of study is far from complete and much work still remains.

Future studies will likely develop and validate existing and newer models of infantile spasms and West syndrome based both on old and new ideas of pathogenesis. This work is important because current treatments are largely ineffective and most children with infantile spasms have long-term risks of severe developmental disabilities and seizures. The key goal in developing animal models of infantile spasms is to discover underlying mechanisms so that more effective treatments can be designed to stop this

highly disabling seizure disorder. Children with infantile spasms deserve the opportunity to grow up with as near normal cognitive development as possible.

Acknowledgements

This work was supported by National Institutes of Health R01 NS38992 and P05 NS02808 to GWM.

See also: **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood.

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Cellular, Molecular and Behavioral Consequences of Early-Life Seizures

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Introduction

Rationale for Studying the Consequences of Early-Life Seizures

There is a predilection for children to develop seizures at an early age. The incidence of epilepsy peaks in the first year of life. Various etiologies cause seizures during the neonatal and early childhood periods. The majority of seizures in this age group are symptomatic of a remote or recent insult. Young children are at higher risk than adults to develop seizures after various insults such as strokes, demyelination, or trauma, reasons for which are not yet fully elucidated (but are likely related, at least in part, to developmental differences in synaptic connectivity and neurotransmitter receptor expression). Of the remaining idiopathic cases, many will fit into an epilepsy syndrome (a constellation of clinical seizure type, developmental stage, EEG findings, and outcomes). Some of these seizure syndromes are benign (i.e., the child's development remains normal in the face of recurrent seizures) but many of these

highly disabling seizure disorder. Children with infantile spasms deserve the opportunity to grow up with as near normal cognitive development as possible.

Acknowledgements

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See also: **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood.

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Cellular, Molecular and Behavioral Consequences of Early-Life Seizures

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Introduction

Rationale for Studying the Consequences of Early-Life Seizures

There is a predilection for children to develop seizures at an early age. The incidence of epilepsy peaks in the first year of life. Various etiologies cause seizures during the neonatal and early childhood periods. The majority of seizures in this age group are symptomatic of a remote or recent insult. Young children are at higher risk than adults to develop seizures after various insults such as strokes, demyelination, or trauma, reasons for which are not yet fully elucidated (but are likely related, at least in part, to developmental differences in synaptic connectivity and neurotransmitter receptor expression). Of the remaining idiopathic cases, many will fit into an epilepsy syndrome (a constellation of clinical seizure type, developmental stage, EEG findings, and outcomes). Some of these seizure syndromes are benign (i.e., the child's development remains normal in the face of recurrent seizures) but many of these

syndromes are malignant (i.e., the development of the child either slows or regresses). How seizures in any of these settings affect the brain acutely and/or later on as development proceeds is an important clinical question. Indeed, understanding why seizures in the benign syndromes do not cause later developmental problems, but seizures in the more malignant syndromes cause irreparable damage, would help neurologist understand who and what to treat and how to direct future therapies.

The questions of how seizures affect the brain can be asked on many levels.

From the subcellular: How do seizures alter the molecular machinery of neurons? Do seizures alter transcription profiles or neurotransmitter receptors? Do seizures alter the cell cycle or neurogenesis?

To the cellular: How do early-life seizures, whether symptomatic, benign, or malignant, affect the histology and regional anatomy?

And finally to the entire organism: How do seizures alter the development of the brain? Does motor, sensory, and spatial learning change?

The answers to these questions could direct scientist to develop new therapies targeted at the changes that occur after seizures in order to prevent detrimental consequences.

Unfortunately, attempting to answer any of these questions in patients is very difficult. There are many factors that could affect the way seizures alter the developing brain. First and foremost, all children with seizures are treated with the currently available antiepileptic drugs. These drugs may have an independent impact on the brain, or at least interact with how the seizures affect the brain. Second, the child's environmental exposure and/or genetic background can have a profound effect on any developmental changes or how seizures could interact with the developing brain. Third, the etiology of the seizures, which is often not known, most likely affects the impact a seizure can have on early brain maturation. Therefore, neuroscientists, epileptologists, and neurologists have been asking the above questions using various developmental animal models of epilepsy with the hope of developing novel therapeutics to protect the developing brain and to more effectively prevent recurrent seizures.

Background

Various animal models have been developed to study the cellular, molecular, and behavioral consequences of early-life seizures. All the methods used to study early-life seizures produce either prolonged or repetitive seizures by using a chemoconvulsant (lithium pilocarpine, kainate, flurothyl), hyperthermia, or electrical kindling. Details of these models are presented later. Not surprisingly, many studies, particularly those of Greg Holmes and colleagues in the 1980s and 1990s, have found significant differences in the effect that seizures produce in the adult vs. the developing brain. Early studies suggested that very young rats or mice (postnatal day 15 or less), although easily induced to develop prolonged seizures (status epilepticus; SE), did not go on to later develop spontaneous seizures. These early studies also found few or no morphological or histological changes after early-life SE. Some of the studies from Holmes and Stafstrom did show, however, behavioral change following the SE – but without the animals developing spontaneous seizures or having a lowered seizure threshold.

More recent studies from multiple labs suggest that the initial reports underestimated the changes induced by SE. Many authors have confirmed that compared to the adult rodent there is minimal cell loss after prolonged or recurrent seizures in the immature brain, but changes in mossy fiber sprouting, receptor expression (both excitatory and inhibitory), and neurogenesis have all now been documented after early-life SE; the degree and nature of changes seen in the immature animal may be quite different from what occurs in the adult (as detailed later).

Methods

Overview of Models and Techniques

There are many models used to study how seizures can affect the developing brain. Indeed, almost all models currently implemented in adult rodents have been attempted in developing animals. Briefly, these models include using chemoconvulsants, electrical activation of the brain, trauma, and ischemia. Various chemoconvulsants have been studied, including lithium pilocarpine, kainic acid, pentylenetetrazol, tetanus toxin, flurothyl, and penicillin. Lithium pilocarpine and kainic acid are the two most frequently used chemoconvulsants. Both methods are used in developing animals in a similar manner as in adults, although the dose required to produce SE is lower in young animals. The compounds are typically injected via an intraperitoneal route, but kainate can also be given intraventricularly or intravenously. Both drugs cause the initiation of SE if given in sufficient doses, and both are believed to act by activating limbic structures, therefore modeling temporal lobe epilepsy. The electrical methods of inducing seizures include hippocampal kindling and perforant path stimulation. In hippocampal kindling, electrodes are placed in the hippocampus (usually CA1 region) and brief rapid low current electrical pulses are given for a few seconds. These stimuli induce after-discharges, which with repeated stimulation become longer, higher voltage, and eventually self-sustaining electrical seizures. If sufficient stimuli are given, then spontaneous recurrent seizures begin to occur. Perforant path stimulation is applied using a similar protocol, except that the perforant path is stimulated to induce electrical discharges.

Other methods used to generate spontaneous seizures in immature animals include hyperthermia (febrile seizure model), trauma, and hypoxia–ischemia. The febrile seizure model, as employed most often by the laboratory of Baram, uses hot air to increase the body temperature of a very young rat until the rat begins to have seizures; the seizures persist as long as the temperature is maintained. A substantial fraction of these animals (35%) develop spontaneous recurrent seizures weeks to months after the febrile SE, making it of particular interest to the pediatric epilepsy research community. All of the non-chemoconvulsant early-life seizure models are extremely important as they reproduce acute symptomatic etiologies that are frequent causes of seizures in human neonates and infants. Many of these models will be discussed in detail elsewhere in this volume.

The methods used to induce seizures in the developing brain are typically administered between postnatal day (P) 7 and 20 in rats and mice, a time period that is thought to roughly parallel a term human neonate (P7) through mid-childhood. After the administration of drugs or hot air or other insults, the animals are monitored, either with video or both video and EEG, to document the duration and severity of the status. The animals are often then monitored again, after the latent period to determine the latency and severity of the spontaneous seizures if they occur. Then either during or after status, or after the onset of spontaneous seizures, the animals are tested for cellular, molecular, and behavioral consequences of the induced seizures. Many different methodologies have been used to test for the impact of the seizures. For example, to investigate the cellular and molecular consequences of seizures on learning, changes in long-term potentiation (LTP) has been examined in hippocampal slices from controls and in animals that experienced seizures. Learning capabilities after seizures have also been examined using a variety of behavioral tests (e.g., the Morris water maze or elevated T-maze). To study seizure-induced molecular changes, all the currently available molecular, biochemical, and genetic methods have been employed. For example, RT-PCR and reverse Northern blots have been employed to look at receptor changes in particular brain regions and cell types. Finally, to investigate cellular changes, many laboratories have used various histological procedures, including the Timm stain to look for mossy fiber sprouting. In addition, immunohistochemical and electrophysiological techniques are used to test for the cellular alterations induced by seizures.

Recent Results

Seizures and the Developing Brain

Researchers from a number of laboratories have made significant advances in detailing the impact of early-life seizures on development. Although early reports from the laboratory of Holmes and colleagues suggested a preservation of cognitive development after early seizures, more recent studies have uncovered age-specific behavioral and molecular effects. Impaired learning and memory in rodents has now been demonstrated after early-life seizures induced by a variety of methods, including tetanus toxin, corticotropin-releasing hormone (CRH), lithium pilocarpine, kainate, and flurothyl. These results suggest a consistent, model-independent impact of seizures on the developing brain. Potential mechanisms of these behavioral effects include deficits found in hippocampal LTP and 'place cells' in animals that experience early-life seizures. These mechanisms are being actively investigated.

Unlike prolonged seizures in adult animals, which produce significant hippocampal CA1 and CA3 cell loss and dentate granule cell mossy fiber sprouting, most models of early-life seizures, both prolonged and repetitive, do not demonstrate significant cell loss or mossy fiber sprouting. Some models, however, do show some degree of cell loss or mossy fiber sprouting. For example, both CRH-induced and recurrent flurothyl seizures in the first week of life result in moderate cellular and synaptic changes in the hippocampal CA3 region. Damage outside the hippocampus, in various thalamic nuclei (as reported by Druga *et al.*), has also been reported in the lithium-pilocarpine model as early at P10. Taken together, these studies suggest that although cell death and synaptic reorganization can be seen after some types of early-life seizures, it is likely not necessary for early-life seizure-induced memory and learning deficits, as many models show none of these changes yet still demonstrate learning and memory disruption.

If structural changes are not the cause of learning and memory deficits or of recurrent seizures, then what is? One possibility is the impact of early-life seizures on dentate granule cell neurogenesis. Dentate neurogenesis is hypothesized to be important for the formation and stabilization of memories. Any alteration by seizures of this process could potentially result in cognitive changes. In adult animals, seizures have been shown to both induce neurogenesis in the dentate gyrus acutely, but inhibit neurogenesis chronically. In the immature animal, our laboratory and others have shown an increase in cell birth following seizures at postnatal days 14–21; this increase is also accompanied by an increase in cell death of both mature and immature granular neurons. One report by McCabe *et al.* details a reduction in neurogenesis after repetitive flurothyl seizures at postnatal days 1–5. These data in immature animals, along with much of the adult rat literature, suggest that seizures could alter dentate neurogenesis and impact on the process of epileptogensis as well as on learning and memory.

The effects of early-life seizures on neurotransmitter systems and intrinsic neuronal properties are another potential cause of seizure-induced cellular, molecular, and

behavioral changes. Inhibitory and excitatory neurotransmitter receptors and voltage-gated ion-channels all have activity-dependent developmental programs that could be altered by seizures during development. Changes in inhibitory neurotransmission have been studied by several laboratories because of the known association between GABAergic function and memory. Enhancing $GABA_A$ receptor-mediated inhibition with benzodiazepines can disrupt LTP, memory formation, and cortical plasticity. All these cognitive processes could be impaired after early-life seizures if inhibition were enhanced. Increased inhibition after postnatal seizures has been shown on a circuit level after both hyperthermic- and kainate-induced seizures by the Sutula laboratory. Molecular changes reported by our laboratory after early-life lithium-pilocarpine-induced seizures includes increased expression of the $GABA_A$ receptor $\alpha 1$ subunit in dentate gyrus, both immediately after early-life seizures and when the animals reach adulthood. The increase in $\alpha 1$ subunit after seizures was shown to be functionally important and associated with enhanced type I benzodiazepine augmentation of the receptor. The early-life increase of $\alpha 1$ subunit expression following pilocarpine-induced seizures is opposite to the decrease in $\alpha 1$ subunit expression seen after seizures in adult rats. Seizures in young animals also affect transcription of some neurotransmitter receptors differentially between mature and immature dentate granule neurons. Clearly, seizures during development can alter expression of $GABA_A$ receptor subunits in an age-dependent manner, with the $GABA_A$ receptor expression changes after early-life seizures contributing to enhanced inhibition that could lead to cognitive and memory deficits.

Excitatory neurotransmission is also impacted by early-life seizures. Changes in expression of glutamate receptors, the primary excitatory neurotransmitter in brain, may contribute to abnormalities in learning and behavior after early-life seizures. Excitatory signaling through both the AMPA and NMDA receptors are critical for different types of LTP and hippocampal learning. Many of the models described above have reported deficits in AMPA- and NMDA-receptor-mediated excitatory synaptic signaling. In particular, lowered expression levels of the GluR2 subunit was reported by our laboratory in the lithium-pilocarpine model and by Jensen and colleagues in the postnatal hypoxia model. The Swann laboratory has used a postnatal tetanus-toxin model to show a decrease in hippocampal CA3 dendritic spine density and a decrease in hippocampal NR1, NR2A, and NR2B receptor subunits. Recent work (e.g., Cornejo and colleagues) has also shown NR subunit decreases after single seizures induced by kainic acid.

Baram and colleagues, working on the febrile seizure model, reported an impact of early-life seizures on voltage-gated ion-channels – a long-lasting enhancement of the intrinsic hyperpolarization-activated membrane current, I_h. The seizures were shown to decrease hippocampal CA1 neuronal hyperpolarization-activated, cyclic nucleotide-gated channel-1 (HCN1) mRNA with simultaneous increase of HCN2 mRNA expression. Persistent limbic hyperexcitability and a 35% incidence of later spontaneous seizures is hypothesized to be due changes in the I_h current. These data suggest that early-life seizures may permanently affect inhibitory and excitatory neurotransmission as well as intrinsic neuronal properties in the developing and adult brains.

Changes in neuromodulatory pathways have also been reported after early-life seizures. One neuromodulator believed to be critically important for learning and memory is cyclic AMP, the action of which is mediated, in part, by the cAMP response element binding protein (CREB). CREB is a key mediator of changes in gene expression necessary for nervous system plasticity. Chronic decreases in learning-specific CREB phosphorylation has been reported after recurrent febrile seizures. Another neuromodulator that is affected by early-life seizures is corticotropin-releasing hormone (CRH). Hippocampal CRH mRNA expression is chronically elevated into adulthood following early-life seizures, and excess corticotropin-releasing factor and early-life stress have been shown to affect both dendritic arborization patterns and long-term cognitive function.

The earlier-mentioned studies highlight the advances made in understanding how early-life seizures may impact the developing brain. One interesting addition to the earlier-mentioned studies is how these behavioral and cellular changes are altered by changes in the animals' environment. In the Holmes laboratory, timed periods of environmental enrichment given to rats exposed to early-life seizures (lithium pilocarpine and kainic acid) resulted in significantly better performance than that seen in normally-housed rats, on multiple learning tests. This difference in behavioral and learning performance was independent of seizures, as all rats developed seizures and similar hippocampal damage. These studies highlight that seizures are not the only factor that determine the course of development for a child with epilepsy. Aspects of environmental exposure may contribute to the impact of seizures on cognitive outcome.

Future Directions

Significant progress has been made in our understanding of how seizures from different etiologies – genetic, traumatic, ischemic, exposure, and infectious – affect brain development and the resulting change in cognitive potential. More research is needed to fully elucidate the molecular, cellular, and circuit mechanisms that contribute to cognitive dysfunction after early-life seizures or possibly that protect against it. Once these mechanisms are identified, and it has been determined which are causal and

which compensatory, then they can be targeted pharmacologically to prevent or correct seizure-associated learning and memory deficits. As more studies are performed and newer technologies – e.g., protein arrays, conditional transgenic animals, and siRNA – are applied, the missing details can be added to our understanding. As this additional information is accumulated, the potential for new pharmacologic or genetic therapies that could help protect scores of children with epilepsy will become possible.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; Cognitive Effects of Seizures in Animal Models; Language and Communication Disorders; **Epileptogenesis**: Multi-Hit Mechanisms Involved in Epileptogenesis: Role of Early-Life Seizures; **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; Mechanisms of $GABA_A$ Receptor Gene Regulation in Epilepsy; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations; **Pediatric Epilepsy**: Seizure-Induced Injury and the Two-Hit Hypothesis.

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Cortical Dysplasia and Catastrophic Epilepsies of Childhood

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Introduction

As described by Shields, catastrophic epilepsies of childhood (CEC) are defined by seizure onset in the first few years of life, by seizures that are refractory to medical therapy, and by a strong association with mental retardation. In addition, CEC often results in frequent seizures, with some affected children experiencing up to 100 seizures per day. In addition to the direct, immediate impact of these severe seizures, there are also concerns about the effect of the seizures on

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subsequent brain development. Not surprisingly, several studies have shown that children who experience seizure control, through medical and/or surgical therapies, have much better ultimate clinical and cognitive outcomes compared with those who cannot be controlled. The two most common clinical syndromes found in CEC are West syndrome (infantile spasms with mental retardation; typical onset within first year of life) and Lennox–Gastaut syndrome (multiple seizure types, mental retardation, slow spike and wave on EEG; typical onset 1–3 years of age).

In the early 1980s, two important clinical developments altered our understanding of CEC. First, some epilepsy surgery centers began to perform resections on affected children. The surgical decision was based on a variety of diagnostic tests, including MRI, EEG, ECoG (EEG recorded from the surface of the brain), and PET. Subsequent analysis of the brain tissue removed during these surgeries provided a more comprehensive pathological picture of the brains in these children. Malformations of cortical development formed the pathological substrate for a significant number of these cases. The other major development was improved methods for detecting subtle cortical malformations using MRI. This enhanced sensitivity allowed for the diagnosis of these conditions prior to surgical resection. These clinical advances set off a minor explosion in the scientific study of malformations of cortical development, with investigators using both human tissue (from surgical resections) and animal models.

Malformations of cortical development (MCDs) comprise a large number of brain disorders that share the common feature of some perturbation of cortical development during the pre- or perinatal period. Clinical syndromes include lissencephaly, agyria/pachygyria, hemimegalencephaly, subcortical band heterotopia, periventricular nodular heterotopia, polymicrogyria, focal cortical dysplasia, cortical tubers of tuberous sclerosis, and acquired cortical dysplasia associated with perinatal stroke. Over recent years, some of these syndromes – including tuberous sclerosis, type I lissencephaly, X-linked lissencephaly, subcortical band heterotopia, and X-linked periventricular nodular heterotopia – have been found to be the results of single gene defects,. However, the etiology for the majority of malformations of cortical development remains unknown.

At the microscopic level, cortical dysplasia (CD) is characterized by loss of lamination, loss of spatial orientation, abnormalities of neuronal morphology, and abnormalities of cell commitment. Human focal cortical dysplasia has been classified by Palmini and colleagues into three types: Type I shows signs of abnormal lamination and spatial orientation only; Type IIA shows large, dysmorphic neurons in addition to the abnormalities seen in type I; and Type IIB shows all of the above plus 'balloon cells' – large, dysmorphic cells thought to be of glial origin that can, in some instances, stain for markers of both neuronal and glial lineage. A large number of studies have been performed directly on human CD tissue acquired from surgery. The results of these important studies are too numerous to cover in this article, but include abnormalities of excitatory and inhibitory neurotransmitter receptors, abnormalities in genes that regulate cell migration and growth, and loss of interneurons in regions of CD. One study of particular relevance to this article came from Calcagnotto and colleagues, who recorded from pyramidal cells in human type II CD. They found a significant reduction in the frequency of spontaneous and miniature inhibitory postsynaptic currents (sIPSCs and mIPSCs, respectively). As discussed below, this result is very similar to the key findings from CD in irradiated rats.

Animal models of CD fall into two broad categories, genetic and injury-based. Our laboratory focused on in utero irradiation because the histological features of this model are similar to many types of human CD. In this model, pregnant females are exposed to about 200 cGy of external radiation on E17. Other injury-based models use DNA-alkylating agents, methylazoxymethanol (MAM) or BCNU, administered to the pregnant dam. Many of the histological effects in the offspring are similar to those seen in utero irradiation, but physiological features differ across the various models. The perinatal freeze-lesioned rat serves as a model of focal CD and polymicrogyria. Genetic models are numerous and varied. Some are the products of spontaneous mutations (i.e., flathead rat and *tish* rat) while others are modeled after genes known to produce human disease (such as the knockout mouse for the *Lis1* gene, known to be involved in human lissencephaly type I). Other relevant genetic models include animals with alterations in genes known to be important in neuronal migration; that is, *reeler* and *p35*−/− mice. Particularly relevant to the current topic are animals with gene defects that alter migration and development of cortical interneurons; these models include the flathead rat, *Dlx1/2* mutant mice and *uPAR*−/− mice. Although a fascinating topic, a comparison of the various animal models of CD is outside the scope of this article.

Background

As mentioned above, the in utero irradiation model involves exposing a pregnant rat to 200 cGy of external radiation on E17. The offspring exhibits microcephaly, diffuse CD, heterotopic gray matter, heterotopic neurons in the hippocampus, and hypoplasia/agenesis of the corpus callosum. Najm and colleagues have shown that these animals demonstrate spontaneous seizures. However, their seizures are not as frequent or severe as those seen in some of the genetic mutations that may die from seizures early in life. The seizure disorder in these rats is also not as severe as many forms of CEC. At a microscopic level, the involved cortex shows reduced thickness

(i.e., pia to white matter) dyslamination, loss of spatial orientation of neurons, and subcortical heterotopic gray matter. Giant, dysmorphic neurons or balloon cells are not apparent. Therefore, using the Palmini classification system, the irradiated rat would be a model of type I human CD.

CD in the irradiated rat is the end result of two distinct but interactive processes: the initial cell death produced by the radiation and the subsequent continued attempt to build a cortex in a radically altered cellular environment. Radiation exposure occurs at a time (E17) of maximal generation of cortical neurons and neuronal migration. Within 24 h after exposure, extensive cell death is seen throughout the developing telencephalon, being most severe in the intermediate zone – which includes radial glia and migrating, immature neurons. Indeed, using an antibody for nestin to label radial glia, we have documented severe disruption of the radial glia after irradiation on E17. The progenitor cells in the ventricular zone, and the more mature cells in the cortical plate, are relatively spared. After the injury, cortical neurons continue to be born and migrate into the cortical plate. However, cell counting studies from our lab suggest that the neurons that arrive in the cortical plate after the injury are almost exclusively excitatory cells. That is, cortical interneurons either quit being born after the injury or their migratory pathways are so altered that they simply never reach the cortical plate. The fact that interneurons (generated in the ganglionic eminence) have a much longer distance to travel than pyramidal cells (generated in the ventricular zone of the telencephalon) in order to reach the cortical plate may explain why the cortical interneurons are more severely disturbed in this model of CD.

We have discovered a number of other abnormalities in CD from irradiated rats. Most of them reflect a severe disruption of the development of the cortical inhibitory interneurons and their incorporation into the circuitry of the neocortex. Early cell counting studies in adult animals showed a selective reduction in the density of parvalbumin- and calbindin-immunoreactive neurons (markers for two types of interneurons) in CD from irradiated animals. In subsequent physiological studies, we recorded excitatory and inhibitory postsynaptic currents (PSCs) from layer V pyramidal cells (using in vitro brain slices); we found a reduction in frequency of spontaneous inhibitory PSCs (sIPSCs) and miniature inhibitory PSC (mIPSCs), but no reduction in excitatory PSCs (EPSCs). This result, suggesting that there was impaired inhibition in dysplastic cortex at the presynaptic level, was consistent with our cell counting study. If there were fewer inhibitory interneurons, it is likely that there would be fewer inhibitory terminals on the neighboring pyramidal cells and thus a reduced frequency of IPSCs. We also recorded from pyramidal cells in subcortical heterotopic gray matter and obtained similar results.

Methods

Most of our findings have come from physiological study of neurons, using in vitro brain slices. We obtain 400 μm-thick coronal slices through the sensorimotor cortex. The slices are maintained in a submerged slice chamber, where they are perfused with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 26 $NaHCO_2$, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, and 10 D-glucose. Individual neurons are imaged in the living slice using infrared-differential interference contrast (IR-DIC) microscopy (Zeiss Axioskop 2 FS and a Nikon FN1 fixed stage microscopes). We obtain whole-cell recordings in both voltage-clamp and current-clamp configurations. The target cells vary depending on the goals of the particular experiment. Earlier studies from our laboratory focused on layer V pyramidal cells and 'comparable' large pyramidal cells from irradiated rat cortex, and inhibition was examined by studying mIPSCs, sIPSCs, and stimulus-evoked IPSCs in these cells. In later experiments, we recorded directly from inhibitory interneurons to examine membrane properties and excitatory connections in these cells.

In many of our studies, we have used standard analysis techniques – of membrane properties, kinetics of miniature and spontaneous EPSCs and IPSCs, and frequency and amplitude of PSCs – to examine function within the cortical circuit. Because many of our findings suggested a presynaptic impairment of inhibition, we also monitored short-term plasticity (STP) as a surrogate measure of release probability within a group of presynaptic terminals.

Histological and immunohistochemical techniques have been employed to study the dysplastic cortex in the irradiated rat model. Early studies used antibodies for parvalbumin and calbindin to identify two types of cortical interneurons in adult animals. For perinatal studies, we have used an antibody for GABA to identify immature interneurons (since the calcium binding proteins mentioned above are not expressed until later in development). For some cell counting studies, we have used the optical fractionator method of stereological analysis; this technique has been described in detail in many other sources.

Recent Results

We have recorded directly from inhibitory interneurons in layer IV/V of CD and control sensorimotor cortex, concentrating on two broad categories of interneurons that were present in both control and irradiated tissue. Type I interneurons corresponded to fast-spiking (FS) interneurons that have been well-characterized in many

other laboratories. They generate rapid, nonadapting action potentials when the cell is depolarized and show short-term depression of stimulus-evoked EPSCs. Many type I interneurons contain parvalbumin; the category includes basket cells that form dense axonal contacts onto the soma of neighboring pyramidal cells. Our type II cells generate slower action potentials, with frequency-adaptation when the cell is depolarized and short-term facilitation of stimulus-evoked EPSCs. This type II category consists of a mixed group of cells, including low-threshold spiking (LTS) interneurons and regular spiking nonpyramidal cells (RSNPs) that have been described by other investigators. Our findings were the same for both types of interneurons. In CD tissue, they all showed a reduction in frequency, but not amplitude, of mEPSCs and sEPSCs. STP did not differ between irradiated and controls for either type and Sholl analysis did not show a difference in dendritic arborization between the controls and irradiated samples. These findings suggest that there are fewer excitatory terminals onto the interneurons in dysplastic cortex in this model. This profile, in turn, raises the interesting possibility that these CD interneurons might be the 'dormant interneurons' that have been proposed to be the basis of hyperexcitability in other models of epilepsy. According to this scenario, the interneuron is present but does not mediate a sufficient level of

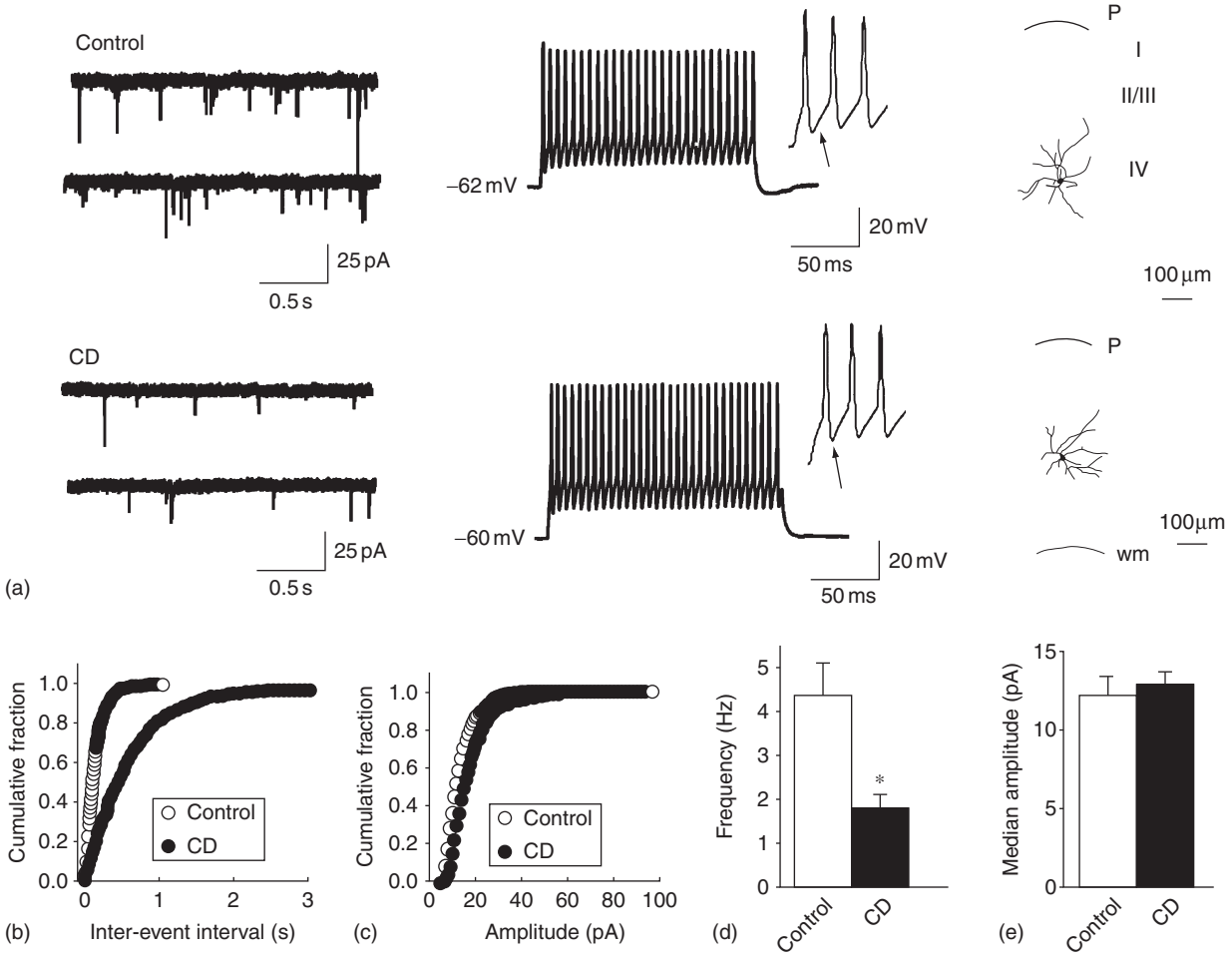


Figure 1 Type I (fast-spiking) interneurons in CD demonstrate a reduction in frequency of sEPSCs compared to controls. (a) The left column shows continuous recordings of sEPSCs from representative Type I interneurons in normal control cortex (upper traces) and CD (lower traces). The middle column shows the typical spiking patterns to current pulses (300 pA, 300 ms) of Type I interneurons from control cortex (upper) and CD (lower). Insets: expanded traces showing large, fast AHPs indicated by arrows. The right column shows camera lucida drawings of biocytin-filled interneurons from control cortex (upper) and CD (lower). (b, c). Cumulative probability curves of sEPSC inter-event interval (b) and amplitude (c) for the two representative Type I cells from control cortex (open circles) and CD (filled circles). (d) Histograms of pooled data from dysplastic and control Type I interneurons showing a reduction in frequency of sEPSCs (control, $n = 16$; CD, $n = 15$) in CD. (e) Histograms of pooled data showing no difference in median amplitude of sEPSCs between control and CD. Asterisk denotes statistical significance ($p < 0.05$). Reproduced from Xiang H, Chen HX, Yu XX, King MA, and Roper SN (2006) Reduced excitatory drive in interneurons in an animal model of cortical dysplasia. *Journal of Neurophysiology* 96: 259–278, with permission from American Physiological Society.

inhibition because it receives a reduced excitatory drive. Interestingly, this reduced excitatory drive appears to be specific to interneurons; our earlier study showed no reduction in frequency of sEPSCs or mEPSCs in excitatory pyramidal cells. These newer findings, coupled with the earlier findings of reduced number of cortical interneurons in irradiated rats, suggest a profound impairment of inhibition. Not only are there fewer interneurons present, but also the surviving interneurons have reduced excitatory drive (**Figure 1**).

Another recent finding involves an alteration in excitatory synapses onto pyramidal cells in irradiated rats. In normal postnatal development, STP of EPSCs in layer V pyramidal cells switches from short-term facilitation to short-term depression between 2 and 4 weeks of age. This change is due, in large part, to a developmental reduction in release probability in the excitatory presynaptic terminals. In earlier studies, we had noted that this switch did not seem to occur in irradiated rat. We performed initial studies in control rats that showed that the developmental switch from facilitation to depression was due largely to increased activation of type 2/3 metabotropic glutamatergic receptors (mGluR 2/3). Because the receptors are already present at 2 weeks, their increased activation between 2 and 4 weeks appears to result from increasing concentrations of ambient glutamate during this period.

Next, we performed detailed analysis of STP in controls and irradiated rats at 2 weeks, 4 weeks, and adults (>60 d). We found that, as expected, control cortex switched from short-term facilitation to short-term depression between 2 and 4 weeks. In contrast, irradiated rats showed short-term facilitation in all age groups, including adults. That is, the normal developmental switch does not occur in dyplastic cortex in this model, reflecting the persistence of an immature increased release probability in the excitatory terminals onto pyramidal cells. The ultimate importance of these findings remains to be seen; however, the literature suggests that factors that increase release probability in excitatory terminals also increase seizure susceptibility.

Future Goals

The work in irradiated rats raises many interesting questions for future research. First, we would like to follow-up the finding of reduced excitatory drive onto interneurons with anatomical studies. We are using antibodies for excitatory and inhibitory presynaptic terminals (VGlut1 and VGAT), in conjunction with biocytin labeling of specific interneurons, to see if anatomical studies will support our physiological findings that suggest a reduced number of excitatory terminals on these interneurons. We would also like to know the identity of the 'lost terminals' (e.g., are they from neighboring pyramidal cells or corticothalamic fibers?) We are performing experiments in slices that maintain both the barrel field structure of the sensory cortex and the ventral thalamus to address this question.

A second, larger issue of interest revolves around the physiological effects of reduced interneuron number and reduced excitatory drive onto remaining interneurons. In particular, how do these features affect the generation of seizures and epilepsy? We are performing slice experiments in the presence of the convulsant, 4-aminopyridine (4-AP), to see how synaptic activity and action potentials are affected in interneurons and pyramidal cells (before and during an epileptiform burst), and how these processes are altered in dysplastic cortex with impaired inhibition. In collaboration with other laboratories, we plan to examine how cortical activity is affected in vivo in irradiated rats, focusing not only on spontaneous seizure activity but also on analysis of single unit activity under baseline conditions and during interictal and ictal events.

See also: **Development/Malformations**: Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Animal Models of Infant-Onset Epileptic Encephalopathy; Epileptogenesis in Childhood; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation.

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Developmental Aspects of Seizures

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Introduction

During the first months of life children are particularly at a high risk for seizures, and largest number of new-onset seizure disorders occurs during this time. Because of the birthing process, the infant is at risk for several insults that can result in seizures. These insults include birth trauma and hypoxic-ischemic insults, as well as perinatally-acquired infections, intracranial hemorrhages, and metabolic disturbances. There is a considerable evidence to show that the normal immature brain is more susceptible to seizures than the mature brain.

In addition to being quite common, neonatal seizures are associated with a significantly high risk for subsequent neurological sequelae, including epilepsy, cognitive dysfunction, and motor deficits. While the cause of the seizures is the major determining factor for outcome, there is also evidence suggesting that seizures per se increase the risk of brain injury, regardless of etiology.

Neonatal seizures are associated with an extensive synaptic reorganization of the axons and terminals of the dentate granule cells, reduction in dendritic spines, decreases in neurogenesis, alterations in expression of AMPA and NMDA receptors, and changes in GABA subunit configuration. When animals that had neonatal seizures are tested as adults, deficits in spatial memory and auditory discrimination are found.

The particularly detrimental effects of neonatal seizures may relate to their timing with respect to brain development. Neonatal seizures occur during periods of marked changes in neuronal connectivity and synaptic physiology. For example, the number of inhibitory and excitatory synapses increases; GABA action shifts from excitation to inhibition; the percentage of glutamatergic NMDA-receptor-based 'silent' synapses decreases; the decay of NMDA-mediated postsynaptic responses becomes faster; and some membrane ionic channels and transporters appear and others disappear. Paroxysmal activity may alter normal physiological patterns, and thus influence the developmental process. Increasing evidence suggests that seizures can modify – slow down or accelerate – a wide range of unique processes that take place during development and that are essential for the correct formation and wiring of brain circuitry.

Background

The propensity for seizures or seizure-like activity in the immature brain has been demonstrated in several experimental models, including kainic acid, electrical stimulation, hypoxia, penicillin, picrotoxin, GABA(B) receptor antagonists, and increased extracellular potassium. The underlying mechanisms responsible for increased excitability during this period of life are not completely understood, but are clearly age-dependent. In general, immature neurons and networks tend to oscillate, and this inherent property facilitates the generation of pathological and pathogenic activity such as seizures. This oscillatory propensity is due to the higher input resistance of immature neurons, which enhances the action potential generation and consequent excitability.

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During the early postnatal period, at a time when the immature brain is highly susceptible to seizures, GABA exerts paradoxical excitatory action in all animal species and brain structures (including primates in utero), suggesting that this pattern has been preserved throughout evolution. GABA is depolarizing to approximately postnatal day 14 in rats and until the third trimester in primates. Immature neurons accumulate a high intracellular concentration of chloride, so that there is a chloride efflux – instead of an influx – when GABA receptors are activated to open chloride channels. The lack of efficient GABAergic inhibition enhances excitability and may facilitate synchronicity of epileptic activity.

Although some of the reasons for increased brain excitability in young animals are reasonably well-established, the effects of early-life seizures on subsequent brain excitability are less clear. It is known that following neonatal seizures in the rat there are changes in brain excitability, with a long-standing reduction in seizure susceptibility. Intracellular recordings from hippocampal CA1 and CA3 pyramidal neurons, from rats subjected to a series of 25 neonatal seizures and then studied as adolescents, revealed no significant differences in resting membrane potential, input resistance, membrane time constant, or action potential characteristics. However, CA1 pyramidal cells from these flurothyl-treated rats had marked reductions in spike frequency adaptation and reduced amplitudes of the afterhyperpolarizing potential following a spike train – factors likely to increase excitability of the hippocampal circuitry.

On the basis of this altered excitability, the long-term consequences of neonatal seizures on inhibitory and excitatory neurotransmission, as well as the possible effects of neonatal seizures on the timing of the switch from excitatory to inhibitory effects of GABA has been evaluated.

Methods

Induction of seizures: Flurothyl is used to induce seizures in immature rats. The volatile agent flurothyl (bis-2, 2, 2-triflurothyl ether) is a potent and rapidly acting central nervous system stimulant that produces seizures within minutes of exposure. It has been previously demonstrated that flurothyl seizures during the first days and weeks of life in rats result in several morphological and behavioral changes. Recurrent flurothyl seizures during the neonatal period result in subsequent increases in mossy fiber growth in both the supragranular region and CA3 hippocampal subfield. This sprouting occurs without any cell loss of the principal neurons. Rats are placed in a plastic container and liquid flurothyl is delivered through a plastic syringe and dripped slowly (3 cc/h) onto filter paper in the center of the container, where it evaporates. Rats are exposed to this volatile flurothyl until tonic extension of both the forelimbs and hindlimbs is observed.

Patch-clamp Recordings

Spontaneous inhibitory and excitatory postsynaptic currents (EPSCs) were recorded from visually-identified hippocampal CA3 pyramidal cells in in vitro slices, using voltage-clamp technique in a whole-cell configuration. Spontaneous $GABA_A$ receptor-mediated postsynaptic currents (PSCs) were recorded at a reversal potential of EPSCs (0 mV). In experiments studying the effects of flurothyl treatment on spontaneous EPSCs (sEPSCs), cells were held at 0 mV and incubated in ACSF containing 15 μM bicuculline until detectible spontaneous synaptic activity was blocked; recordings of sEPSCs were then made at −80 mV and +40 mV to separately detect AMPA and NMDA sEPSCs (respectively) on the same cell. Previous studies have shown that at a holding potential of −80 mV, the NMDA component of EPSCs is negligible in extracellular solution containing more than 2 mM Mg^{2+} so we did not used NMDA receptor blockers when measuring AMPA currents. At a holding potential of +40 mV, the sEPSCs consist of both AMPA and NMDA components, so recordings were made in the presence of the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (10 μM) in the extracellular solution. The remaining sEPSCs were abolished with the bath application of the NMDA receptor antagonist D-APV (50 μM), indicating that those sPSCs were mediated by NMDA receptors.

Extracellular Recordings

Extracellular recordings of multiple unit activity (MUA) were obtained from the CA3 pyramidal cell layer using metal electrodes of 50 μm diameter; the signal was amplified using a custom-made amplifier with enhanced electromagnetic interference noise suppression (bandpass 0.1 Hz–4 kHz; amplification 1000). Peak-to-peak noise was in the range of 20 μV. After 10–15 min of multiple MUA recording, the selective $GABA_A$ receptor agonist isoguvacine was briefly applied on the slice (10 μM for 1 min, bath application) to activate $GABA_A$ receptors. In control experiments using whole cell recordings from CA3 pyramidal cells we found that brief bath application of isoguvacine induced a transient chloride-dependent current sensitive to the $GABA_A$ receptor antagonist bicuculline. In extracellular recording, the effect of isoguvacine was associated with transient changes in the frequency of MUA and was completely reversible. We determined the effect of isoguvacine on MUA using a ratio based on the MUA frequency at the peak of the isoguvacine response to the MUA frequency in control. For the purposes of fit, we normalized the effect of isoguvacine by assigning values +1 and −1 for all values >100% (excitation) and <100% (inhibition), respectively.

Recent Results

To study the long-term effects of neonatal seizures on GABA currents, we induced a series of 25 flurothyl seizures in rat pups from postnatal days (P) 0 to 4. Whole-cell patch clamp and extracellular recordings were then made from the CA3 hippocampal pyramidal cells in slice preparations, from 3 to 11 days after the last seizure in flurothyl-treated rats; control rats (no seizures) of comparable age were also examined. We compared sPSCs recorded from pyramidal cells in CA3 in control and flurothyl-treated rats at P8–10 and P15–17. The frequency of sIPSCs in control rats increased significantly over the second and third postnatal weeks (**Figure 1(a)**) ($p < 0.005$). A similar increase in frequency of sIPSCs during this developmental period was also observed in flurothyl-treated rats. As shown in **Figure 1(b)**, on average the amplitude of sIPSCs in control rats also changed with advancing postnatal age ($p < 0.05$). In the flurothyl-treated rats, the amplitude of the sIPSCs did not differ from the control values at P8–10. However, sIPSCs amplitude did not increase during the third postnatal week, and was 27% lower in amplitude than sIPSCs in age-matched control animals. These results show that neonatal seizures are associated with subsequent decreases in GABA currents in the hippocampus.

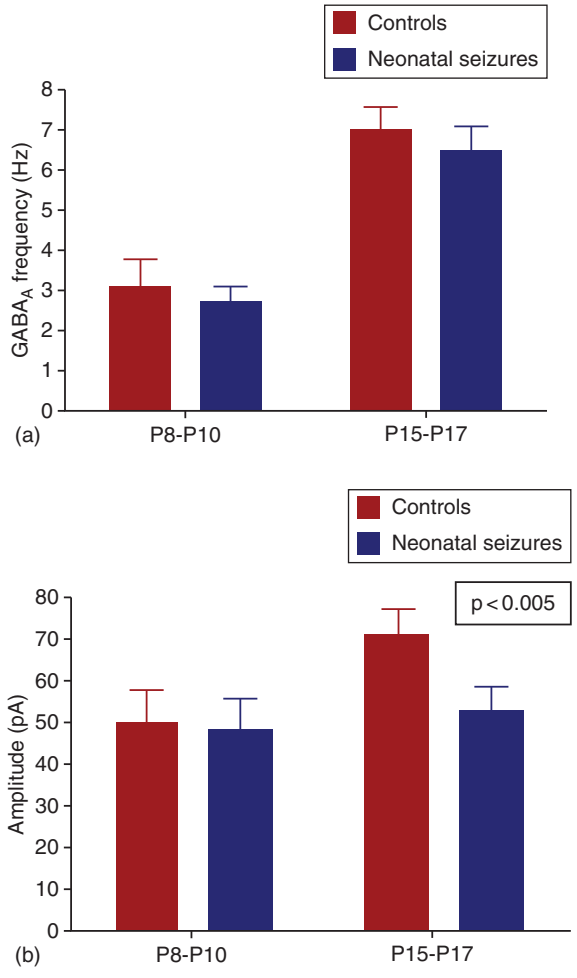


Figure 1 Effect of neonatal flurothyl-induced seizures on GABAergic synaptic transmission in CA3 pyramidal cell at P8–10 and P15–17. (a) GABA(A)-PSCs frequency increased with maturation without a significant difference between the control and flurothyl-treated rats. (b) Amplitude of the GABA(A)-PSCs increased with advancing postnatal age in control rats but not flurothyl-treated rats.

To investigate whether neonatal flurothyl-induced seizures influence excitatory synaptic transmission, recordings were made in the presence of 15 μM bicuculline and at holding potentials −80 and +40 mV to separately detect AMPA and NMDA sEPSCs, respectively. The frequency and amplitude of AMPA and NMDA sEPSCs recorded from control and flurothyl-treated rats increased significantly ($p < 0.05$) and to much the same extent over the second and third postnatal weeks. However, there were no significant differences between AMPA and NMDA sEPSCs amplitudes at any of the ages studied in the control and flurothyl-treated rats. Glutamate neurotransmission does not appear to be permanently altered following neonatal seizures.

In the next series of experiments, we asked whether recurrent neonatal flurothyl-induced seizures affect the developmental excitatory-to-inhibitory switch in the action of GABA. We used non-invasive extracellular recordings of MUA in the CA3 pyramidal cell layer. This method allowed us to record action potentials from tens of neurons in the vicinity of the recording electrode without altering the intracellular chloride concentration. To activate $GABA_A$ receptors, we applied the selective $GABA_A$ receptor agonist isoguvacine (10 μM for 1 min). Day-by-day analysis of the age dependence in the effect of the $GABA_A$ receptor activation, as reflected in MUA frequency, is presented in **Figure 2**; the effect of isoguvacine is presented as a ratio between MUA frequencies at the maximum of the isoguvacine-induced response and during baseline control conditions (prior to isoguvacine application). Age-dependence of the effect of isoguvacine on MUA was approximated with a Boltzman function. A developmental switch in the action of isoguvacine from excitation to inhibition, as determined by the midpoint of the Boltzman fit (i.e., the age at which half of the slices showed an isoguvacine-induced increase in MUA and in the other half showed an isoguvacine-induced decrease in MUA) was estimated to be postnatal day P 13.7 ± 0.3 in control rats and P14.0 ± 0.7 in fluorthyl rats. This difference was not statistically significant, indicating that neonatal seizures did not alter the timing of the switch of GABA from excitation to inhibition.

Down-regulation of GABAergic synaptic transmission by flurothyl-induced seizures can be explained by different mechanisms. Recurrent seizures induced by flurothyl during the neonatal period result in a long-term decrease in the expression of the $GABA_A$-α1 receptor subunit expression.

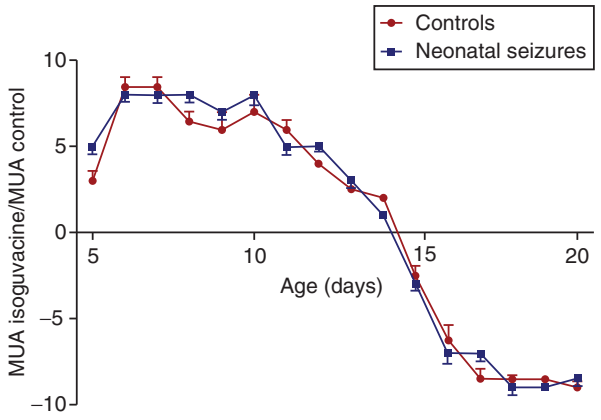


Figure 2 Flurothyl-induced recurrent neonatal seizures in vivo do not affect the developmental switch in the action of GABA from excitatory to inhibitory. Plot of the age dependence in the effect of the GABA(A) agonist isoguvacine on neuronal firing. Each point represents the mean ratio of the multiple unit (MUA) frequency at the peak of isoguvacine effect to the MUA frequency in control conditions. No differences were noted in the control animals and those subjected to recurrent flurothyl seizures.

It has also been shown that increased neuronal activity in hippocampal pyramidal cells in culture increased the percentage of internalized $GABA_A$ receptors. Decreasing of $MgCl_2$ or increasing KCl in the culture medium led to a 30% reduction in mIPSC amplitude. It has been shown that $GABA_A$ receptor endocytosis can last at least several days after low-magnesium treatment of hippocampal cultures, and thus may contribute to a decrease in receptor function and surface expression of $GABA_A$ receptors.

Another possible explanation for the reduction of GABAergic synaptic transmission arises from studies demonstrating a postsynaptic decrease in $GABA_A$ receptor-mediated inhibition secondary to several different metabolic processes. In the low magnesium model of epilepsy, investigators demonstrated that a postsynaptic decrease in $GABA_A$ receptor-mediated inhibition was ATP dependent. It also has been shown that after hypoxia-induced seizures, CA1 pyramidal neurons exhibit a downregulation of $GABA_A$ receptor-mediated inhibition that can be reversed by phosphatase calcineurin inhibitors.

Future Goals

Our study adds further evidence that a seizure during early development alters synaptic function in the developing brain. We propose that the early pathophysiological changes could contribute significantly to the long-term consequences of seizures that occur early in life. Since GABA signaling is important in a wide range of developmental functions, alterations of GABA neurotransmission may have other long-lasting detrimental effects.

Our next major goal is to understand the mechanisms by which neonatal seizures alters inhibitory neurotransmission. The consequences of neonatal seizures appear to be life-long, so that developing the strategies to prevent these long-term changes has major health care implications. Current studies have demonstrated that seizures can alter the functions of brain but the challenge is to determine why such impairment occurs.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; **Pediatric Epilepsy**: Age-Specific Responses to Antiepileptic Drugs; Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; Epileptogenesis in Childhood; Quantitative and Qualitative Changes of Action of Antiepileptic Drugs in Developing Experimental Animals.

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Epileptogenesis in Childhood

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Introduction

Although 'epilepsy' is referred to as a single disorder, other articles in this encyclopedia highlight that there are many types of epilepsies, involving different neuronal circuits. In addition, these epilepsies are a result of many differing causes and mechanisms. In some instances, the reason for the development of the spontaneous seizures (i.e., epilepsy) relates to abnormal brain structure (malformation) that can be local or generalized. In others, mutations of ion channel genes or of other genes confer a genetic basis of the epilepsy. In still other cases, infection, trauma, and probably also some very long febrile seizures provoke epileptogenesis, the process of epilepsy generation, in a brain that otherwise would not develop epilepsy without this insult. Commonly, the timing of epilepsy onset is during the first decade in humans, even though the responsible mutation, malformation, or other insult may be congenital or occur much earlier in postnatal life. Why do the first seizures that herald the onset of epilepsy occur in infancy and childhood?

Background

Basis of Susceptibility of the Early Postnatal Brain to Seizures

During the first months to years in humans, and the comparable first days and weeks in rodents, several factors coalesce to render the brain, and particularly the limbic circuit, susceptible to the occurrence of single and recurrent seizures. Much of the information about the mechanisms that contribute to this fact has arisen from rodent animal models, and it is therefore important to realize that direct comparison of the developmental stages between human and rodent brain are inexact. For examples, the gestation and postnatal development of rodents are much shorter, and rats reach puberty at ~6 weeks and adulthood at about two months of age. Comparative anatomy suggests that in rats and mice, cortical neuron birth and migration are completed shortly after birth, and brain growth takes place largely within the first 2–3 weeks of life. Particularly considering hippocampal development, a newborn rat pup's developmental stage is comparable to that of a premature baby, a rat at 5–7 days of age has similar development to that of a full-term newborn, and at 3 weeks that of an older child.

During the first postnatal week, excitatory neurotransmitter development and maturation precedes that of inhibition, mainly because activation of the $GABA_A$ receptor by endogenous γ-aminobutyric acid (GABA) produces depolarization. This is a result of the immature state of chloride transporters, resulting in higher intracellular chloride. In addition, expression of certain glutamate receptor subunits 'overshoots' adult levels during the first two postnatal weeks. This combination increases relative excitatory synaptic transmission, promoting hyperexcitability and the susceptibility to seizures.

The unique developmental profile of neurotransmitter expression is accompanied by similar age-specific expression patterns of neuropeptide modulators and intrinsic neuronal ion channels. Like 'classical' neurotransmitters, neuropeptides are released from neurons and can influence the excitability of a neuronal network, acting typically via metabotropic receptors at postsynaptic or presynaptic sites. Inhibitory neuropeptides, including for example neuropeptide y (NPY), are expressed in cortex and hippocampus in humans (and rodents), and reduce network excitability and seizure susceptibility. NPY levels are relatively low in early postnatal hippocampus, which may contribute to the enhanced susceptibility to seizures. In contrast, the excitatory peptide corticotropin-releasing hormone (CRH), and its major receptor, CRFR1, are expressed in developing hippocampus at levels higher than in the adult. This is particularly true during the second postnatal week in the rodents, a developmental epoch characterized by high susceptibility to seizures. CRH increases network excitability by reducing afterhyperpolarizations.

In addition to age-dependent expression patterns of glutamate, GABA, and neuropeptides, and hence in synaptic neurotransmission, developmental expression patterns of ion channels govern age-specific intrinsic properties of neurons, promoting network excitability. Even minor changes in the function of these channels, resulting from mutations or altered expression levels, may promote seizures and epilepsy. For example, mutations in 3 of the 13 known sodium channel genes in mammals (*NaV*1.1, *NaV*1.2, β1) have been associated with epileptic phenotypes, typically arising during infancy or early childhood, and often disappearing later in life. Potassium channels are particularly important during the developmental period when GABA is depolarizing, because they are among the few cellular components that promote repolarization. Indeed, dysfunction of two members of the voltage-gated potassium channels, KCNQ2 and KCNQ3, that carry the

M-current lead to benign familial neonatal convulsions, seizures that occur during the first days and weeks of life, and disappear later. For the Ca^{2+}-channels, age-specific contribution of the CaV2.1-subunit to seizure susceptibility has been delineated in mice. In thalamic neurons of the neonatal mouse, either N- or P/Q-type channels support neurotransmitter release. However, later in life, this function is taken over exclusively by the P/Q-type channels, formed by CaV2.1 subunits, so that dysfunction of these channels provokes seizure vulnerability.

The repertoire of cellular components, including ion channels, transporters, and receptors, that are differentially expressed during development and may influence susceptibility to seizures is constantly enlarging. Recent additions include the chloride transporter NKCC1.

Conceptual and Methodological Considerations

Susceptibility to Seizures Vs. Vulnerability to Epileptogenesis

Although a significant body of information exists that clarifies, or at least is consistent with, the increased likelihood of seizures early in postnatal life, little is known about the basis for the vulnerability to epileptogenesis during this epoch. Indeed, seizure susceptibility is not synonymous with epileptogenesis. An excellent example for this dichotomy involves the consequences of trauma in children and adults. Children are much more likely to experience a seizure in response to even modest trauma, compared with adults. However, the probability of developing posttraumatic epilepsy is at least threefold lower. This example suggests that the developing brain might not be more vulnerable to epileptogenesis compared with that of a mature organism. In this scenario, epileptogenesis might occur more commonly early in life because the incidence of inciting insults, and especially seizures and status epilepticus (SE), is higher. This higher incidence, in turn, is dependent on the factors described earlier.

Age-Specific Epileptogenesis

Epileptogenesis is a term defining a series of complex processes leading to the occurrence of spontaneous seizures. The determinant of epileptogenesis varies widely. The process may arise on an almost purely genetic basis, for example, in the setting of a mutation of a critical gene such as an ion channel. In contrast, environmental input might play a key role in certain epilepsies, such as trauma or stroke. Generally, it is believed that gene–environment interaction underlies the majority of epileptogenic events.

Most of our knowledge of the epileptogenic processes is derived from studies in animals. These have been helpful for both 'genetic' and 'acquired' epilepsies. For example, transgenic mice carrying ion channel mutations, or natural mutations in calcium and other channels, have developed epilepsy, usually around puberty or later. In this case, the effects of the mutation promote seizures, and are typically, but not always, unaccompanied by neuronal death or other anatomical changes. In several animal models, the recurrent spontaneous seizures themselves, i.e., the epilepsy, eventually lead to cell death and often compensatory 'sprouting' of axons of remaining neurons.

Animal models of human epilepsies that do not seem to have strong familial or genetic component, including temporal lobe epilepsy, have often relied on SE as an inciting insult. As described in other chapters, SE leads to loss of neurons in vulnerable brain regions. In the hippocampus, loss of pyramidal cells in areas CA3 and CA1, and loss of neuronal population in the hilus of the dentate gyrus, are typical. The cell loss is classically followed by sprouting of granule cells and other neurons, formation of abnormal circuitry, and – after a latent period – the evolution of spontaneous seizures (i.e., epilepsy). There is considerable evidence that supports the notion that the cell death provoked by the original SE is required for the development of epilepsy.

Recent Results

Seizure-Induced Plasticity May Contribute to Epileptogenesis during Infancy and Childhood

Early in life, however, the story is quite different. In the 1980s it became clear that during the first two weeks of the rodent life, even severe seizures lead to little neuronal death. This finding was originally interpreted to suggest that early postnatal seizures might be benign, and indeed, gross cognitive sequelae as well as epileptogenesis were rare. However, more recently, more refined assessment of cognitive function has suggested that recurrent short seizures or prolonged single seizures might lead to impaired function. Still, the majority of the conventional chemically- and electrically induced SE and prolonged seizures do not seem to result in epileptogenesis.

In recent years, several animal models have shed light on the mechanisms of early-life epileptogenesis. Remarkably, although highly diverse, they provide consistent information. Spontaneous seizures have been reported after early-life experimental prolonged febrile seizures, after tetanus toxin, focal TTX injection, or endothelin-1 injections. In these models, cell death was not prominent and, in contradistinction to the mature brain, does not appear to be required for epileptogenesis. 'Sprouting' and neurogenesis were also not consistently present in the brain of immature rodents that developed epilepsy after these provoking insults. These data suggest that the mechanisms of epileptogenesis during early life require

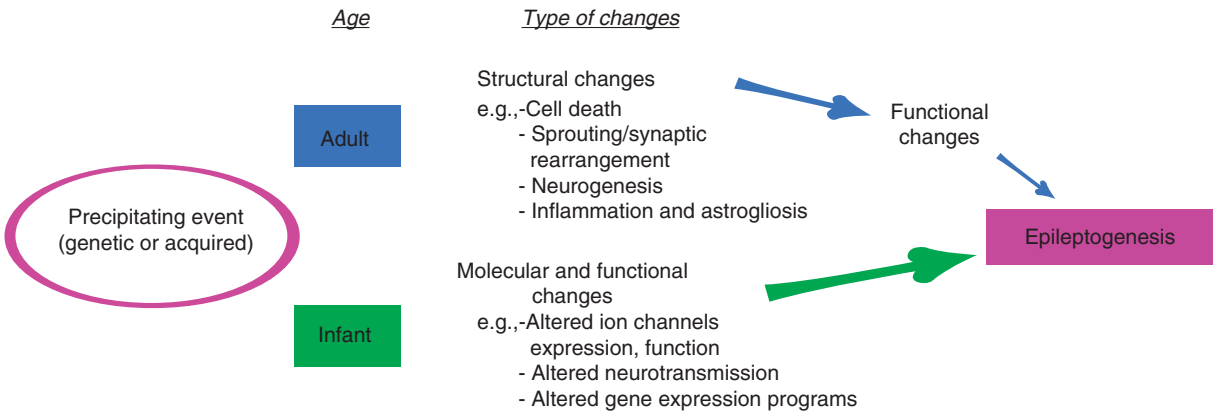


Figure 1 Distinguishing characteristics of epileptogenesis in the mature vs. the immature brain. The schematic shows how an original insult (e.g., genetic mutation of an important gene or severe long seizures) leads to epileptogenesis through different type of processes in immature and adult brain. An adult brain typically requires structural changes such as cell death and sprouting to become epileptic (as best defined in models of acquired epilepsy). In contrast, during childhood, neuroplasticity, often governed by altered programs of gene expression, is a leading mechanism of epileptogenesis.

functional rather than structural changes in neurons and neuronal circuit (Figure 1).

Several candidate mechanisms exist for epileptogenesis without cell death, and generally involve enduring changes in expression programs of key genes. Early-life seizures lead to persistent changes in the expression of several $GABA_A$ receptor subunits, which profoundly alter the properties of the receptors, rendering the brain more conducive to 'runaway excitability.' *N*-methyl-D-aspartate (NMDA)- and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors are also changed. The expression of intrinsic ion channels, notably the hyperpolarization-activated ion channels, is also changed, leading to currents that increase the probability of rebound depolarization. Other receptors (e.g., the endocannabinoid receptor type 1) are also modulated long-term. The nature of the regulatory factors that are activated by early-life seizures to orchestrate these coordinate alterations of gene expression programs are not yet known. Altered signaling through the cAMP system and the neuronal suppressor NRSF have been found. One theory proposes that, at least in the case of long experimental febrile seizures, induction of endogenous inflammatory mediators and specifically IL-1β leads to altered gene expression via the NF-kB cascade, and endures long-term.

Future Directions

Although much has been learned from these studies and others about the pathways that convert a normal developing brain into an epileptic brain, many questions remain unanswered. For example, following a given insult, such as hypoxia, trauma, or complex febrile seizure, an individual rat or human may or may not develop epilepsy. The ability to predict who will develop epilepsy after an initiating event would be highly beneficial, because it will enable targeting of future interventions to the population at risk. Current and future studies aim to determine genetic and acquired susceptibility to epileptogenesis. In parallel, investigators are attempting to define 'biomarkers' of the epileptogenic process. Molecular arrays, or fingerprints, at mRNA or protein levels are under investigation. In addition, imaging methods (including MRI) are being used to examine whether changes visible in vulnerable brain areas denote regions that are undergoing changes conducive to the development of epilepsy at a later date.

In summary, significant evidence supports the independence of epileptogenesis in childhood from the need for cell death. However, relatively little is known about the nature and basis of the functional neuronal changes (neuroplasticity) that promote the epileptogenic process early in life. Future studies should help clarify these processes and, optimally, predict in advance who will develop epilepsy, and enable intervention and prevention of this disorder.

See also: **Epileptogenesis**: Multi-Hit Mechanisms Involved in Epileptogenesis: Role of Early-Life Seizures; **Glutamate**: Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Animal Models of Infant-Onset Epileptic Encephalopathy; Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; Developmental Aspects of Seizures; Seizure-Induced Injury and the Two-Hit Hypothesis.

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Hippocampal EEG in the Neonatal Rat: Development of Normal and Pathological Activity

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Introduction

Children are particularly susceptible to seizures during the first few months of postnatal life. During this period, a clinician may examine a child's background (nonparoxysmal) EEG activity for abnormal patterns that might predict pathological outcomes. In this context, 'abnormal' background EEG patterns (e.g., abnormalities of amplitude and frequency) are defined in relation to 'normal' age-specific patterns, which change continuously across this period as the complexity of the EEG increases.

The most common cause of seizures during the neonatal period is hypoxic–ischemic encephalopathy (HIE). To study the age-dependence of vulnerability to HIE and its associated neurological damage, an animal model was developed by Jensen and her colleagues. This experimental model involves the exposure of neonatal rats to graded global hypoxia in an airtight chamber. Studies utilizing this paradigm have reported age-dependent effects in the developing neocortex and hippocampus, with seizure being most readily induced at approximately postnatal day (P)10. The model therefore appears to capture a period of heightened seizure susceptibility in the neonatal rat that is analogous to that of the human infant. Yet, if the prognostic background EEG correlates of the HIE-like state in rats are to be properly studied, we need to better understand what 'normal' background activity entails.

Recent work in our laboratory has systematically assessed the development of the 'normal' hippocampal EEG across the first three postnatal weeks in unanesthetized rats. In addition, we have correlated the developmental changes in spontaneous activity with the age-dependent emergence of evoked pathological activity. In this article, we describe our findings, with special emphasis on the dramatic changes occurring during the second postnatal week.

Background

In rats, peak susceptibility to hippocampal seizures occurs during the second postnatal week. The increased excitability of the hippocampus during this period appears to be attributable to several factors. One of the more extensively studied of these factors is the ontogenetic shift in intracellular Cl^- concentration, which causes $GABA_A$ receptor activation to transition from having a depolarizing effect to a hyperpolarizing effect on the cell. This shift occurs as the Cl^- accumulating cotransporter NKCC1 is down-regulated, and the Cl^- extruding cotransporter KCC2 is up-regulated. A separate factor thought to contribute to the increased excitability of the neonatal hippocampus is that the effects of the excitatory neurotransmitter glutamate are potentiated by a transient up-regulation of AMPA and NMDA receptors. In addition, electrical coupling between neurons appears to be more abundant during the second

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postnatal week, as the proteins that form gap junctions are transiently up-regulated. Finally, it is during this period that the recurrent collateral branches that interconnect pyramidal cells in the CA3 region reach levels greater than those seen in adulthood, before gradually being refined. Thus, it appears that during the second postnatal week, the hippocampus is characterized by conditions that favor both excitation and population synchrony.

The excitability and synchrony of the adult hippocampus are reflected in the frequency and duration of hippocampal sharp waves (SPWs), large field potential events that reflect a synchronous discharge of neurons in the CA3 network that is then conveyed to the CA1 field via the Schaffer collateral system. Accordingly, the transiently increased tendency of the neonatal hippocampus toward excitability and synchronization should be reflected in the expression of SPWs, and perhaps also their associated high-frequency oscillations. Indeed, there is currently a great amount of interest in the developmental relationship between the emergence of spontaneous physiological high-frequency activity (>40 Hz) and the emergence of the pathological high-frequency oscillations (≥60 Hz) that characterize epileptogenic activity. Although two recent studies have examined neonatal SPWs in vivo, neither study examined subjects during the developmentally important second postnatal week. We therefore sought to determine how age-dependent changes in hippocampal activity relate to the developmental onset and offset of the period of hyperexcitability.

Methods

In our laboratory, we have taken advantage of 16-site linear silicon probes (100-μm vertical separation between recording electrodes; NeuroNexus Technologies, Ann Arbor, MI) to simultaneously monitor the activity of multiple hippocampal layers within the dorsal CA1–dentate gyrus axis. These probes also allow the investigator to distinguish locally generated events and oscillations from passively conducted activity, because the former exhibit polarity reversals across the appropriate layers.

To determine the normal course of postnatal EEG development, the hippocampal activity of unanesthetized rats at P2–18 was monitored. Under isoflurane anesthesia, subjects' heads were secured for electrophysiological recordings by surgically attaching a custom-built, stainless steel apparatus that was designed to attach to the ear-bar and nose-bar holders of a stereotaxic apparatus. This preparation allowed access to the dorsal hippocampus while minimizing movement artifacts. Nuchal and vastus lateralis EMG electrodes were implanted and used to identify behavioral state. Finally, to inhibit movement and calm the subjects, they were wrapped gently in gauze. During recovery, acclimation, and testing, the subjects' body and brain temperatures were maintained at thermoneutrality (35 °C and 37 °C, respectively).

A subset of subjects received intrahippocampal infusions of kainic acid (KA) to assess seizure susceptibility across the first three postnatal weeks. After baseline recording periods, a 30-gauge needle attached to a 10-μl Hamilton syringe was lowered slowly into the contralateral CA1 pyramidale-radiatum region at approximately the same coordinates as the recording electrode. Next, 1 μl of KA (200 ng/μl; dissolved in sterile saline) was manually infused (1 μl per 10 s). After each infusion, hippocampal activity was continuously recorded for 10 min. Each subject received a maximum of 10 infusions (1 infusion per each of ten 10-min recording sessions). The general experimental design that we employed is illustrated in **Figure 1(a)**.

Results

At the earliest age examined (P2), the hippocampal EEG consisted of SPWs and brief bursts of 'slow' gamma (20–50 Hz) rhythms. These two patterns predominated until P6, when we observed the emergence of 'fast' gamma (60–100) rhythms, which were initially observable only as 'tails' of activity following SPWs. At P7, we observed the emergence of the SPW-related 'ripple' (140–200 Hz), as well as dentate spikes. P7 was also the age at which KA infusion was first able to elicit interictal spikes (IISs; **Figure 1(b)** and **1(c)**) and associated pathological high-frequency oscillations (60–200 Hz). Theta activity (4–12 Hz) emerged on P8, coincident with the period of peak susceptibility to KA-induced seizure activity. In general, the amplitude and incidence of all events and oscillations increased across development; the exception was gamma tails, which continued to occur with low probability after their emergence.

As illustrated in **Figure 1(d)**, the sensitivity to KA infusion increased dramatically between P7 and P11, and declined thereafter such that, in most subjects, even 10 infusions of KA were no longer sufficient to evoke seizure activity.

Importantly, we found that in addition to the emergence of ripples and dentate spikes, there were two changes in SPW activity that reflected the onset, as well as duration, of the period of increased seizure susceptibility, between P7 and P11. These included (1) an increase in the occurrence of SPW doublets (that is, two SPWs occurring within a 400-ms window) and (2) a decrease in the duration of SPWs. Our results are summarized in **Figure 2**. Note that the period of greatest seizure susceptibility is predicted by the onset of several EEG patterns, as well as the transient modification of SPWs. The physiological significance of the latter is discussed below.

It was recently suggested that the development of pathological high-frequency oscillations at P6–7 precedes

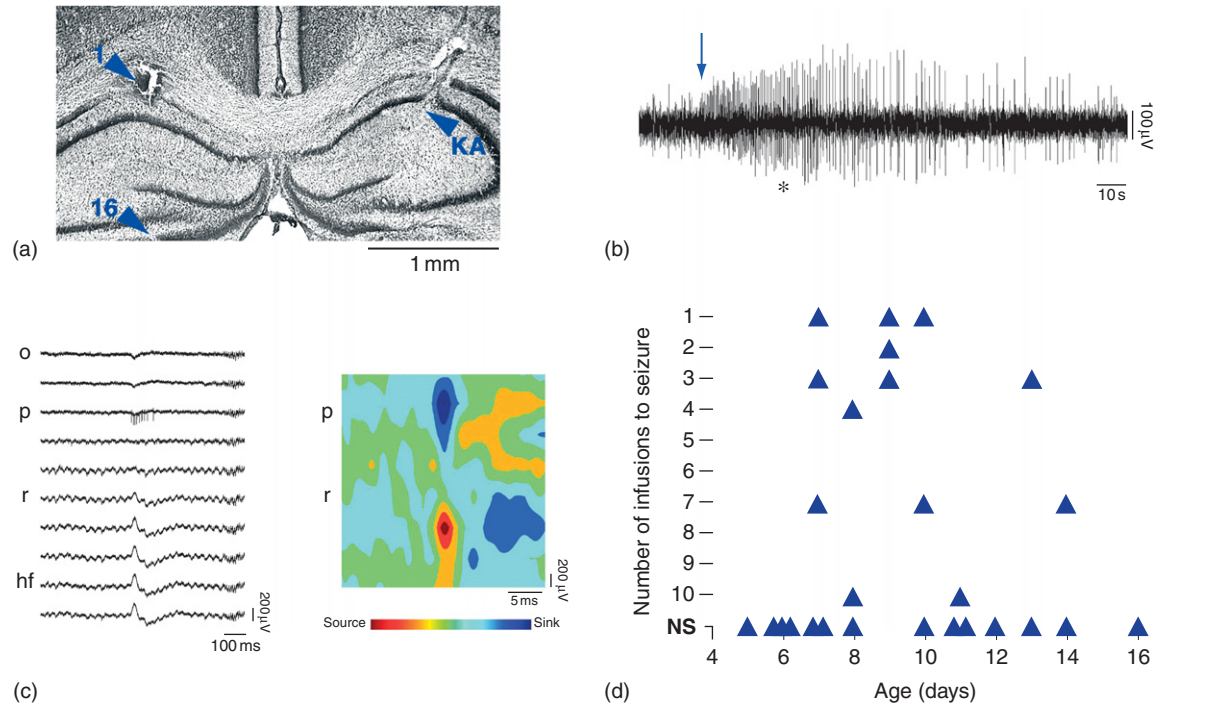


Figure 1 Hippocampal activity following contralateral kainic acid (KA) infusion. (a) Histological section from a P8 subject, illustrating the design of the experiment. In the left hippocampus, a marking lesion indicates the location of the most dorsal recording site (site 1). The most ventral recording site is also indicated (site 16). In the right hippocampus, the location of the KA infusions is indicated by the needle track penetrating CA1. (b) For the subject in (a), this recording illustrates the onset (indicated by arrow) of seizure activity following KA infusion. Asterisk indicates the location of the interictal spike (IIS) presented in greater detail in (c). (c) Left panel: Representative depth profile (wide-band, 1–5000 Hz) of a type 2 IIS, and associated neuronal discharge in stratum pyramidale (p). Right panel: Current source density analysis, derived by averaging across many IISs comprising the interictal episode from which the IIS in the left panel was selected. (d) For each subject, the number of KA infusions required to evoke seizure activity. NS, no seizure activity detected after a maximum of 10 infusions. o, stratum oriens; r, stratum radiatum; hf, hippocampal fissure. Reproduced from Mohns EJ, Karlsson KÆ, and Blumberg MS (2007) Developmental emergence of transient and persistent hippocampal events and oscillations and their association with infant seizure susceptibility. *European Journal of Neuroscience* 26: 2719–2730, with permission from Blackwell.

the development of physiological high-frequency oscillations in the ripple frequency range at approximately P14. While we indeed found that pathological high-frequency oscillations emerge around P7, we also found that physiological high-frequency oscillations in the gamma-frequency range emerge at P6 and in the ripple range as early as P7. Thus, we offer the alternative view that pathological and physiological high-frequency oscillations emerge at approximately the same time.

Gamma-frequency rhythms have been described in head-fixed and freely moving rats as early as P2–5, but it is important to note that these rhythms are unrelated to SPWs, and are of lower frequency (i.e., 20–60 Hz) than the SPW-related high-frequency (60–100 Hz) gamma tails that we have found at P6. These findings reflect an important interaction between subject age and oscillation frequency. Indeed, because high-frequency gamma oscillations are dependent, in part, upon AMPA receptor-mediated excitation, it is noteworthy that the emergence of 60–100-Hz oscillations at P6 is coincident with the up-regulation of AMPA receptors that takes place at this age.

Our study also indicates that the onset and duration of the period of greatest susceptibility to seizure activity are evident in baseline, nonpathological EEG patterns. In particular, this period is characterized by a decrease in the duration of the SPW waveform, and by an increase in the occurrence of SPW doublets. SPW duration in CA1 indicates the degree of synchrony among CA3 pyramidal neurons; a CA1 SPW of shorter duration reflects more rapid recruitment of cells within the CA3 recurrent collateral network, and thus greater network synchrony. Our finding that SPW duration was shortest during the second postnatal week is therefore consistent with the finding that CA3 pyramidal-cell collateralization is greatest during this period. In addition, because seizures are produced by neuronal network hypersynchrony, it is noteworthy that the period of shortest SPW duration was also coincident with the period of greatest seizure susceptibility. It is equally noteworthy that there was an increased rate of SPW doublets during this period; as SPWs occur during periods of reduced cortical and subcortical inhibition, an enhanced rate of occurrence of SPWs and SPW doublets is, in adult

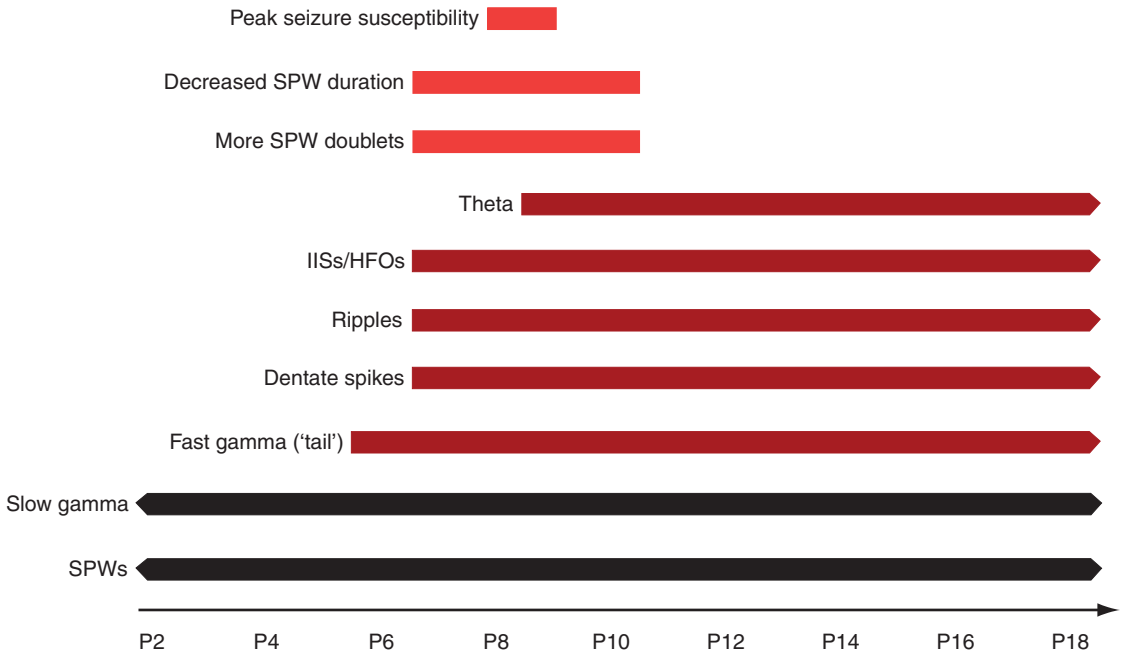


Figure 2 Summary of results. Presented is a schematic illustration of the development of transient and persistent hippocampal events and oscillations. SPWs and slow gamma oscillations (20–50 Hz) occurred at all ages examined. SPW-related fast gamma tails (60–100 Hz) and ripples (140–200 Hz) were first detected at P6 and P7, respectively. Interictal spikes (IISs) and associated pathological high-frequency oscillations (HFOs; 60–200 Hz) were first evoked by kainic-acid (KA) infusion into the contralateral hippocampus at P7. Dentate spikes were also first observed at P7, while theta activity (4–12 Hz) did not emerge until P8. The occurrence of SPW doublets increased, and SPW duration decreased, transiently between P7 and P11. Finally, peak susceptibility to seizure following KA infusion, as indicated by the number of infusions required to evoke seizure activity, occurred at approximately P9. Reproduced from Mohns EJ, Karlsson KÆ, and Blumberg MS (2007) Developmental emergence of transient and persistent hippocampal events and oscillations and their association with infant seizure susceptibility. *European Journal of Neuroscience* 26: 2719–2730, with permission from Blackwell.

rats, thought to reflect a state of increased network excitability.

To test whether the EEG characteristics we had identified did indeed correlate with increased seizure susceptibility, we developed a method for inducing seizure activity in vivo that is based on an in vitro method for induction of epileptogenic mirror foci. This method utilizes the early postnatal development of functional hippocampal commissural projections to induce seizure activity via contralateral KA infusion. Using this experimental preparation, we found that the fewest number of infusions required to produce electrographic seizure activity occurred at approximately P9, with decreased efficacy at younger and older ages. This result indicated that there was, in fact, a strong relationship between the transient SPW-related changes and seizure susceptibility, as illustrated in Figure 2. Figure 2 also illustrates how much the hippocampal EEG increases in complexity during this very brief period.

Our finding that seizure susceptibility reached a peak at P9 in response to KA infusion is consistent with the results of several other experimental models developed to examine neonatal seizure susceptibility. These models include in vivo approaches utilizing hypoxia and hyperthermia to induce seizures, which reveal peak susceptibilities at P10–12 and P8–11, respectively. Our data are also consistent with the high potassium in vitro model of neonatal seizure induction, which reaches peak effectiveness at approximately P11.

Future Goals

One of the primary goals of our ongoing research is to distinguish the epigenetic factors that influence the normal and pathological development of the neonatal brain. We hypothesize that the well-established influence of maternal care on brain development may have a great influence on how prone to seizure a rat will become, particularly during the second postnatal week. Moreover, we believe that the mechanisms underlying postnatal hyperexcitability will be elucidated by further studies addressing the individual and synergistic contributions of recurrent collateral branches, gap junctions, glutamate receptors, $GABA_A$ receptors, and other factors that are developmentally modulated during this period.

The results presented here identify several transient SPW-related events that are characteristic of a period

of increased synchrony and excitability in the neonatal hippocampus. That the period delineated by these events coincides with the down-regulation of NKCC1 and the up-regulation of KCC2 may suggest a mechanistic connection. Specifically, if the shift from GABAergic excitation to inhibition is mediated by an activity-dependent mechanism, then perhaps the increased activity associated with hyperexcitability promotes a self-limiting process by which the infant transitions to a nonhyperexcitable state.

See also: **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; **Glutamate**: Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain; **Oscillatory Activity**: Seizures Beget Seizures in the Developing Brain: Central Role of GABA and High Frequency Oscillations; **Pediatric Epilepsy**: Developmental Aspects of Seizures; Epileptogenesis in Childhood; **Transporters**: Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

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Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation

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Introduction

That malformations of cortical development (MCD) are associated with epilepsy was first reported over 130 years ago. Malformations are primarily associated with severe pediatric seizure disorders and are often resistant to conventional antiepileptic drugs. Driven, in large part, by the advent of sophisticated neuroimaging techniques, MCD-associated seizure disorders are now recognized as a fairly common form of pediatric epilepsy. Indeed, recent clinical estimate indicates that nearly 30% of all epilepsy surgeries involve removal of dysplastic tissue. Consequently, a critical issue facing investigators is to determine mechanisms through which dysplastic tissue can function in a hyperexcitable manner that would, ultimately, contribute to a proepileptic phenotype.

Clinical studies already indicate that (1) malformations are regions of interictal hypometabolism and ictal hypermetabolism, i.e., typical features of epileptogenic foci, (2) malformed brain regions exhibit paroxysmal activity in corticography or synoperative deep electrode recordings, and (3) surgical resection of focal malformations

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leads, in many cases, to suppression of seizure activity. To further highlight the proepileptogenic nature of MCDs, human dysplastic tissue maintained in vitro was shown to generate ictal-like epileptiform activity when exposed to a common convulsant, 4-aminopyridine (4-AP). From these observations, an experimental interest in how malformed neurons function (and communicate) has emerged. A good starting point for examining how seizures could be generated in malformed tissue is to consider two distinct (but classic) hypotheses, namely, that hyperexcitability is the result of a defect in (1) the intrinsic firing properties of individual neurons or (2) the synaptic properties of a network of neurons – or perhaps both.

Background

At present, a great deal of clinical information is available regarding brain malformations. This information is extremely useful in guiding the development of appropriate animal models. For example, from morphological studies we know that MCDs cover a wide neuropathological spectrum, including patients with subtle (i.e., single aberrant or dysplastic cells) up to severe malformations such as having two separate cortices (i.e., subcortical band heterotopia) or large clusters of dysplastic neurons (i.e., nodular heterotopia). From histological data, we know that MCDs are typically encountered in cortex, but can also be observed as 'tectonic' clusters of pyramidal cells in hippocampal areas CA1 or CA2. Although the precise identity of dysplastic cells is debated, it is often the case that these cells are neurons and not aberrant glia, oligodendrocytes, or astrocytes.

In contrast to our understanding of human MCD anatomy, very little is known about the electrophysiological characteristics of dysplastic tissue. Where sophisticated patch-clamp analysis of tissue derived from surgery was performed, emerging data suggest diverse and, in some cases, clearly hyperexcitable firing properties for subpopulations of cells located within a malformation. Recent single-cell studies further describe (1) N-methyl-D-aspartic acid (NMDA)-mediated currents with decreased Mg^{2+} sensitivity and reduced current density and (2) reduced γ-aminobutyric-acid (GABA)-mediated inhibition onto dysplastic neurons and altered transporter function. Unfortunately, these types of studies are rare, controls are difficult to assign, and this tissue is not always amenable to the wide range of experimental manipulation necessary for testing mechanistic hypotheses.

To address this problem, several animal models have been proposed. Broadly, these animals can be classified as injury-based (e.g., irradiation, teratogen exposure, or freeze-lesion) or genetically-based (e.g., knockout mice, naturally occurring mutants, RNAi knockdown) (**Table 1**).

Table 1 Animal models featuring malformations of cortical development

Rodent model	*Malformation*
Postnatal FL rats	Polymicrogyria
In utero gamma irradiation rats	Nodular heterotopia Loss of lamination Cortical dysplasia Periventricular heterotopia
In utero methylazoxymethanol exposure rats	Nodular heterotopia Loss of lamination Cortical dysplasia Periventricular heterotopia
In utero N,N′-bis(2-chloroethyl)-N-nitrosurea (BCNU) exposure rats	Nodular heterotopia Loss of lamination Cortical dysplasia
In utero cocaine exposure rats	Microdysgenesis
Ihara rats	Microdysgenesis Heterotopia
Ibotenate injection rats	Periventricular heterotopia Band heterotopia
TISH rats	Band heterotopia
SRK	Loss of lamination Cortical dysplasia
RNA interference knockdown of *DCX* in the rat	Band heterotopia
p35 knockout mice	Granule-cell dispersion
Lis1 heterozygote mice	Cortical disorganization Loss of lamination
Otx1 heterozygote mice	Cortical disorganization
MEKK4 knockout mice	Periventricular heterotopia
NXSM and NZB autoimmune mice	Neocortical ectopia
Flathead (fh/fh) mutant mice	Lissencephaly Hydrocephaly
Presenilin-1-deficient mice	Cortical dysplasia
Fukutin-deficient mice	Neocortical ectopia Loss of lamination

MAM, methylazoxymethanol; FL, freeze-lesion; TISH, telencephalic internal structural heterotopia; SRK, shaking rat Kawasaki; *DCX*, *doublecortin*.

In many cases, these animals exhibit robust signs of hyperexcitability and even evidence for spontaneous electrographic seizures in a few examples. It is important to remember that many of these models have been developed or described in the last 10 years, and are not as widely studied as conventional kindling or temporal lobe epilepsy (TLE) animals; thus less information on the phenotypes is available. As these are rodent models with a central nervous system somewhat different from humans, it is not altogether surprising that no single animal model can reproduce the precise anatomical disorganization and behavioral symptoms observed clinically. Fortunately, recapitulation of the entire spectrum of abnormal cells found in human MCDs may be neither an animal model 'short-coming' nor a 'litmus' test requirement. Indeed, it is perhaps unnecessary

to reproduce in an animal model some of the cell types associated with MCDs in human. For example, balloon cells (a hallmark feature of human dysplasia) do not exhibit active firing properties and are likely to be electrically silent nonepileptogenic cells; thus, their absence from animal models should not reduce the usefulness of the model for studying mechanisms underlying epileptic discharge.

Methods

In our laboratory, we have chosen a rodent model with a readily identifiable brain malformation, e.g., prenatal methylazoxymethanol (MAM) exposure. MAM injected into pregnant rats is transported across the placenta into the fetus where it causes DNA methylation and primary damage to newly born cells. MAM-exposed offspring exhibit microencephaly and, if injections occur on embryonic day 15 (E15) in the rat, hippocampal nodular heterotopia with loss of cortical and hippocampal lamination (**Figure 1(a) and 1(b)**). Within the hippocampus, heterotopic neurons exhibit reduced dendritic growth (**Figure 2(a)**), decreased spine density, abnormal connectivity with neocortex, and are hyperinnervated by catecholaminergic fibers. Heterotopic neurons do not express cell-type-specific markers of hippocampal pyramidal or granule cells, but do express a tumor suppressor factor (*Id-2*) normally found in superficial layers of neocortex. White-matter abnormalities and periventricular nodular heterotopia are also present in MAM offspring from single and double prenatal injections, but are highly variable and not as reproducible as the hippocampal heterotopia. The consistent presence of a clear nodule(s) of heterotopic hippocampal cells in MAM animals presents a unique opportunity to identify and study the function, integration, or development of dysplastic neurons.

For most electrophysiological studies, acute hippocampal slices containing heterotopia are prepared from MAM-exposed rats. To efficiently study the intrinsic and synaptic properties of individual neurons within the regions of heterotopia, an infrared differential interference (IR-DIC) video microscopy set-up is commonly used. However, random sampling of neurons in acute slices using a sharp intracellular electrode filled with biocytin (and post hoc analysis of filled neurons) is also possible. Histopathological features of the malformed hippocampus have largely been studied using conventional single- and double-label immunohistochemical protocols. Tract tracing studies have been performed using in vivo injections of biotinylated dextran amine. Analysis of seizure threshold has largely relied on behavioral observation following acute administration of a convulsant (flurothyl, kainic acid, pentylenetetrazole, or kindling) and, in some cases, video-encephalographic (EEG) recording (**Fig. 1(c)**).

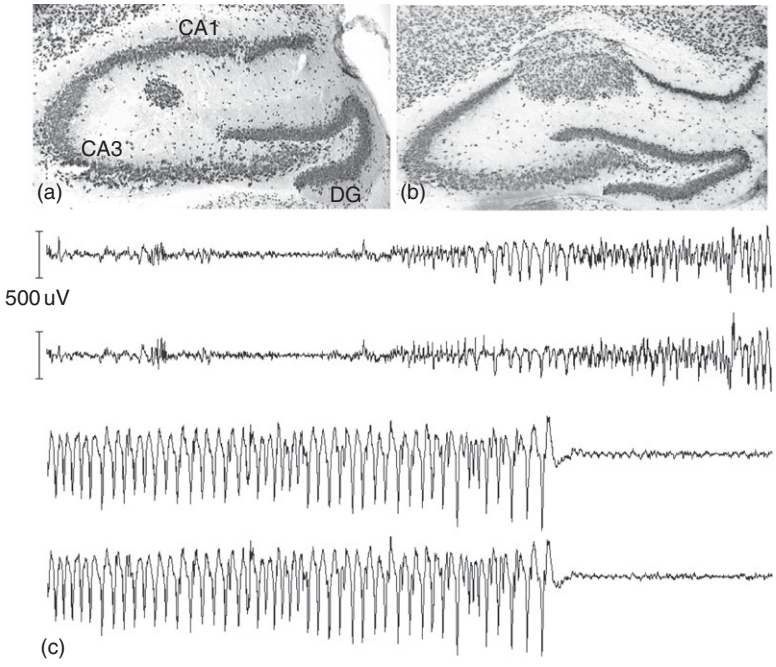


Figure 1 NeuN antibody staining to identify neuronal disorganization in the hippocampus of young adult rats exposed to methylazoxymethanol (MAM) in utero. A small nodule of pyramidal neurons in stratum lacunosum is shown in (a) and a larger cluster of heterotopic neurons disrupting stratum pyramidale (CA1) is shown in (b). Spontaneous seizure episode recorded from an MAM-exposed rat (c).

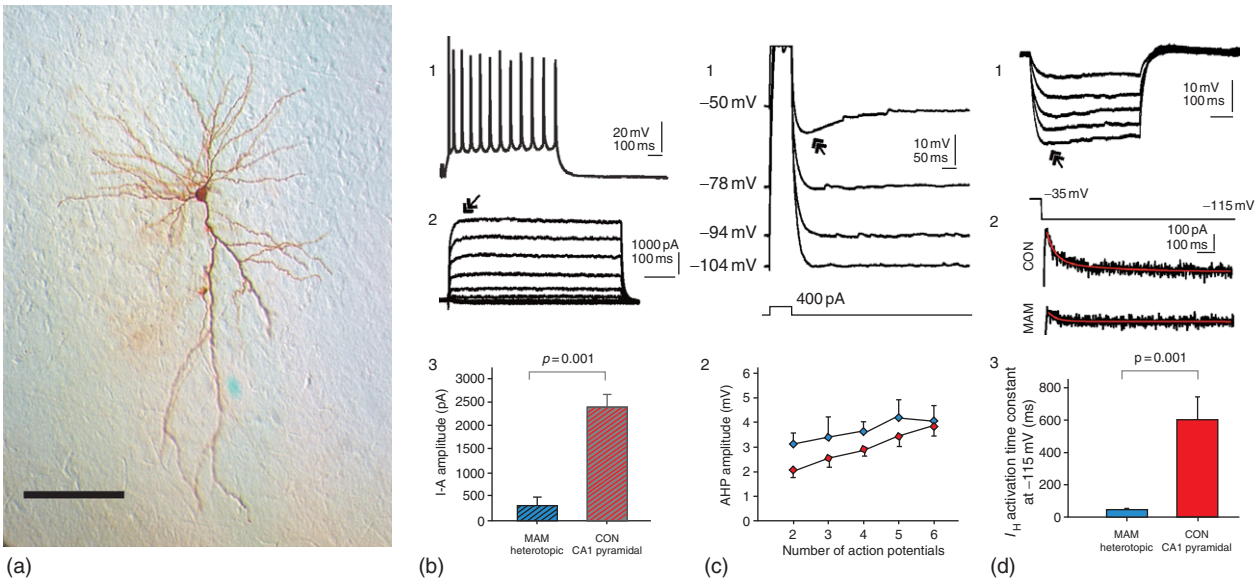


Figure 2 Representative biocytin-filled heterotopic neuron recorded under infrared differential interference (IR-DIC) visualization (a). A current-clamp recording from an identified heterotopic neuron showing increased firing activity (b1); a voltage-clamp recording showing a lack of fast A-type potassium current (arrowhead) is shown for a second heterotopic neuron (b2). A plot comparing the peak amplitude of A-type current for heterotopic ($n = 23$; blue bar) and age-matched normotopic CA1 pyramidal neurons ($n = 17$; red bar) (b3). A current-clamp recording showing spike-train elicited afterhyperpolarizations (AHP, arrowhead) at various holding potentials for a heterotopic neuron. Note the relative absence of an AHP current at more hyperpolarized potentials (c1). A plot comparing peak AHP amplitude following generation of increasing numbers of action potentials for heterotopic ($n = 12$; blue diamonds) and normotopic ($n = 8$; red diamonds) neurons. A current-clamp recording showing membrane sag (arrowhead) at hyperpolarizing step potentials for a heterotopic neuron. Note the relatively small sag current (d1). Voltage-clamp recordings of I_H and a fit of the activation time constant (red line) for age-matched heterotopic (MAM) and normotopic (CON) neurons (d2). A plot of the activation time constant for I_H measured at a step to −115 mV for heterotopic ($n = 7$; blue bar) and normotopic ($n = 7$; red bar) neurons (d3).

Recent Results

Abnormal clusters of pyramidal neurons (heterotopia; Figure 1(a) and 1(b)) first appear postnatally in the hippocampus of rats exposed to MAM in utero. Studying the evolution of hippocampal heterotopia, we noted that disruptions in the development of the embryonic neocortex contribute to this process. For example, marginal zone thickness and an increase in the density of reelin-positive Cajal-Retzius cells within the MZ (and in deeper areas) were observed 24–48 h after embryonic MAM exposure. Interestingly, these defects were associated with decreased chemokine stromal-derived factor-1 (SDF1) expression, and could be 'rescued' in vitro by the addition of this diffusible factor to the culture medium. Increased numbers of reelin-positive cells have also been reported in the tissue from patients with focal cortical dysplasia and cerebral microdysgenesis. Embryonic disruption of radial glial scaffolding noted in MAM-exposed rats (and ferrets) is another mechanism quite likely to contribute to loss of lamination and abnormal cell clustering observed in adult MAM rats. These results suggest that the MAM model may provide valuable insights to the etiology of clinical neuronal migration disorders.

By using in vitro and in vivo manipulations, abnormal clusters of hippocampal pyramidal neurons have consistently been shown to contribute to a hyperexcitable and proepileptic circuit. In these studies MAM animals, or hippocampal slices prepared from these rats, exhibit robust epileptiform activity following exposure to common convulsant manipulations. In nearly all cases, the activity was (1) easier to generate and/or (2) more robust when hippocampal heterotopia was present. Recent in vivo hippocampal recordings showed that afferent activation results in long-latency epileptiform afterdischarge in MAM rats but not in controls; similar results were reported in vitro. Despite these findings, obtained in several different laboratories and using a variety of manipulations, clear evidence for spontaneous seizures has been lacking. Similar to many new animal models of epilepsy, this putative deficiency is probably related more to a lack of good long-term video-EEG monitoring than to the absence of seizure-like activity. We recently addressed this problem in a relatively small sample of adult MAM rats ($n = 11$). Electrographic seizures were observed in two animals (Figure 1(c)). Seizures, classified as Level 3 on the Racine scale, were characterized by a sudden arrest in movement, face twitching, rearing, loss of posture, and hindlimb clonus.

Given the presence of identifiable clusters of dysplastic neurons and convergent evidence indicating hyperexcitability associated with these malformations, the MAM model emerges as a reasonable choice to study seizure generation in a malformed brain. This question is best addressed by considering (1) the intrinsic properties of dysplastic neurons and (2) synaptic communication among dysplastic neurons. Early studies indicated that heterotopic hippocampal neurons display bursts of action potentials in response to intracellular current injection. These findings led to a hypothesis that ion channels mediating these membrane properties were altered in the MAM model. In a series of voltage- and current-clamp studies on visually identified hippocampal heterotopic neurons (**Figure 2(a)**), we uncovered three different aspects of ion channel function that appear abnormal in the context of normotopically placed CA1 pyramidal neurons. First, a fast transient A-type potassium current thought to regulate the processing of synaptic inputs, the amplitude of back propagating action potentials, and action potential firing properties at the soma was nominal or nonexistent in these neurons (**Figure 2(b)**). This reduction in A-type current was associated with markedly reduced expression of Kv4.2 (*Shal*) potassium channel subunits in these neurons. Interestingly, reduced Kv4.2 function was later reported in a rodent model of TLE, and a channel truncation was identified in a patient with TLE, further implicating this current in an epileptogenic process. Second, a Ca^{2+}-activated potassium current responsible for hyperpolarizing the membrane after action potential firing (e.g., afterhyperpolarization, AHP) was found to be diminished in heterotopic neurons (**Figure 2(c)**). AHP currents are thought to play a critical role in preventing abnormal burst firing, and the presence of a robust AHP current can reduce epileptiform discharge. Third, a voltage 'sag' mediated by noninactivating depolarizing current activated upon membrane hyperpolarization (e.g., I_H) is markedly decreased in heterotopic neurons (**Figure 2(d)**). Similar to observations after perinatal seizure-inducing hypoxia and a mouse model of febrile seizures, it is likely that decreased I_H promotes epileptogenesis. Although several hyperexcitable and proepileptic alterations in the membrane properties of heterotopic neurons have been noted, defects in ion channel function for these cells are not global as we could find no evidence for changes in properties related to calcium or sodium channels.

Neurons communicate primarily via glutamate-mediated excitation and GABA-mediated inhibition. Given the severe disorganization of the canonical hippocampal trisynaptic circuit observed in MAM rats, changes in synaptic function are likely. Examination of excitatory neurotransmission in heterotopic hippocampi revealed a low threshold for the generation of abnormal discharge. Using a stimulating electrode or focal glutamate application, heterotopic neurons exhibited a prolonged depolarization with multiple action potentials in current-clamp mode (**Figure 3(a)**) or burst-like excitatory postsynaptic currents in voltage-clamp mode. These responses were blocked by D-2-amino-5-phosphonovaleric acid (D-APV), indicating a role for NMDA-type glutamate receptors. Although we could find no evidence for altered NMDA receptor subunit expression in immunohistochemical studies, single-cell electrophysiology recordings suggest an enhancement of the late NMDA-mediated evoked excitatory postsynaptic current (EPSC) component and reduced sensitivity to ifenprodil (an NR2B subunit antagonist). In contrast, voltage-clamp recordings and focal GABA application did not reveal gross changes in the properties of GABA-mediated synaptic inhibition. However, analysis of inhibitory postsynaptic currents (IPSCs) onto heterotopic neurons revealed a decrease in inhibitory tone consistent with hyperexcitability. We also uncovered a potentially compensatory prolongation of GABA-mediated currents linked to a reduction in GABA reuptake and diffuse expression of the hippocampal GABA transporters, GAT-1 and GAT-3, within the regions of malformation. These inhibitory changes and alterations in GAT expression were confirmed in tissue samples obtained from young adult epilepsy patients undergoing surgery for focal cortical dysplasia. Taken together, these results suggest that synaptic communication is disrupted in the hippocampal formation of rats featuring abnormal clusters of pyramidal neurons.

An additional observation from immunohistochemical studies was a profound reduction in GluR1 expression in and around hippocampal heterotopia. Reduced expression of this alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor subunit was observed as early as postnatal day 7 and persisted into adulthood. Because GluR1 subunits are essential to the extrasynaptic population of AMPA receptors and could play a critical role in synaptic plasticity, we performed slice electrophysiology studies to analyze long-term potentiation (LTP) in regions of heterotopia. Typical 100-Hz stimulation produced a strong several-fold increase in synaptic response recorded from normotopic CA1 pyramidal neurons, which decays within the next few minutes to a stable enhancement of synaptic transmission, i.e., LTP (**Figure 3(c)**). In contrast, the same stimulation protocol failed to induce LTP in any of the hippocampal heterotopic neurons tested. These findings are consistent with clinical observation of cognitive deficit associated with MCD and recent behavioral studies suggesting working memory deficits in MAM-exposed rats.

Future Directions

A major challenge in understanding epilepsy syndromes associated with MCD is to identify unifying principles of

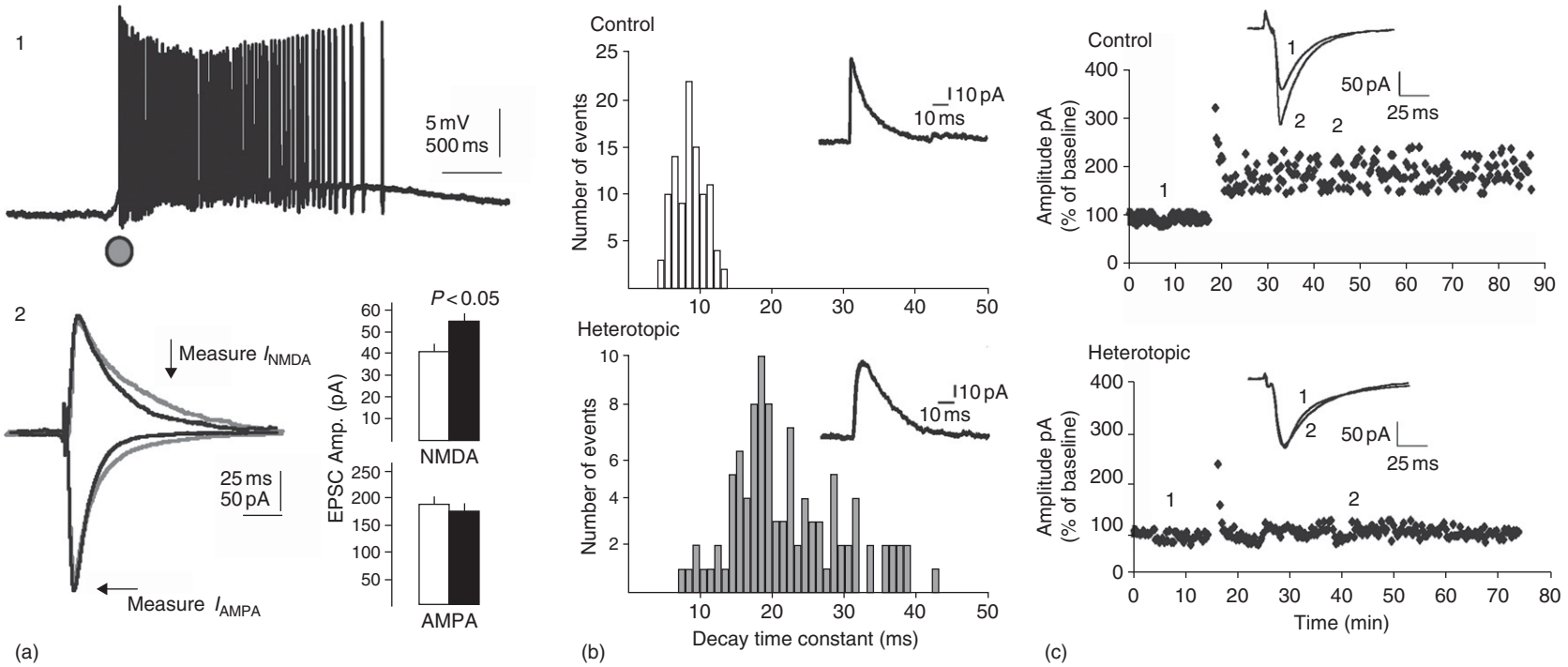


Figure 3 A current-clamp recording from an identified heterotopic neuron showing burst activity in response to focal glutamate application (circle) (a1). Two sets of overlayed evoked EPSCs obtained from normotopic (black traces) and heterotopic (gray) neurons held at −70 mV (to measure I_{NMDA}) and +40 mV (to measure I_{AMPA}) (a2). Plots comparing NMDA and AMPA current components for normotopic ($n = 60$; white bar) and heterotopic ($n = 90$; black) neurons. Representative inhibitory postsynaptic current (IPSC) decay time constant histograms and individual IPSCs (insets) for a normotopic (control CA1) and heterotopic neuron. Note the shift toward longer decay times (b). Absence of LTP onto heterotopic synapses in age-matched recordings (insets) of evoked EPSCs on a heterotopic and normotopic neuron following 100-Hz stimulation (c). Note the enhancement of evoked synaptic activity in control slices.

how seizures are generated in these pathological conditions. From acquired and genetic insults there exists a wide spectrum of MCDs. Although structurally diverse the mechanisms by which abnormal clusters of neurons generate seizure discharge may be similar. From MAM studies, we can predict several defects that directly influence excitability, e.g., loss of A-type potassium current, altered NMDA receptor function, and changes in GABA-mediated inhibition. Whether these defects are specific for this animal model, or universal to all MCD, remains to be systematically evaluated. Should unifying principles emerge from this line of investigation, then appropriate therapeutic treatments targeted to this form of epilepsy can be developed. If not, it is quite likely that each malformation subsyndrome will require individual analysis and syndrome-specific therapy. Animal models mimicking these various forms of MCD epilepsies would be an appropriate starting point for both types of research.

As regards treatment of intractable epilepsies associated with MCD, the MAM model may provide a few starting points. One can imagine designing a viral vector incorporating Kv4.2 potassium channel subunits and then targeting this vector for infection of hippocampal heterotopic neurons. This form of targeted 'gene therapy' would replace a channel shown to be lacking in these cells and could directly lead to restoration of heterotopic cell firing to 'normal' levels. If reduced A-current was a precipitating factor in the reduced seizure thresholds observed in MAM rats, then viral expression of this subunit would 'rescue' this phenotype. Similarly, the MAM model, similar to any rodent model of a human neurological disorder, could also be used for drug screening strategies. In vitro and in vivo testing of conventional antiepileptic drugs indicated that MAM rats, similar to clinical MCD disorders, are resistant to a range of standard antiepileptic drugs. As such, an anticonvulsant drug screening approach utilizing this model could uncover novel compounds effective against epilepsy syndromes associated with brain malformation.

See also: **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; Morphological and Electrophysiological Properties of Abnormal Cells in Pediatric Cortical Dysplasia Tissue; The Tish Rat: An Animal Model of Cortical Malformation in the Study of Epilepsy; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; Animal Models of Infant-Onset Epileptic Encephalopathy; Cortical Dysplasia and Catastrophic Epilepsies of Childhood; Epileptogenesis in Childhood.

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Quantitative and Qualitative Changes of Action of Antiepileptic Drugs in Developing Experimental Animals

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Introduction

Approximately 50% of epilepsies start at infancy or childhood. These epilepsies are treated with antiepileptic drugs. Most often, these drugs have been tested preclinically in adult laboratory animals and clinically in trials in adult patients. New antiepileptic drugs (AEDs) are, when first introduced, used for patients older than 12 years. However, after clinicians have some experience with these drugs, they are often introduced into pediatric epileptology by a 'trial-and-error' method. This approach works with many but not all drugs. Therefore, it is important for laboratories to study the action of anticonvulsant drugs (not only of new AEDs but also of those already used in patients) in the immature laboratory animal models.

Background: Mechanisms of Action of AEDs

Many AEDs possess more than one mechanism of action. For example, phenytoin, one of the most frequently studied drugs in the past, exhibits 11 possible actions which might play a role in its anticonvulsant effects. Therefore, the following discussion reflects a considerable oversimplication, but is designed to focus on the main/most important mechanisms of action relevant to seizure activity.

Of the AEDs in current use, there are two primary mechanisms of action: (1) Effects on ion channels and (2) Effects on neurotransmitter systems (mostly on receptors). With respect to the ion channel effects, use-dependent block of voltage-gated sodium channels has been described as a mechanism of action of phenytoin, carbamazepine, oxcarbazepine, lamotrigine, valproate, and zonisamide. Effects on calcium channels have also been described for phenytoin and carbamazepine, and a positive effect on potassium channels for retigabine. Ethosuximide, and to a lesser extent valproate and zonisamide, have specific effects on T-type calcium channels important for generation of the spike-and-wave EEG rhythm.

With respect to effects on neurotransmitter systems, the most common target has been the GABAergic inhibitory system. Potentiation of its action represents the main mechanism of action of phenobarbital, valproate, benzodiazepines, tiagabine, and vigabatrin. In the case of the last three drugs, the drug's effect on GABAergic inhibition represents the only documented mechanism of anticonvulsant action. Drug effects to suppress excitatory neurotransmission has been compromised by serious, unwanted side effects of excitatory amino acid receptor antagonists; therefore, if this action is possessed by an AED, it does not represent the drug's main mechanism of action. Antagonism of the glutamatergic system has been described for topiramate (antagonism of kainate type of glutamate receptors), felbamate (effect on *N*-methyl-D-aspartate receptors), and (perhaps questionably) lamotrigine (decrease of glutamate release).

Some of the newer 'third-generation' AEDs exhibit completely different mechanisms of action – for example, regulation of release of transmitters from presynaptic endings (e.g., levetiracetam's effect on SV2A protein, gabapentin, and pregabalin effects on a subunit of calcium channels).

Methods: Pentylenetetrazol-Induced Motor Seizures in Rats

To assess drug effects in immature animals, we have developed a useful and relatively simple approach. We first elicit convulsions with pentylenetetrazol in four age groups of immature rats and a group of adult animals. Age groups were initially selected according to comparative data on brain development: 7-day-old rats correspond to human premature newborns, 12-day rats correspond to the early postnatal stage, and 18- and 25-day-old rats approximate to late preschool and school-age children, respectively. The subcutaneously administered dose of pentylenetetrazol (100 mg kg^{-1}) is able to induce two different types of seizures in adult rats – a 'minimal' response consisting predominantly of clonic seizures (the minimal Metrazol seizures described in the old literature), and after a longer latency, generalized tonic-clonic seizures. The same pattern is found for 25- and 18-day-old rats. Pentylenetetrazol does not elicit minimal seizures but only GTCS in the two youngest groups.

Results

Action of AEDs on PTZ-Induced Convulsions

AEDs can be divided into four groups according to their action in adult rats: (1) drugs suppressing both minimal

clonic and GTCS; (2) drugs suppressing only GTCS; (3) drugs specifically suppressing the tonic phase of GTCS; and (4) drugs without a reliable effect in this PTZ model. The first group is typically represented by antiepileptics acting through potentiation of GABAergic inhibition: phenobarbital; the benzodiazepines clonazepam, midazolam, clobazam, bretazenil, and flumazenil (a benzodiazepine receptor antagonist with mixed agonist properties); tiagabine; and valproate. In addition, a drug with a different mechanism of action; ethosuximide – also suppresses both minimal and generalized seizures. Nearly all these drugs suppress GTCS at low dose, and only when the higher dose is administered abolish minimal clonic seizures. An exception is tiagabine and another GABA reuptake blocker, NNC-711, which are more active against minimal clonic seizures than against generalized tonic-clonic ones. The second group is represented by primidone (effects examined shortly after administration when conversion to phenobarbital does not play a role), vigabatrin, felbamate, and progabide, which were found to suppress GTCS but left minimal clonic seizures intact. This effect is also characteristic for antagonists of *N*-methyl-D-aspartate type of glutamate receptors. The third group consists of phenytoin, carbamazepine, oxcarbazepine, hydroxycarbamazepine, lamotrigine, topiramate, felbamate, and gabapentin. The sole member of the fourth group is levetiracetam, which is inactive against PTZ-induced convulsions.

The action of AEDs in immature rats is summarized in Table 1.

All the drugs of the first group except tiagabine suppress GTCS in all age groups; tiagabine has an irregular action – suppression of GTCS in 7- and 25-day-old rats, but suppression only of the tonic phase of GTCS in the other two age groups (12 and 18 days). Minimal clonic seizures are invariably suppressed in both 18- and 25-day-old rats by all drugs in this group. Pretreatment with some of these drugs leads, paradoxically, to an appearance of minimal seizures in 7- and 12-day-old animals; this effect is common with phenobarbital and with low doses of bretazenil and flumazenil.

The second group exhibits more variable effects in immature animals. Primidone and progabide are without effect on GTCS in 25-day-old rats; primidone suppresses GTCS in the three younger age groups, whereas progabide is effective only against the tonic phase. Specific suppression of the tonic phase of GTCS is also exhibited by felbamate in all studied age groups. Vigabatrin suppresses the tonic phase in all but 7-day-old rats, in which GTCS are completely abolished. With respect to minimal seizures, only high doses of felbamate are able to suppress them in 18-day-old rats; other drugs are without effect in both 18- and 25-day-old animals. Primidone administration in 7- and 12-day-old rats results in an appearance of minimal seizures.

AEDs of the third group abolish the tonic phase of GTCS in all age groups. The exceptions are carbamazepine and oxcarbazepine, which suppress GTCS as a whole in 7- and 12-day-old rat pups, and topiramate, which abolishes the tonic phase of GTCS in 7-day-old animals only. Minimal seizures are left untouched by lamotrigine and topiramate, and suppressed in 18-but not in 25-day-old rats by gabapentin. Carbamazepine pretreatment induces minimal seizures in 7- and 12-day-old rat pups; phenytoin does not have this action in the two youngest groups, but it markedly potentiates these seizures in 18-as well as 25-day-old rats. The potentiation is so marked that it is possible to speak about the 'status' of minimal seizures.

Table 1 Effects of antiepileptic drugs in developing rats

Seizure type	*Generalized tonic-clonic seizures*				*Minimal clonic seizures*			
Age (postnatal day)	P7	P12	P18	P25	P7	P12	P18	P25
Phenobarbital	↓	↓	↓	↓	↑	↑	↓	↓
Primidone	↓	↓	↓	0	↑	↑	0	0
Valproate	↓	↓	↓	↓	0	0	↓	↓
Clonazepam	↓	↓	↓	↓	0	0	↓	↓
Clobazam	↓	↓	↓	↓	0	0	↓	↓
Midazolam	↓	↓	↓	↓	0	0	↓	↓
Bretazenil	↓	↓	↓	↓	↑	↑	↓	↓
Flumazenil	↓	↓	↓	↓	↑	↑	↓	↓
Tiagabine	↓	↓T	↓T	↓	0	0	↓	↓
Vigabatrin	↓	↓T	↓T	↓T	↓	↓	0/↑	0/↑
Felbamate	↓T	↓T	↓T	↓T	0	0	↓	0
Progabide	↓T	↓T	↓T	↓	0	0	0	0/↑
Carbamazepine	↓	↓	↓T	↓T	↑	↑	0	0
Oxcarbazepine	↓	↓	↓T	↓T	0	0	0	0
HydroxyCBZ	↓T	↓T	↓T	↓T	0	0	0	0
Phenytoin	↓T	↓T	↓T	↓T	0	0	mMS status	mMS status
Lamotrigine	↓T	↓T	↓T	↓T	0	0	0	0
Etosuximide	↓	↓	↓	↓	0	0	↓	↓
Topiramate	↓	↓T	↓T	↓T	0	0	0	0
Gabapentin	↓T	↓T	↓T	↓T	0	0	↓	0

There are no data on possible actions of levetiracetam in immature animals; it has not been tested in this context because of its inactivity against pentylenetetrazol-induced convulsions in adult rodents.

Summarizing the action of AEDs in immature rats it is necessary to conclude that the action is quite variable – both quantitative and qualitative changes are possible.

Quantitative Changes of Anticonvulsant Action

There are old data on hexobarbital and phenobarbital action against pentylenetetrazol-induced seizures, demonstrating only quantitative changes of their action. These barbiturates are more potent in immature rodents, not only in their anticonvulsant action but also in their effects on sleep time. Similar effects were demonstrated for the anticonvulsant action of benzodiazepines. A common finding is that there is a longer duration of action (e.g., as seen with benzodiazepines) in 12-day-old rats than in older animals, probably due to an immaturity of catabolic enzymes and possibly other factors playing a role in the elimination of AEDs.

Qualitative Changes of Action

With respect to minimal clonic seizures, the most marked qualitative change is the appearance of minimal clonic seizures in 7- and 12-day-old rat pups; that is, in age groups where pentylenetetrazol is unable to elicit minimal seizures under control conditions. This effect is exhibited by AEDs classified in all three groups (based on their activities in adult rats). The generator of minimal clonic seizures is localized in the basal forebrain, and can be activated by other convulsant drugs (e.g., bicuculline or isonicotinehydrazide) even at very early stages of postnatal life. The mechanisms by which some AEDs (with various mechanisms of action, including potentiation of GABAergic inhibition, and effects on voltage-gated sodium channels) permit pentylenetetrazol to activate the generator of minimal seizures remain to be studied. Marked potentiation of minimal seizures by phenytoin, leading to nearly continuous seizures, is another phenomenon to be explained, as is the difference of effects of felbamate and gabapentin between 18- and 25-day-old rats.

Effects on GTCS appear to be less variable than those on minimal seizures. The relatively common qualitative difference between adult and immature rats is suppression of GTCS in the youngest animals, replaced during development by a specific action against the tonic phase. A lack of effect of primidone and progabide in 25-day-old rats, in contrast to their efficacy in adult as well as younger rats, is still to be explained. The effect may be related to the action of the drug on $GABA_B$ receptors in the case of progabide, but such a mechanism has not been demonstrated for primidone.

Future Directions

Data on the action of AEDs in the model of pentylenetetrazol-induced convulsions in the developing rats do not present a simple picture and indeed give rise to many more new questions than answers. At present, it is impossible to predict the efficacy of AEDs in pediatric neurology from preclinical experiments or from clinical data in mature brain. Even drugs with similar primary mechanisms of action can exhibit different effects in immature laboratory animals (e.g., appearance of minimal clonic seizures in 7- and 12-day-old rats after some, but not all, benzodiazepines). More studies are necessary to elucidate the age-related changes in anticonvulsant action, even in the present model. To predict clinical efficacy of a given drug at a given age, more models must be examined, including a model of complex partial seizures (e.g., hippocampal afterdischarges) and a model of absence seizures (e.g., pharmacologically induced spike-and-wave episodes). All these models give information about anticonvulsant, but unfortunately not about the antiepileptic action of these drugs. To study this antiepileptic phenomena, it is essential to examine drug effects in chronic models of epilepsy.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy; **Models**: Model Characterization in Relationship to Human Disorders; **Pediatric Epilepsy**: Age-Specific Responses to Antiepileptic Drugs; Developmental Aspects of Seizures.

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Seizure-Induced Injury and the Two-Hit Hypothesis

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Introduction

Repeated or prolonged seizures may result in a striking behavioral syndrome comprised of cognitive decline and selective impairment of declarative memory. This consequence of seizures is a major cause of long-term morbidity in patients with epilepsy and in patients with prolonged or repeated convulsions that occur in the setting of metabolic disturbance. Elegant neuropsychological studies have implicated the hippocampus and related structures in the pathophysiology of this acquired behavioral disorder. Human neuropathological studies and examination of animal models of epilepsy have repeatedly demonstrated evidence of selective neuronal vulnerability in hippocampus and other limbic structures after seizures. Clinical and experimental studies suggest that seizure-induced neuronal injury may lead to altered network properties and the establishment of a chronic epileptic condition. The molecular and biochemical mechanisms of neuronal injury following seizures, and the precise relationship between seizure-induced neuronal injury and subsequent recurrent seizures, remain unclear.

Background

Seizure-Induced Neuronal Injury is Anatomically Selective

An important feature of seizure-induced neuronal injury is its striking anatomic selectivity. Clinical studies have shown that pyramidal cells of the CA1 and CA3 hippocampal regions, as well as hilar neurons, are selectively vulnerable; in contrast CA2, subiculum, and certain peptidergic classes of interneurons are relatively resistant to injury. Dentate granule cells, once thought to be resistant to seizure-induced injury, have more recently been shown to be at least partially vulnerable. Animal models have been variably successful in recapitulating these findings. For example, KA-induced seizures result in injury in CA1 and CA3, but do not appear to affect granule cells, whereas pilocarpine-induced seizures or chronic perforant path stimulation clearly result in both the injury of the Cornu Ammonis and granule cell loss.

Vulnerability to Seizure-Induced Injury is Age-Dependent

Another key characteristic of seizure-induced neuronal injury is its striking age-dependence. Young children and immature rodents (<P21, postnatal day 21), while highly susceptible to seizures, are largely resistant to seizure-induced neuronal injury. While the precise mechanism of age-dependent resistance to injury remains unclear, it is likely that several characteristics of the developing nervous system contribute to it. Maturation of the nervous system involves a series of steps that occur postnatally in the rodent. Neurogenesis is followed by the development of dendrites – a process that peaks at about P15 – and then the elaboration of dendritic spines – which peaks at about P20. Interestingly, it is the appearance of spines that seems to correlate most closely with the development of vulnerability to seizure-induced neuronal injury. All these developmental structural events take place in a changing neurochemical milieu. For example, neurotrophic factors and their receptors, particularly NGF, reach their maximum levels at P15. Subsequent decreases in NGF levels parallel the decline in synapse number that is thought to be mediated by activity-dependent processes. These observations suggest three distinct hypothetical mechanisms that could mediate early-life resistance to activity-dependent neuronal injury: (1) neurons may be protected by an immature dendritic arbor lacking spines; (2) they may be protected by an excess of synapses and arbors; and finally (3) they may be protected by an endogenously generated abundance of trophic or protective factors. In preliminary studies, we have found evidence that the latter mechanism is, at the very least, an important contributor. Another important possibility is that during development, neurons are protected against seizure-induced cell death by a failure of activation, transduction, or execution of cell death programs. As apoptosis is a ubiquitous and normal phenomenon in the CNS during early post-natal life, it is unlikely that the required molecular elements are absent. Rather, we have hypothesized that seizures in the immature brain are either incapable of or inadequate to activate existing apoptotic pathways. Our preliminary observation that seizures lead to an increased abundance of the pro-enzyme procaspase-3, but not its active cleavage product caspase-3, supports this hypothesis.

Methods

Sprague-Dawley rats were designated; K (Kainate (KA) on P15 only), SS (saline on P15 and P45), SK (saline on P15 and KA on P45), KS (KA on P15 and saline on P45), and KK (KA on P15 and P45). On P15, 5–8 rats from each litter were treated with KA (5 mg/kg intraperitoneally

[ip] in phosphate-buffered saline [PBS], pH 7.4). Littermate controls received equal ip volumes of PBS. On P45, animals in the SK and KK groups received KA (15 mg/kg ip), whereas animals in the SS and KS groups received equal volumes of PBS (200–250 mL i.p.). Age-specific doses of KA used in the present study were previously determined to result in 25–40% mortality while inducing seizures in over 60% of animals. On P50, all surviving animals began testing in a modified Morris water maze. Animals were killed on P55 by transcardiac perfusion and brains were processed for histology and for DNA fragmentation assayed by in situ nick translation. We have previously shown that seizure-induced DNA fragmentation is maximal by 48–72 h and remains detectable for 10 days after KA-induced seizures.

Behavioral Observation and Seizure Grading

After KA injections, animals were closely observed and their behavior was recorded for 6 h, as previously described. Latency to the first clinical manifestation of seizure activity was recorded. A seizure severity grade was assigned based on the maximal response achieved, on a scale from 0 to IV as follows: 0, no response; I, wet dog shake (WDS) and/or behavioral arrest; II, WDS, staring, pawing, and clonic jerks; III, WDS, staring, pawing, clonic jerks, rearing, and falling; and IV, continuous grade III seizures for longer than 30 min (status epilepticus).

Behavioral Testing

A modified Morris water maze was used to assess visuospatial learning and memory. A circular steel tank (117 cm diameter) was filled with water to a depth of 25 cm. The water was made opaque by an addition of 100 mL of evaporated milk. On day 1, each rat was placed in the pool for 60 s with no platform present for a "free swim." On days 2–5, rats were trained for 24 trials, 6 trials per day, to locate and escape onto a plexiglass platform (8 × 8 cm) placed 1.5 cm under the water surface. For each rat, while the quadrant in which the platform was located remained constant, the point of immersion into the pool varied in a quasi-random order for the 24 trials. The latency from immersion into the pool to escape onto the platform was recorded.

Detection of DNA Fragmentation by in situ Nick Translation Histochemistry

Animals were killed by transcardiac perfusion with 50 mL of PBS followed by 200 mL of ice-cold 4% paraformaldehyde. Brains were removed, postfixed, and then cryoprotected in 20% sucrose overnight. Fifty-micrometer horizontal sections were cut and processed for in situ nick translation to detect fragmented DNA. Adjacent sections from each animal were stained with cresyl violet to allow assessment of neuronal loss.

Cell Counts

To quantify DNA fragmentation, sections were analyzed by light microscopy using a grid reticule. Counts of positively stained cells in each hippocampal subfield (hilus, CA4, CA3, and CA1) were made from both right and left hippocampii and averaged. A total of 204 hippocampal sections were analyzed from 34 animals (three sections per animal, two hippocampi per section).

Recent Results

Early-Life Seizures Increase Vulnerability to Later Life Seizure-Induced Injury

Considerable evidence suggests that seizures occurring in the immature brain, while not resulting in overt injury, nonetheless, result in a long term change in the nervous system that renders the animal more susceptible to seizure-induced neuronal injury, with associated behavioral deficits, later in life. We have shown that kainic acid (KA)-induced seizures on P15 result in neither immediate (48 h) nor delayed (35 days later, on P50) detectable injury. Animals subjected to KA on P15, however, showed potentiated behavioral (convulsive) responses to KA when re-challenged on P45. Furthermore, these animals showed enhanced cellular injury and striking behavioral abnormalities compared to naïve controls and to animals treated with KA only on P15 or P45.

Similar findings have been reported from other groups using a variety of models. For example, Holmes and colleagues showed that flurothyl seizures early in life potentiated the effects of pilocarpine, KA, and kindling later in life. Baram and colleagues showed that hyperthermic seizures induced on P10–P12 had a similar result. Jensen and colleagues have shown that hypoxia-induced seizures on P10 increase susceptibility to spontaneous and kainate-induced seizures later in life. We have referred to this phenomenon as the "two-hit phenomenon," but perhaps a more descriptive term is that events occurring early in life "prime" the central nervous system, making it especially susceptible to later insults. Notably, the first "hit" need not be epileptic. Previous studies have shown that in rats with a toxin-induced neuronal migration defect, hyperthermia may result in hippocampal injury independent of seizures. Moreover, animals with neuronal migration defects are more susceptible to kainate and kindling, while immature and also as adults. Together with our investigations, these studies suggest that a variety of insults (including but not limited to seizures) during development can increase the susceptibility to seizures in adults and enhance their injurious effect. Of note, some researchers have described a protective effect of mild seizure activity with respect to subsequent seizure-induced injury. It is possible that the severity of the initial

event is critical in determining whether it protects against or predisposes to future injury.

The Mechanism of the Two-Hit Phenomenon is Unknown

The two-hit phenomenon implies that a mechanism exists to transduce brief episodes of paroxysmal activity into long-lasting changes in excitability. Potential underlying mechanisms include, but are not limited to, long-lasting changes in connectivity, cellular phenotype, receptor phenotype, or biochemical milieu – each induced by the early-life event and persisting into adulthood.

In our studies, the detrimental long-term pathologic and behavioral consequences of early KA seizures became evident only when the animals were subjected to another insult later in life. This observation highlights the possibility that early KA seizures alter neuronal connectivity or function, even in the absence of detectable neuronal injury or cell death, in a manner that increases susceptibility to later seizure-induced damage. During normal development, neurons as well as axons and synaptic terminals are overproduced and subsequently eliminated, apparently as a result of a competition between afferents for a limited supply of target-derived substance. In rat CA3 hippocampal pyramidal cells, for example, an exuberant outgrowth of axons takes place by P10, and axon length, number of varicosities, and axon branch points reach up to 225% of mature levels during the second postnatal week. This exaggerated development coincides with the period of enhanced seizure susceptibility. Synaptic activity is critical for the process of refinement of connections between axons and their appropriate targets. It is thus conceivable that a prolonged seizure, via intense synchronized neuronal activation, could have a permanent effect at this critical time of afferent-dependent synaptic remodeling. A precedent for this notion can be found in a study in which continuous infusion of penicillin was used to induce epileptiform activity in the visual cortex of rabbits from P5 through P21. In penicillin-exposed rabbits, visual callosal projections persisted through most of area 17 in a pattern characteristic of neonatal rabbits, rather than being restricted to a narrow zone at the lateral border of area 17 as seen in normal mature animals. These results suggest that epileptic cortical activity stabilized immature callosal projections that are normally eliminated during development. An alternative mechanism has been demonstrated by Jensen and colleagues, who have shown that early life seizures lead to an altered expression of glutamate receptor (GluR2) subunits, resulting in an increased expression of Ca^{2+}-permeable AMPA receptors on hippocampal neurons. In their study, treatment with the AMPA-antagoists NBQX or topiramate reduces or prevents the expression of this two-hit phenomenon.

Endogenous Neuroprotection and the Two Hit Phenomen May Share Common Mechanisms

We have been particularly interested in the hypothesis that early seizures, perhaps by stabilizing immature synaptic connections, result in the persistence of a hyperexcitable neuronal network later in life. Such a process could be the result of paroxysmal seizure-induced trophic factor synthesis occurring in the setting of an already high ambient level of NGF. Numerous studies have demonstrated hat NGF synthesis is increased by seizures, and furthermore that a host of NGF-inducible genes are regulated by seizures in anatomically relevant structures. One might predict that synaptic connectivity would demonstrate increased complexity in this scenario, but evidence to support that notion has not been forthcoming. In fact, Swann and colleagues have shown that under certain circumstances, early seizures appear to result in a simplification of synaptic connectivity with reduced spine number and arborization. It remains unclear just how far this finding can be generalized, and (more importantly) what its effect on subsequent excitability might be. Nonetheless, there is clear evidence that, regardless of its effects on spine complexity, NGF may be protective against seizure-induced neuronal loss and therefore lead to stabilized or persistent neuronal circuits that could mediate the two-hit phenomenon.

To investigate the mechanism of NGF-mediated protection against seizure-induced neuronal injury, we have queried whether the protective effect of NGF is unique or is shared with other neurotrophins. We reasoned that if BDNF and NGF share a neuroprotective property in our models, then that property could not be dependent on specific Trk receptor coupling, but would more likely represent either a pan-Trk effect or a specific effect of NGF mediated through the shared low affinity p^{75ntfr}. In our hands, infusions of BDNF were not protective, nor were bFGF infusions. These results forced us to focus on TrkA or a unique NGF effect mediated through p^{75ntfr}. Because in immunohistochemical studies we could find no significant expression of TrkA (the preferred target for NGF) in adult hippocampus, we chose to focus on the low affinity receptor instead, which is expressed in basal forebrain and in cholinergic terminals projecting to hippocampus.

We initially attempted to test the role of p^{75ntfr} utilizing transgenic mice expressing a defective receptor. Those experiments were unsuccessful for two reasons. First, we had considerable difficulty establishing a breeding program to produce adult animals, mainly due to maternal rejection of the pups. Second, we have found that mice respond to convulsant drugs with an extraordinarily steep dose—response relationship, resulting in either inadequate seizures or early mortality in a high percentage of (often scarce) animals. We, therefore, abandoned these

studies and shifted our attention to animals with an acquired defect in p^{75ntfr}. We chose to by lesion $p^{75ntfr+}$ neurons in immature animals and then looking for a loss of resistance to injury. These studies proved to be fortuitous and dramatic. We found that in animals treated with IgG-192-saporin, a selective immunotoxin that lesions $p^{75ntfr+}$ neurons, an adult pattern of injury resulted from KA-induced seizures on P15. We confirmed that immunotoxin lesions resulted in abolition of $p^{75ntfr+}$ neurons and terminals using both p^{75ntfr} immunohistochemistry and AChE staining. We also saw well preserved cholinergic neurons in these animals in striatum, indicating that the toxin was not lesioning cholinergic neurons indiscriminately. These studies have led us to conclude that high ambient levels of NGF, present during development, serve as an endogenous neuroprotectant.

Conclusions and Directions for Future Research

It will be important to follow up this preliminary observation with two key experiments. First, we must show that NGF levels are indeed high on P15 in our animals. Second, we must go back to the adult animals and show that lesioning $p^{75ntfr+}$-neurons block the neuroprotective effect of NGF against seizure-induced hippocampal injury. If these points can be adequately demonstrated, then we will be in a position to test the hypothesis that the two-hit phenomenon is a maladaptive consequence of an endogenous neuroprotective system mediated by NGF effects on neurons expressing the p^{75ntfr}.

In conclusion, substantial evidence supports the existence of a two-hit phenomenon to which early-life seizures and/or other specific pathological abnormalities confer, and it also supports enhanced susceptibility to later life seizures and their consequences. NGF appears to have an important neuroprotective role, and selective lesions of $p^{75ntfr+}$ neurons confer a unique vulnerability to seizure-induced injury on immature brain. It is tempting to speculate that normal actions of $p^{75ntfr+}$-neurons, perhaps by providing enhanced or persistent direct cholinergic input to the hippocampus, underlie at least part of the two hit phenomenon.

If, in fact, the two-hit hypothesis represents a general mechanism of epileptogenesis, it supports the notion that the development of epilepsy is a multistep processs that occurs in a temporally distributed fashion. If true, it may be possible to intervene at many points during the process of epileptogenesis to effectively prevent the development of the epileptic phenotype. This view of epileptogenesis has obvious ramifications for the development of anti-epileptogenic treatments and the need to treat pre-symptomatic individuals.

See also: **Epileptogenesis**: Multi-Hit Mechanisms Involved in Epileptogenesis: Role of Early-Life Seizures; **Glutamate**: Glutamate-Mediated Excitation and Mechanisms of Epileptogenesis in the Immature Brain; **Imaging**: Seizure-Related Acute MRI Changes; **Neurotrophic Factors**: Role of BDNF in Animal Models of Epilepsy; **Pediatric Epilepsy**: Cellular, Molecular and Behavioral Consequences of Early-Life Seizures; Developmental Aspects of Seizures.

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PEPTIDES

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Cannabinoids and Epileptiform Activity

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Introduction

Marijuana (*Cannabis sativa*) has been used recreationally by human beings for millennia. Historically there also has been interest in possible medicinal or therapeutic applications of *Cannabis*, including its possible effects on seizures in patients with epilepsy. Presumably because it is so widely used and readily available (albeit illegal), *Cannabis* has become an attractive candidate for development as an antiepileptic drug. Anecdotal reports from patients about the effects of *Cannabis* on their epileptic seizures have been mixed: Although some have reported that their seizures are suppressed or 'made better,' others have reported that their seizures become worse. Unfortunately, there have been no publicly accessible controlled laboratory studies of the effects of *Cannabis* or of its major active ingredient, tetrahydrocannabinol (THC), on epileptic activity in humans, although proprietary clinical trials with cannabinoids may be underway. The only controlled study published in any detail examined the effects of a cannabinoid that is supposedly not psychoactive, cannabidiol (CBD), in a group of patients whose epilepsy was resistant to conventional antiepileptic drugs; this study reported that CBD produced significant beneficial effects in a majority of the patients. However, the neural bases of the actions of CBD are unknown, and the observation has apparently not been replicated in the ensuing 27 years. So nearly all of what we know about the potential effects of cannabinoids on seizures has come from studies in animals experiencing various forms of experimentally triggered epileptiform activity.

Background

The availability of pure samples of THC in the 1970s resulted in a spate of studies of the effects of THC and other cannabinoids on seizures in animals. In those studies, researchers triggered convulsions in rats and mice by electroconvulsive shock (ECS) or convulsant drugs, and in epileptic baboons by strobe light; other studies employed the kindling preparation, in which generalized seizures develop gradually in response to intermittent electrical stimulation of forebrain sites. The results were generally disappointing. Although THC exerted anticonvulsant effects against several types of convulsion, these effects were usually at high and toxic doses. THC could delay the progression of kindling, but it was unclear whether this result was due to a genuine prophylactic effect or merely to acute suppression of early-stage seizures. Because of the relatively unimpressive results, and in part because of our lack of knowledge about the biological mechanism of cannabis's actions, research on potential antiepileptic effects had largely ceased by the early 1980s.

Recent Knowledge of Cannabinoid Action

Understanding of the neurobiological basis of cannabis's activity has increased significantly in the past 15 years or so. It is now known that the body contains several different endogenous cannabis-like compounds, the endocannabinoids, which bind to specific receptors. The most thoroughly investigated of the cannabinoid receptors are

the CB1 and CB2 receptors, although other types of CB receptor are probably present. Cloning of CB receptors has led to the development of artificial cannabinoid drugs that either mimic cannabis and activate the receptors (agonists) or bind to the receptors and block the actions of endocannabinoids (antagonists). WIN 55,212-2 and HU210 are examples of potent agonists at CB1 and CB2 receptors, and AM251, AM281, and SR141716A are selective antagonists of CB1 receptors.

One of the important recent discoveries about the action of endocannabinoids in the hippocampus and elsewhere is that they are released from depolarized postsynaptic membranes, and bind in a retrograde fashion with presynaptic CB1 receptors, resulting in decreased release of the neurotransmitter from the presynaptic membrane. Experimentally, endocannabinoids at higher concentrations can suppress release of the excitatory neurotransmitter glutamate; however, much lower concentrations of endocannabinoids suppress release of GABA at inhibitory GABAergic synapses, suggesting that cannabinoid action at inhibitory synapses is more significant functionally. The phenomenon at inhibitory synapses has been referred to as depolarization-induced suppression of inhibition (DSI). When cannabinoids act at CB1 receptors on GABAergic terminals, the consequence of their actions is disinhibition, or increased excitability.

The availability of selective agonists and antagonists for CB receptors has sparked a resurgence of research on the potential antiepileptic effects of cannabinoids. From the forty or so laboratory studies of the effects of synthetic cannabinoids on epileptiform activity published over the last decade, we will focus on experiments from two laboratories that have produced somewhat divergent results. These experiments provide the context for our own research, described subsequently.

A comprehensive series of experiments has recently emerged from De Lorenzo's laboratory at Virginia Commonwealth University, characterizing the effects of cannabinoids on convulsions in several preparations of experimental epilepsy. He and his colleagues have shown that the CB agonist WIN 55,212-1 suppresses convulsions induced by maximal electroshock, as does the endocannabinoid anandamide. These effects are blocked by the antagonist SR141716A, implicating specific action at the CB1 receptor. Furthermore, when administered by itself, the CB antagonist reduces seizure threshold, implicating endocannabinoid tone in the ongoing modulation of seizures. Cannabinoids have similar effects on spontaneous seizures produced by treatment with pilocarpine, and acute pilocarpine-induced seizures are associated with increased concentrations of the endocannabinoid 2-arachidonylglycerol (2AG) and increased expression of the CB1 receptor protein. In another preparation of acquired epilepsy, WIN 55,212-2 abolishes seizure discharge provoked by low Mg^{2+} in primary hippocampal cultures. Because CB antagonists enhance epileptiform activity in cultures treated with low Mg^{2+}, it has been concluded that CB1 receptor-dependent endocannabinoid tone plays an important role in modulating seizures and preventing epileptic status. Collectively these results suggest that endocannabinoids dampen seizure activity, and that cannabinoids might be useful pharmacotherapies in epilepsy.

The results of experiments from Soltesz's laboratory at the University of California, Irvine, with a different preparation of acquired epilepsy, present a somewhat divergent picture. He and his colleagues have found that a single episode of a prolonged febrile convulsion in neonatal rats results in a long-lasting enhancement of cannabinoid-induced suppression of GABA release in the hippocampus – that is, a potentiation of DSI measured at adulthood. The potentiation of endocannabinoid-mediated DSI is associated with increased density of presynaptic CB1 receptors on GABAergic neurons containing cholecystokinin. Thus a sustained epileptiform event early in life results in a persistent proepileptic effect mediated by endocannabinoids. Subsequent research by Soltesz's group has shown that administration of the CB1 antagonist SR141716A during febrile episodes blocks the potentiation of DSI, the upregulation of CB1 receptors, and the enhancement of seizure susceptibility detected later in life. These results are a further demonstration of a long-lasting proepileptic effect established in the hippocampus by the action of endocannabinoids. Note, however, that Soltesz's group observed that, notwithstanding the long-term proepileptic action of endocannabinoids, the CB1 antagonist SR141716A itself produces a proepileptic effect against kainic acid-induced convulsions in neonatal rats, indicative of an acute antiepileptic effect of endocannabinoids. These potentially contradictory results can be reconciled by considering that cannabinoids exert divergent effects over time: The acute or immediate actions of endocannabinoids can be antiepileptic, whereas the chronic or long-term actions of endocannabionids can be proepileptic, presumably as a consequence of the induction of a plastic response such as a long-lasting upregulation of presynaptic CB1 receptors.

Methods

Our own recent research on cannabinoids and seizures was an attempt to extend earlier work on the effects of THC on kindling, a popular preparation of acquired epilepsy. We have been examining the potential antiepileptic effects of the potent and selective CB1/CB2 agonist HU210 and the selective CB1 antagonist AM281 on generalized seizures kindled with stimulation of the

amygdala. The procedure for kindling typically involves application of a 1-s train of electrical stimulation through an implanted electrode once daily until a predetermined endpoint is reached. Standard dependent measures include duration and intensity of the convulsion and duration of the epileptiform afterdischarge (AD) recorded in the EEG. We applied daily kindling stimulation until five generalized stage 5 seizures had been evoked, at which point seizures were well-developed and consistent. We then began drug trials. Rats received intraperitoneal injections of a variety of doses of either HU210 or AM281, at 20 min before each amygdaloid stimulation, one injection daily for 5 consecutive days.

Results

Previous research showed that acute administration of THC produces, at best, weak antiepileptic effects against kindled amygdaloid seizures in rats, cats, and baboons, generally at toxic doses. The same applies to HU210: Figure 1 shows that significant antiepileptic effects occurred only at the highest doses, which were markedly toxic. Furthermore, with chronic administration of HU210, a proepileptic effect emerged at the high doses and even at the more moderate doses (Figure 1). We obtained generally similar results when we examined seizures kindled from the dorsal hippocampus or anterior neocortex in other rats, and with another cannabinoid agonist, WIN 55,212-2. Thus, with clonic forebrain seizures kindled by focal electrical stimulation, we found no evidence of promising antiepileptic effects of cannabinoid agonists, and instead observed a distinct proepileptic effect over time.

We also wanted to determine whether endocannabinoid tone modulates kindled amygdaloid seizures, by examining the effects of a variety of doses of the CB1 antagonist AM281. Figure 2 shows that acute administration of AM281 produced modest, but at some doses significant, proepileptic effects on amygdaloid AD duration, whereas it was without effect on the accompanying behavioral seizures (data not shown). Note that proepileptic effects of the agonist HU210 emerged only with chronic administration, whereas no additional effects were seen with chronic administration of the antagonist (Figure 2). These results are generally consistent with the view that endocannabinoid tone dampens acute susceptibility to kindled seizure

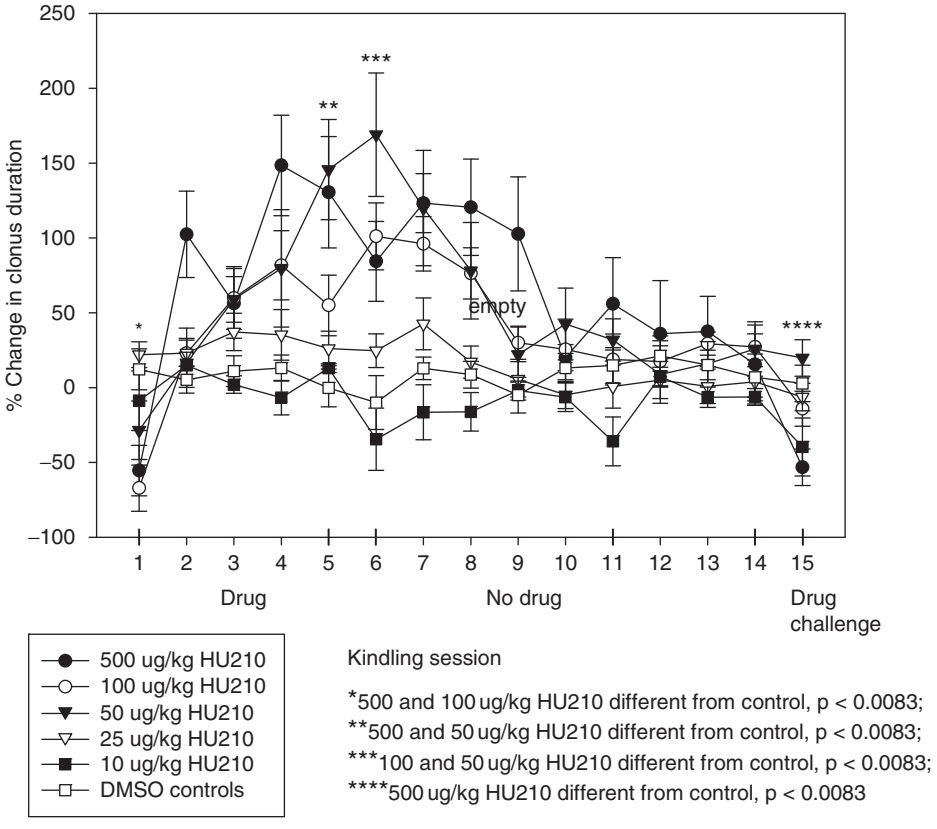


Figure 1 Dose-related effects of acute and chronic administration of the cannabinoid agonist HU210 on kindled generalized seizures, expressed as percentage change in duration of clonus.

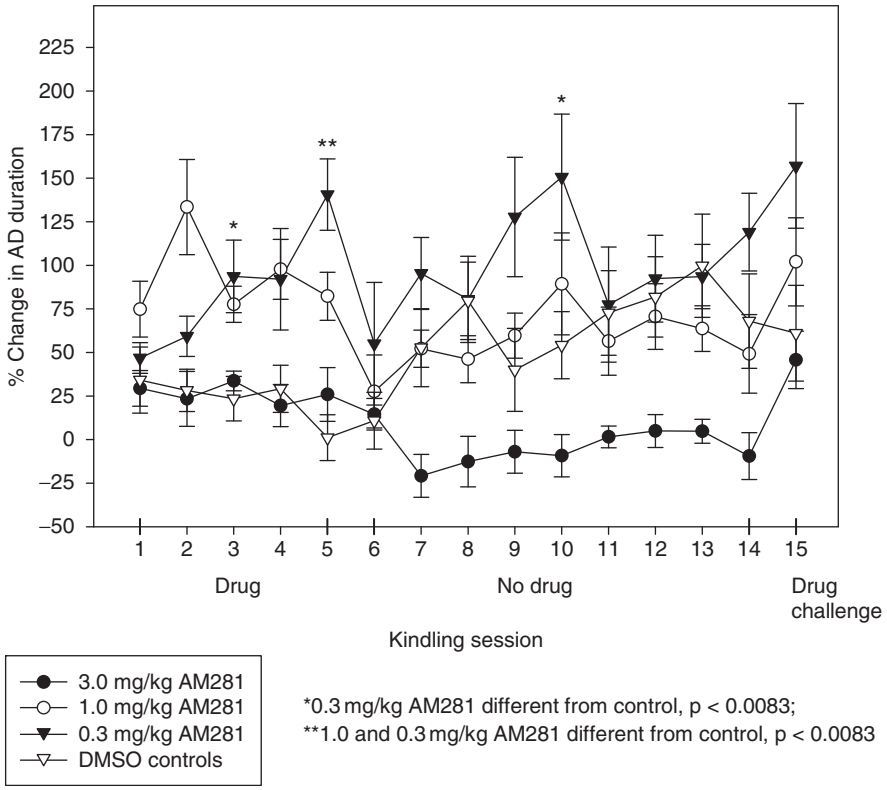


Figure 2 Dose-related effects of acute and chronic administration of the cannabinoid antagonist AM281 on kindled generalized seizures, expressed as percentage change in duration of AD.

discharge, measured electrographically but not behaviorally. In an attempt to determine whether the acute antiepileptic effect of HU210 is due to action at the CB1 receptor, we treated some rats with a cocktail of HU210 and a high dose of AM281 ($3\,mg\,kg^{-1}$) that by itself had no effect on kindled seizures (Figure 2). We found that the antagonist blocked the antiepileptic effect of a high dose of agonist (Figure 3), thereby implicating the CB1 receptor in the acute antiepileptic action of HU210. Surprisingly, however, the antagonist had no effect on the emergence of the proepileptic effect consequent to repeated administration of the agonist. This result suggests that the proepileptic effects of HU210 are produced at a site other than the CB1 receptor.

An important feature of kindling is that one can determine the effects of drug treatment during kindling itself, to test for effects on epileptogenesis. Figure 4(a) shows that amygdaloid kindling was markedly accelerated by treatment with moderate doses of the agonist HU210, whereas Figure 4(b) shows that the antagonist AM281 had no effect on the rate of kindling. Thus chronic administration of a selective cannabinoid agonist potentiates both kindled seizures and epileptogenesis itself.

Our results are generally compatible with Soltesz's findings that a severe febrile episode produces a lasting increase in seizure susceptibility dependent on a proepileptic effect of endocannabinoids. Our data diverge from De Lorenzo's findings that acute administration of cannabinoids suppresses a variety of seizure types. Why do our results point to a proepileptic effect of CB agonists against kindling and kindled seizures, whereas other research points to antiepileptic effects? One possibility is that antiepileptic effects are observed against convulsions induced in the normal brain, whereas proepileptic effects are seen against acquired epilepsies, as in kindling or febrile-induced susceptibility. This explanation is unlikely, however, given that CB agonists exert antiepileptic effects against other models of acquired epilepsy, such as the pilocarpine preparation. Or perhaps cannabinoids suppress tonic (brainstem) seizures but not clonic (forebrain) seizures, such as those triggered by amygdaloid kindling. However, we have determined that the tonic seizures associated with brainstem kindling are unaffected by an injection of HU210, arguing against this explanation.

A third possibility is, as discussed earlier, that acute and chronic administration of cannabinoids evoke different mechanisms. To determine whether the acute–chronic dimension is critical, we have compared the effects of acute and chronic administration of HU210 in rats challenged with a dose of pentylenetetrazol (PTZ) that

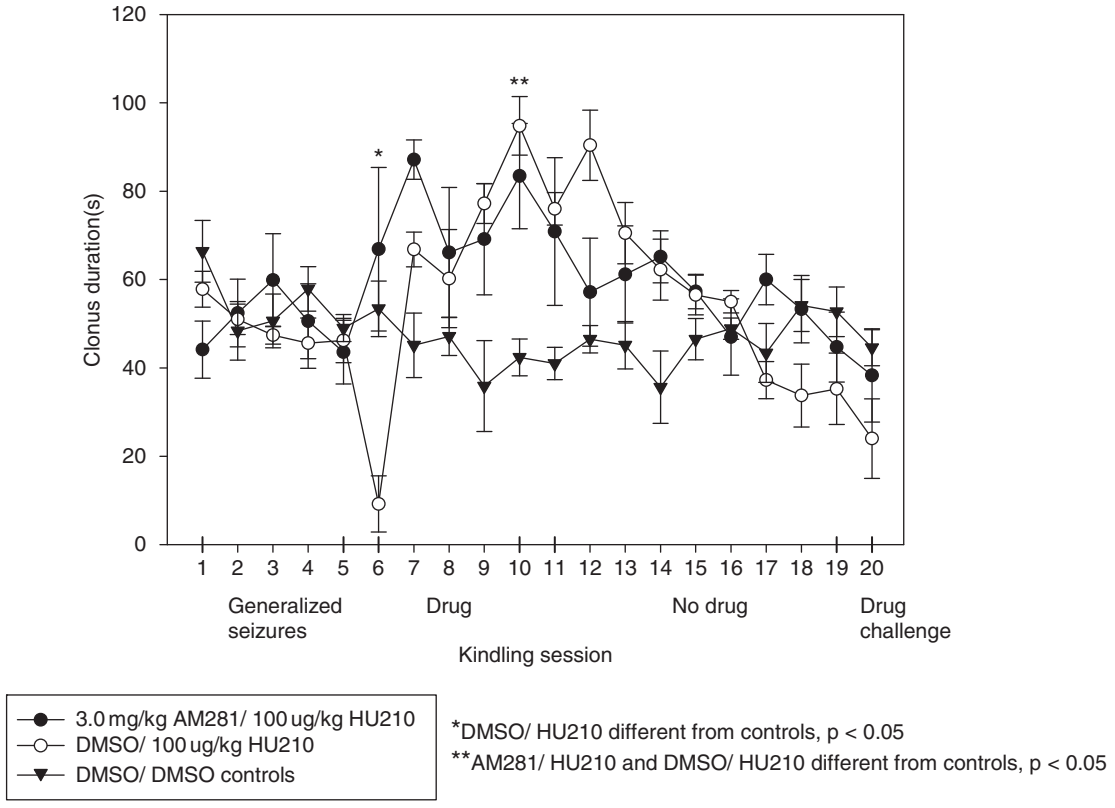


Figure 3 Effects of HU210 on generalized kindled seizures after pretreatment with AM281, expressed as percentage change in duration of clonus.

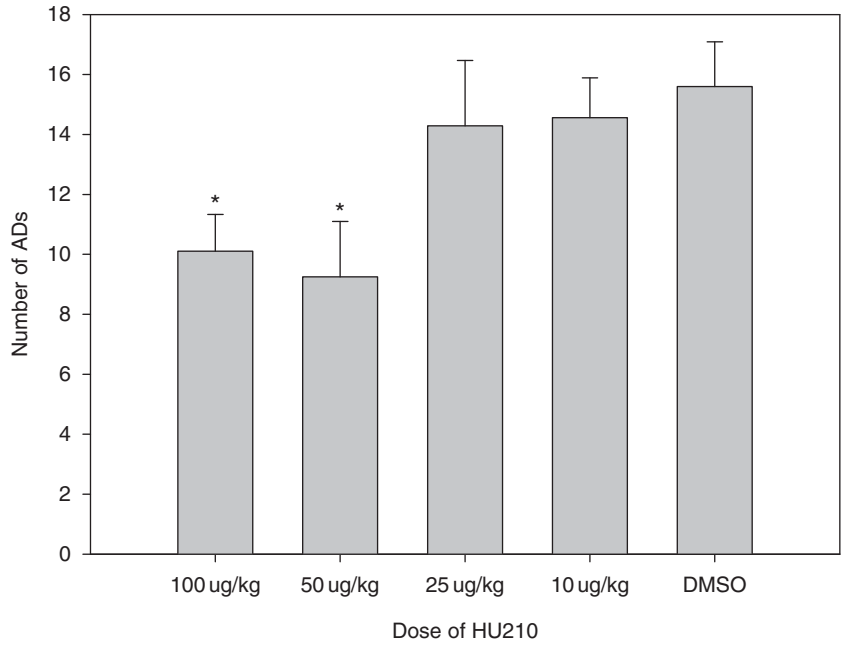
produces recurrent clonic convulsions. Rats in the chronic group received five injections of 100 $\mu g\,kg^{-1}$ of HU210, one each day, with the final injection followed 20 min later by a subcutaneous injection of PTZ at 65 $mg\,kg^{-1}$. Rats in the acute group received only a single injection of HU210, followed by challenge with PTZ. Chronic treatment with HU210 resulted in more intense convulsions as compared to controls – a proepileptic effect – whereas acute treatment with HU210 resulted in less intense convulsions as compared to controls – an antiepileptic effect.

Thus current data from two different preparations of acquired epilepsy support the interpretation that proepileptic effects of cannabinoids are a function of chronic administration of the drug. Note that some previous studies uncovered proepileptic effects of cannabinoids in particular preparations of experimental epilepsy. What is novel about our findings, however, is that the proepileptic effects against kindling and kindled seizures emerge after repeated injections. Presumably, chronic administration triggers a plastic process that exacerbates epileptogenesis and kindled seizures, analogous to the increased density of presynaptic CB1 receptors seen following a sustained febrile episode early in life. It is unlikely that increased density of CB1 receptors underlies the proepileptic effect, however, given that coadministration of the antagonist failed to block it. In contrast, administration of the antagonist did block the weak antiepileptic effect against kindled seizures we observed after acute administration, and this effect may reflect suppression of epileptiform activity in principal cells (e.g., pyramidal neurons in the hippocampus) as a consequence of CB1 receptor activation.

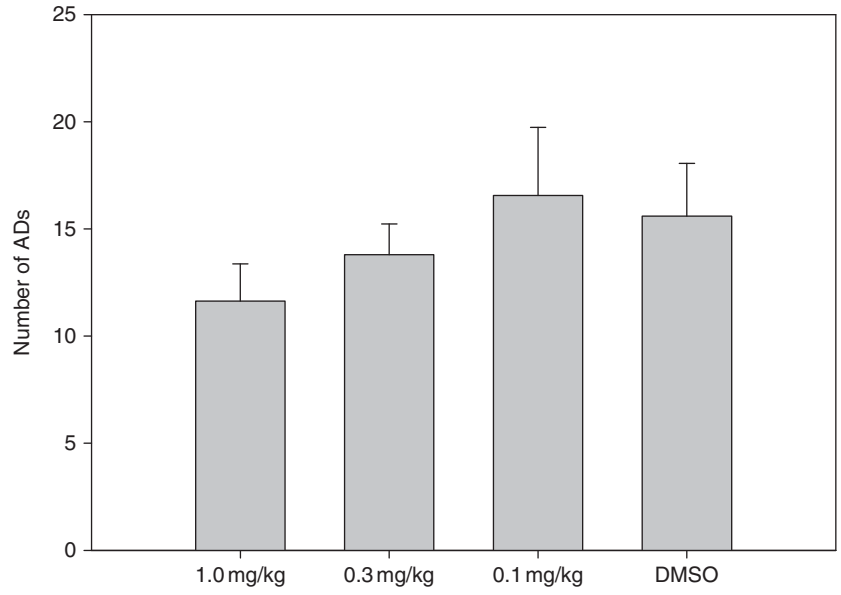
Future Goals

Numerous additional issues remain to be explored. For one, there is no published information about the pharmacokinetics of CB agonists such as HU210 and WIN 55,212-2. It is conceivable that the drugs accumulate with chronic injections at relatively short interinjection intervals, as in our experiments, and the molecular consequences and proepileptic effects of chronic injections may in part be due to failure to clear the drug, resulting in drug accumulation. It also remains to be determined whether chronic injections of HU210 in adulthood result in upregulation of CB1 receptors, as does a single febrile episode in infancy. If upregulation does occur, is it restricted to the hippocampal formation, or is it more widely distributed?

It is certainly conceivable that hyperexcitability induced in the hippocampus by chronic cannabinoid



(a) *100 and 50 ug/kg HU210 different from control, $p < 0.05$



(b)

Figure 4 (a) Dose-related effects of HU210 on the rate of kindling the first generalized stage 5 seizure, expressed as number of ADs to generalization. (b) Dose-related effects of AM281 on the rate of kindling the first generalized stage 5 seizure, expressed as number of ADs to generalization.

administration could accelerate kindling and exacerbate kindled seizures. However, if upregulation of CB1 receptors is not responsible for the proepileptic effect, as suggested by the failure of coadministration of the antagonist to block the effect, other potential mechanisms will have to be investigated. One possibility is that repeated administration of the exogenous agonist prevents the endocannabinoids from binding to the CB1 receptor, resulting in atypical actions at other CB receptors.

Whatever the final explanation for these results, it is clear that caution needs to be exercised in considering pharmacotherapy with cannabinoids. The effects of cannabinoid agonists are different in different preparations of experimental epilepsy, and, unexpectedly, chronic

administration of agonists can exacerbate seizures. These facts raise serious questions about the potential usefulness of cannabinoid drugs in treatment of epilepsy.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy; **Epileptogenesis**: Epileptogenesis and Plasticity; **Models**: Characteristics and Mechanisms of Kindling; **Peptides**: Endocannabinoids, Opioids and the Regulation of Hippocampal Inhibition.

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Dynorphin and Nociceptin/Orphanin FQ

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Introduction – The Possible Implication of Dynorphin A and N/OFQ in Epilepsy

One new therapeutic approach to the epilepsies may be based on the pharmacological modulation of neuropeptide transmitters. In many nerve terminals, neuropeptides coexist and are coreleased with classic small molecule transmitters in a frequency-dependent manner. Neuropeptide release appears to require burst discharge or high frequency activity – that is, events characteristic of epileptiform activity and seizures. Under these conditions, released neuropeptides may act as auxiliary messengers, modulating the efficacy of the principal neurotransmitter and the excitability of the tissue. Indeed, a large body of data supports the view that neuropeptides are involved in seizure mechanisms. Taken together, experimental results have shown that (1) seizures specifically and differentially influence the biosynthesis and release of neuropeptides in various brain areas and (2) neuropeptides exert region- and cell-specific complex effects on seizure expression.

Among the neuropeptides, endogenous opioids have been proposed to play a prominent role in epilepsy. The endogenous opioids are grouped in four systems, based on the existence of four genes coding for four peptide precursors and of four receptor types. The four precursors (pre-proenkephalin, pre-proopiomelanocortin, pre-prodynorphin, and pre-proN/OFQ) can generate many biologically active peptides: [met]enkephalin, [leu]enkephalin, β-endorphin, α-neo-dynorphin, dynorphin A (Dyn A), dynorphin B, nociceptin/orphanin FQ (N/OFQ). This article will focus on dynorphin A and N/OFQ.

Dynorphin A

Electrophysiological, biochemical, pharmacological, and clinical data strongly support the hypothesis that the dynorphinergic system is involved in specific types of seizures. Evidence for this association is particularly strong for complex partial (limbic) seizures – that is, those seizures characteristic of temporal lobe epilepsy.

administration of agonists can exacerbate seizures. These facts raise serious questions about the potential usefulness of cannabinoid drugs in treatment of epilepsy.

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Dynorphin A

Electrophysiological, biochemical, pharmacological, and clinical data strongly support the hypothesis that the dynorphinergic system is involved in specific types of seizures. Evidence for this association is particularly strong for complex partial (limbic) seizures – that is, those seizures characteristic of temporal lobe epilepsy.

In synthesis, the available data suggest that (1) dynorphin is highly expressed in various parts of the limbic system, and particularly in the granule cells of the hippocampus; (2) dynorphin is released in the hippocampus (and in other brain areas) during limbic seizures; (3) released dynorphin inhibits excitatory neurotransmission at multiple synapses in the hippocampus via activation of κ opioid receptors; (4) κ opioid receptor agonists are highly effective in blocking experimental limbic seizures; (5) virus-induced encephalitis causes loss of dynorphin in the hippocampus and epileptic responses that are prevented by κ receptor agonists; and (6) decreased prodynorphin promoter activity increases risk for temporal lobe epilepsy in humans.

Data on generalized tonic–clonic seizures are less straightforward. Dynorphin release appears to occur after electroconvulsive shock seizures, and κ agonists exert a clear anticonvulsant effect in this model. However, inconclusive biochemical data and the lack of κ agonist efficacy in other generalized tonic–clonic seizure models argue against the key involvement of dynorphin in this seizure type. Involvement of dynorphin in generalized absence seizures appears unlikely. Given the presumed origin of absence seizures in alterations of the thalamocortical circuit, and the low representation of dynorphin in the thalamus, this conclusion may not be surprising.

In summary, it appears that dynorphin plays a role as an endogenous anticonvulsant in complex partial seizures and in some cases of tonic–clonic seizures, but not in generalized absence.

N/OFQ

N/OFQ may also be involved in seizure control. The genes encoding the N/OFQ precursor and the NOP receptor are constitutively expressed in forebrain areas thought to be implicated in limbic seizures, such as the dentate granule cell region of hippocampus, as well as in other areas possibly involved in the generation of other seizure types. As detailed below, however, the role specifically played by N/OFQ is still unclear.

Background

Biosynthesis

Only one prodynorphin and one proN/OFQ gene have been reported in man and rodents. The prodynorphin mRNA codes for the biologically inactive peptide precursor pre-prodynorphin, which is posttranslationally processed to generate several functional peptides, including dynorphin A, α-neo-endorphin, dynorphin A 1–32, dynorphin B 1–13 and dynorphin A 1–8. The proN/OFQ mRNA codes for the precursor pre-proN/OFQ, which can also generate multiple functional peptides, including N/OFQ and nocistatin.

Dynorphin A and N/OFQ are both 17 amino acid peptides and share high sequence similarity. However, they belong to clearly distinct systems, based both on biological features (different precursors and receptors, above) and pharmacological properties (different agonists and antagonists, below).

Distribution and Regulation

Cells expressing prodynorphin mRNA in the CNS are found in several regions. The expression is high in the hypothalamus, nucleus accumbens, caudate–putamen, hippocampus, cerebral cortex and spinal cord. In the hippocampus, prodynorphin-derived peptides are synthesized in the granule cells of the dentate gyrus and then transported not only to the axon terminals in the hilus and CA3, but also to the granule cell dendrites.

In the forebrain, proN/OFQ mRNA and N/OFQ peptide levels are prominent in the neocortex, in the reticular nucleus of the thalamus, in the central and medial nucleus of the amygdala, and (at lower levels) in the dentate gyrus of the hippocampus.

Receptors

Four opioid receptor types, μ (MOP), δ (DOP), κ (KOP), and NOP, have been identified, each with distinct distributions and functions.

Dynorphin A shows a relatively high selectivity for the κ opioid receptor, although it can also bind to all other opioid receptor types. Kappa sites are dense in areas that may be directly implicated in some forms of epilepsy, like the cerebral cortex, the hippocampus, the ventromedial part of the caudate–putamen, and some hypothalamic nuclei. In the hippocampus, κ receptors are observed in the pyramidal cell layer and, with lower density, in the granular and molecular layers of the dentate gyrus. Based on different agonist and antagonist sensitivities, the existence of κ receptor subtypes (κ1, κ2, κ3) has been suggested; however, at present there is no molecular basis to support this hypothesis. Like the other opioid receptors, the κ receptors are members of the seven-transmembrane G-protein-coupled receptor family. Activation of κ receptors can cause inhibition of adenylate cyclase, opening of K^+ channels and/or closure of Ca^{2+} channels, with inhibition of transmitter release and of action potential discharge. Pharmacologically, the cloned κ receptor shows high affinity for dynorphin A, for the agonists U69593 and U50488, and for the antagonist nor-binalthorphimine.

N/OFQ shows a relatively high selectivity for the NOP receptor. The NOP receptor, originally cloned using molecular screening methods based on opioid receptor gene sequences, shares a high sequence similarity with κ opioid receptors. Relatively high constitutive NOP receptor levels are found in areas critical for

epilepsies, like the cortex (neo-, entorhinal, and piriform cortex), the hippocampal formation (mainly dentate gyrus and CA3 pyramidal cells), the amygdala (mainly medial and basal nucleus), and various nuclei of the thalamus and of the hypothalamus. The NOP receptor is coupled to the Gi/Go protein-mediated transductions system. Similar to the case with activation of κ receptors, activation of NOP receptors can cause inhibition of adenylate cyclase, activation of K^+ conductances, and/or closure of voltage-sensitive Ca^{2+} channels. These effects can lead to the inhibition of the release of various neurotransmitters and to a reduction in the neuronal firing rate. Pharmacologically, NOP shows high affinity for N/OFQ, for the agonists Ro646198 and UFP102, and for the antagonists J-113397 and UFP101. The NOP receptor can be antagonized by only high concentrations of naloxone.

Effects

Cellular effects

Dynorphin is an inhibitor of excitatory neurotransmission. This effect has been well characterized in the hippocampus where dynorphin is contained in the mossy fibers (the axons originating from the dentate gyrus granule cells) and released together with glutamate. The high frequency stimulation of granule cells causes a transient heterosynaptic inhibition of neighboring glutamatergic mossy fibers, as well as retrograde inhibition of perforant path terminals in the dentate gyrus. These effects are mediated by the release of dynorphin from granule cell axon terminals and dendrites. That release activates κ opioid receptors located presynaptically at the perforant path-granule cell synapse, and both pre- and postsynaptically at the mossy fiber-CA3 synapse. Studies on granule cells of the guinea pig dentate gyrus demonstrated release of the prodynorphin-derived peptides from dendrites as well as from axon terminals. There is evidence that L-type calcium channels mediate dynorphin release from dendrites, whereas the N-type calcium channels control release from axon terminals. Thus, a single cell type can release dynorphin from two different cellular domains (axonal and dendritic) using different release mechanisms.

N/OFQ exerts inhibitory effects on neurotransmitter release, with relevant differences in efficiency depending on the 'target' neurotransmitter and the brain area. Relevant to the epilepsies, N/OFQ inhibits norepinephrine, serotonin and perhaps glutamate release in the cortex, while it does not appear to affect acetylcholine and GABA release. In the hippocampus, N/OFQ inhibits norepinephrine release and excitatory neurotransmission; in the amygdala, it inhibits GABA and glutamate release; and in the thalamus, N/OFQ decreases firing rate in a subset on neurons, but increases it in another subset. These heterogeneous cellular effects underly complex (and sometimes paradoxical) effects at the integrated (intact animal) level.

Behavioral effects

Dynorphin has been proposed to be involved in a range of functions, including nociception, mood, motor activity, and response to injury. N/OFQ may also exert a variety of functions, including modulation of nociception, of locomotor activity, of stress responses and anxiety, and of learning and memory.

Methods

In an attempt to provide some insight into the possible involvement of N/OFQ in seizures and epilepsy, we have investigated the adaptive changes taking place in the N/OFQ-NOP system in epilepsy models, and assessed the functional (pro- or antiseizure) consequences of these changes by using an integrated, pharmacological, and genetic approach. We first analyzed changes in proN/OFQ and NOP mRNA levels (using Northern blot and in situ hybridization), and in receptor binding following acute, kainate-evoked limbic seizures. Second, we studied kainate seizure-evoked N/OFQ release in critical brain areas, namely the hippocampus and the thalamus, using in vivo microdialysis. Third, we evaluated the severity of kainate seizures in mice treated with a NOP receptor antagonist, J-113397, and in mice made N/OFQ-deficient by targeted disruption of the proN/OFQ gene. Fourth, we evaluated amygdala kindling development in N/OFQ-deficient mice.

Recent Data

Alterations in the N/OFQ–NOP System in Response to Seizures

We have studied proN/OFQ gene expression following limbic seizures. Northern analysis revealed increased proN/OFQ mRNA levels in the thalamus (but not in the hippocampus) 3–24 h after kainate administration, with maximal effect (twofold increase over basal levels) at 6 h. In situ hybridization revealed that kainate evokes a dramatic (fourfold) increase in proN/OFQ mRNA levels in the thalamic reticular nucleus and in the medial nucleus of the amygdala. In contrast, NOP mRNA levels were found to decrease within the first 3 h after kainate in many areas, including the amygdala, hippocampus, thalamus, and cortex. Within 3–4 days, NOP receptor density also decreased in the same areas. Thus, the N/OFQ–NOP system appears to undergo adaptive changes following seizures.

One interpretation of these data is that N/OFQ is released during seizures and causes downregulation of NOP receptors and activation of N/OFQ biosynthesis. Therefore, we developed and characterized a microdialysis approach for measuring N/OFQ release in freely moving, awake animals, and used it to study kainate seizure-evoked N/OFQ release in the hippocampus and in the thalamus.

Under basal, unstimulated conditions, more than half of the N/OFQ output was Ca^{2+}-dependent and TTX-sensitive, indicating that it depends on action potential propagation. The Ca^{2+}-independent and TTX-insensitive component of N/OFQ output may be generated by N/OFQ leakage due to the damage produced by the implantation of the microdialysis probe. N/OFQ efflux was greatly increased by depolarization. High K^+ caused a threefold increase in N/OFQ efflux both in the hippocampus and thalamus. Stimulus-evoked release was totally Ca^{2+}-dependent and TTX-sensitive, suggesting a neuronal origin. Importantly, all of these characteristics of N/OFQ release were essentially identical to those reported for other neuropeptides under similar experimental conditions.

The mechanism of N/OFQ release has not been studied yet. However, in analogy with other neuropeptides, it is most likely frequency-dependent, requiring neuronal burst discharge or high frequency activity. Indeed, kainate-induced status epilepticus caused a robust, but transient, increase in N/OFQ efflux in the rat hippocampus and thalamus. This phenomenon displayed several interesting characteristics. First, consistent with the increase of proN/OFQ gene expression, the increase in N/OFQ release began in coincidence with the start of status epilepticus (i.e., approximately 90 min after kainate injection), whereas no significant changes were observed while the animals were experiencing the self-limiting, low-grade seizures that precede status. Although it is tempting to speculate that only prolonged seizures can evoke N/OFQ release, it is also plausible that the technique employed is not sufficiently sensitive to detect less dramatic, fleeting changes.

Second, this early increase in N/OFQ release was transient. It began to decline within 90–120 min, and eventually returned to baseline levels. This decline may depend upon depletion of N/OFQ-containing vesicles in the nerve terminals. Increased release of neuropeptides does prompt new synthesis in the soma and transport along the axons to restore appropriate levels in the nerve terminals, but these events are time-dependent. Indeed, arrival of newly synthesized N/OFQ in the terminals coincided with a second, late increase in its release.

Third, N/OFQ release was not increased compared to prekainate levels on the following day, when animals display only low grade seizures. This observation indicates that N/OFQ release depends on occurrence of generalized seizures. Interestingly, all these characteristics strikingly overlap those reported by others for kainate seizure-induced opioid peptide release in the hippocampus.

Based on these data, we propose the following cascade of events. First, seizures cause N/OFQ release, reducing its levels in the nerve terminals and stimulating new synthesis within the releasing neurons themselves. In turn, released N/OFQ causes downregulation of the NOP receptor. Consistent with this proposal is the chronological sequence of events in the hippocampus and in the thalamus: following kainate administration, peak increase in N/OFQ release occurs at 3 h, peak proN/OFQ mRNA at 6 h (which may cause the late increase in N/OFQ release), decreased NOP mRNA levels in the first day, and decreased NOP receptor density in 3–4 days (consistent with a reported opioid receptor turnover time of 3–4 days in vivo). Furthermore, receptor desensitization and/or internalization may occur at earlier time-points.

Physiopathological Implications

As stated above, the role of released N/OFQ in seizure expression is still uncertain. Initially, the possible involvement of N/OFQ in seizures and epilepsy has been studied by release and electrophysiological studies, which provided information about the basic effects of N/OFQ in the modulation of excitability. As described, N/OFQ exerts inhibitory effects at a cellular level and inhibits the release of excitatory and inhibitory neurotransmitters, with relevant differences in efficiency depending on the neurotransmitter and the brain area under study. It is difficult to speculate about pro- or antiseizure effects of the peptide on this basis, because the net effect of N/OFQ will probably depend on the circuitry involved in the generation or amplification of the specific type of seizure.

Another approach to the problem has been the use of NOP agonists. This approach has given conflicting, inconclusive results. In one study, an N/OFQ analog produced pro-epileptic effects, but these were blocked by naloxone (thus, they were not NOP-mediated); in other studies, intracerebroventricularly injected N/OFQ attenuated bicuculline-, NMDA-, and penicillin-induced seizures and retarded kindling development, but did not attenuate electrical shock and fully kindled seizures.

Finally, by using a combined pharmacological and genetic approach, we have found converging evidence for a proseizure effect of endogenous N/OFQ. Mice treated with the NOP receptor antagonist J-113397 displayed reduced susceptibility to kainate-induced seizures (i.e., significant reduction of behavioral seizure scores). In keeping with these observations, N/OFQ knock-out mice were less susceptible to kainate seizures compared with their wild-type littermates; lethality was reduced, latency to generalized seizure onset prolonged, and behavioral seizure scores decreased. Intracerebroventricular administration of N/OFQ prevented reduced susceptibility to kainate seizures in N/OFQ knock-out mice. We have also found that N/OFQ deficient mice are more resistant to kindling development than their wild-type littermates.

Future Goals

Further investigation is required to characterize the involvement of the N/OFQ-NOP system in epilepsy. Taken together with the other reports, our data suggest

that the role of N/OFQ during seizures is complex, with different effects in different seizure and epilepsy types. Therefore, a careful examination of the precise indications of drugs affecting the N/OFQ-NOP system in the different epilepsy types is warranted. To perform this experimental examination, however, it will be essential to improve the pharmacological tools that are currently available. In particular, there is still a need for highly selective and specific, potent, nonpeptidic NOP receptor agonists and antagonists.

The experimental analysis of the dynorphin involvement in epilepsy is much more advanced. As summarized above, the available experimental evidence convincingly support the view that dynorphin plays a role as an endogenous anticonvulsant in complex partial seizures and in some cases of tonic–clonic seizures, but not in generalized absence. This pattern of effects should coincide with the antiseizure spectrum of selective κ agonists. Although κ agonists have undergone clinical evaluation, primarily for the treatment of pain or of heroin addiction, studies on their effects in epileptic patients have not been performed. Of course, several factors may be expected to limit the use of pharmacological agents acting through the dynorphin system, including psychotropic side effects (dysphoric or psychotic episodes) and nonspecific actions that may lead to hyperexcitability. Furthermore, prolonged treatments with κ agonists may cause receptor downregulation, with attenuation or loss of the clinical effects. Nonetheless, consideration of these obstacles should not discourage investigators from attempting a progress toward clinical testing of such drugs in patients resistant to the currently available antiepileptic agents.

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Granule Cells**: Properties of Dentate Granule Cells and their Relevance to Seizures; **Neurotrophic Factors**: Fibroblast Growth Factor-2; **Peptides**: Participation of Opioid Peptides in Epilepsy; **Seizures**: Seizure Termination and the Postseizure Refractory Period.

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Endocannabinoids, Opioids and the Regulation of Hippocampal Inhibition

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Introduction

The endogenous opioid and cannabinoid systems, while entirely separate and distinct at cellular and molecular levels, share many remarkable similarities. They both represent systems in which endogenous neuromodulators act on the same receptors that are also activated by plant-derived agents. Both classes of receptors are G-protein coupled and, for the most part, located on presynaptic nerve terminals, where their activation reduces transmitter

that the role of N/OFQ during seizures is complex, with different effects in different seizure and epilepsy types. Therefore, a careful examination of the precise indications of drugs affecting the N/OFQ-NOP system in the different epilepsy types is warranted. To perform this experimental examination, however, it will be essential to improve the pharmacological tools that are currently available. In particular, there is still a need for highly selective and specific, potent, nonpeptidic NOP receptor agonists and antagonists.

The experimental analysis of the dynorphin involvement in epilepsy is much more advanced. As summarized above, the available experimental evidence convincingly support the view that dynorphin plays a role as an endogenous anticonvulsant in complex partial seizures and in some cases of tonic–clonic seizures, but not in generalized absence. This pattern of effects should coincide with the antiseizure spectrum of selective κ agonists. Although κ agonists have undergone clinical evaluation, primarily for the treatment of pain or of heroin addiction, studies on their effects in epileptic patients have not been performed. Of course, several factors may be expected to limit the use of pharmacological agents acting through the dynorphin system, including psychotropic side effects (dysphoric or psychotic episodes) and nonspecific actions that may lead to hyperexcitability. Furthermore, prolonged treatments with κ agonists may cause receptor downregulation, with attenuation or loss of the clinical effects. Nonetheless, consideration of these obstacles should not discourage investigators from attempting a progress toward clinical testing of such drugs in patients resistant to the currently available antiepileptic agents.

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **Granule Cells**: Properties of Dentate Granule Cells and their Relevance to Seizures; **Neurotrophic Factors**: Fibroblast Growth Factor-2; **Peptides**: Participation of Opioid Peptides in Epilepsy; **Seizures**: Seizure Termination and the Postseizure Refractory Period.

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Introduction

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release. In the hippocampal CA1 region, the receptors are mainly found on inhibitory interneuron terminals. Enkephalin, endorphin, and dynorphin are examples of endogenous opioid receptor agonists. Extracts of the opium poppy yield the exogenous agonists morphine and codeine, whereas heroin is a synthetic morphine derivative. Anandamide and 2-arachidonyl glycerol (2-AG) are the major endogenous cannabinoids (endocannabinoids), and in the brain they act on the same receptor at which the derivative of the cannabis plants, Δ^9-tetrahydrocannabinol (THC), is a ligand. In both systems the exogenous and endogenous ligands have powerful psychoactive properties.

Opioids and cannabinoids play many behavioral roles. For example, they both modulate reward behaviors, analgesia, and hypothermia. However, while morphine is a highly addictive drug (causing a broad and well-defined spectrum of dependencies and withdrawal symptoms), cannabis products are generally nonaddictive, although a susceptible subgroup of users may develop a psychological dependency on them. Besides dependence, opiate use also leads to tolerance, prompting the use of higher doses and the risk of fatal outcomes, whereas cannabis use does not lead to tolerance. Opiates often induce vomiting, while cannabinoids are antiemetics.

Background

Endogenous Opioid and Cannabinoid Systems-Basic Properties

As suggested earlier, while opioids and cannabinoid systems share some similarities, they differ in several ways. In terms of basic properties, endogenous opioids are peptides that are derived from larger, dedicated proprecursor proteins. In the CA1 region of hippocampus, the principal endogenous opioid is enkephalin, although in other parts of hippocampus dynorphin is present at high levels. Opioids are generally packaged in secretory vesicles in presynaptic terminals, and are released into the synaptic cleft by conventional mechanisms of synaptic transmission. Opioids are soluble in water, and once released diffuse to their postsynaptic receptors. Interneurons are probably the primary source of enkephalin in CA1, although some extrinsic fibers are also weakly immunoreactive for enkephalin. In CA1, endogenous opioids can be released by stimulation of axonal fibers in stratum lacunosum-moleculare in CA1. Opioid receptors have numerous subtypes (perhaps as many as 16), with the mu opioid receptor (MOR) being the most prominent in the hippocampus and other parts of the brain. Different OR subtypes have selective ligand requirements and are distinguishable pharmacologically. Notably, dynorphin is present in dentate granule cells, and can be released when the cells are stimulated vigorously; details of its packaging and release mechanisms in this system are still lacking. Once released from the postsynaptic granule cells, dynorphin acts as a retrograde messenger and activates kappa-opioid receptors that are present on the excitatory perforant path terminals.

Endogenous cannabinoids are small lipid fatty acid derivatives of arachidonic acid, and are only weakly water-soluble. 2-AG and anandamide, which are derived directly by enzymatic action on common molecular components of the cellular plasma membrane: diacylglycerol in the case of 2-AG, and *n*-arachidonyl–phosphatidyl ethanolamine in the case of anandamide. Anandamide was the first identified endocannabinoid, however, a growing consensus is that 2-AG is probably the major endocannabinoid in most of the brain, and we will focus on 2-AG in this discussion. Production of 2-AG is thought to proceed via two enzymatic steps, activation of phospholipase C, which produces diacyglycerol, and activation of diacylglycerol lipase. The product of diacylglycerol cleavage by diacylglycerol lipase is 2-AG. 2-AG is synthesized and released from postsynaptic target neurons either by a large increase in postsynaptic $[Ca^{2+}]_i$, or activation of G-coupled neurotransmitter receptors (GPCRs) (group I mGluRs, and M1/M3 mAChRs, in the hippocampus), or the combination of a small increase in $[Ca^{2+}]_i$ combined with GPCR activation. Present technologies do not allow the rapid experimental distinction between the synthesis and release steps in cellular studies, and the term 'mobilization' is sometimes used to encompass both. Endocannabinoids can be mobilized when synaptic excitation causes a sufficient rise in $[Ca^{2+}]_i$ to occur in the pyramidal cells. The actual change in $[Ca^{2+}]_i$ required for endocannabinoid synthesis and release depends on the particular stimuli and the time course of the stimulation. If a rise in $[Ca^{2+}]_i$ is the only stimulus, then $[Ca^{2+}]_i$ must be in excess of 10 μM; however, if a GPCR is activated at the same time, then much lower levels of $[Ca^{2+}]_i$, (on the order of 1 μM) are sufficient. The time course of the stimulation is also critical. Even submicromolar levels of $[Ca^{2+}]_i$ can be effective if prolonged for many minutes. Endocannabinoids are not packaged in vesicles, and they are lipophilic. Since they are produced in a membrane environment, it is not clear how readily they will simply diffuse into the largely aqueous environment of the synaptic cleft. Hence, the precise molecular mechanism of their release is presently not understood; it could involve unknown mechanisms, such as transporters or chaperone molecules, rather than simple diffusion.

Thus far, only one type of neuronal cannabinoid receptor has been extensively described in the brain, CB1R; a splice variant, CB1aR, seems functionally indistinguishable from CB1R, and the peripheral cannabinoid receptor, CB2R, may be present on certain microglia. Everywhere else, CB1R predominates. Currently, CB1R is considered the most abundant G-protein coupled receptor in the brain. There are effective CB1R antagonists, SR141716A (also called 'rimonabant'), and AM-251, that

permit pharmacological dissection of the system. It should be born in mind that the antagonists most widely used are not neutral antagonists; they are 'inverse agonists' that have effects all their own on the CB1R, even in the absence of a CB1R agonist. Moreover, neither rimonabant nor AM-251 are perfectly selective and do affect other receptors. Rimonabant is also an antagonist at the transient receptor potential (TRP) channel called V1 (or VR1).

Recent evidence for a third kind of cannabinoid receptor, GPR55, has appeared. GPR55 is richly expressed in brain, including hippocampus, and once detailed studies appear, it will certainly force reevaluation of the roles of endocannabinoids. GPR55 reacts with a number of cannabinoid ligands, but in general its pharmacological properties are very different from those of CB1R; e.g., a well established antagonist/inverse agonist at CB1R, AM251, may be an agonist at GPR55. No physiological roles for GPR55 in the brain have yet been identified.

Several lines of CB1R knockout mice have been developed, including some in which the deletion of CB1R has been targeted to specific cells groups in the brain. To avoid the limitations inherent in constitutive knockouts as well as the complexities of the pharmacological antagonists, a comprehensive strategy for investigating endocannabinoid actions would involve using both antagonists and confirming results with a CB1R knockout mouse. An alternative is to inject a Cre-loaded virus into a restricted brain region of a CB1R-'floxed' mouse. In virally transfected cells, the Cre induces CB1R deletion. Local inducible CB1R removal thus avoids some of the problems inherent in either constitutive knockouts or pharmacological approaches.

At the subcellular level, activation of opioid and cannabinoid receptors inhibit adenylyl cyclase, inhibit voltage-gated calcium channels, and increase the activation of voltage-gated potassium channels – all of which lead to a reduction in the amount of neurotransmitter released from presynaptic terminals. Indeed, one of the most important similarities between endogenous opioids and cannabinoids is that their receptors are often located on presynaptic nerve terminals and mediate presynaptic inhibition.

Localization of MORs and CB1Rs

Interneurons

Perisomatic, 'basket cell' synapses are thought to be the most important for regulating pyramidal cell somatic action potentials, and thereby strongly influence axonal conduction and synaptic output. Dendritic synapses are probably the most important for dendritic integration and the regulation of the intrinsic dendritic excitatory properties, rather than network activity. CB1Rs are present on a major class of interneurons that target both somatic and dendritic regions. These cells selectively colocalize the neuropeptide, cholecystokinin (CCK), with GABA.

The other major class of basket cell contains parvalbumin (PV) but not CCK, in addition to GABA. PV immunoreactivity is reduced in the hippocampus of some patients with temporal lobe epilepsy as well as in animal epilepsy models. This reduction may be associated with the neuronal loss that is seen in some cases of epilepsy, even though few studies have shown an actual correlation. A reduction in the expression of PV, even without a decrease in the number of interneurons, could have an effect on the properties of those interneurons, since PV is a calcium buffer and its downregulation will certainly affect the normal properties of those cells. Human studies find fewer changes in PV immunoreactivity than those reported in animal studies, where PV immunoreactive cell loss is extensive. The subiculum of a small group of epileptic patients was reported to have a marked reduction in PV cell density, whereas in CA1 the PV cell number increased. In mouse models, 2 months after the induction of seizures, the percentage of interneurons expressing PV and P/Q type calcium channels increased, suggesting that the switch to a PV and P/Q expressing interneuron could be neuroprotective.

Cellular and subcellular localization of MOR and CB1R

Both MORs and CB1Rs are mainly located on inhibitory, GABAergic interneurons in the hippocampus. In the CA1 region, there are several classes of GABAergic inhibitory interneurons having distinct morphological and electrophysiological characteristics. As indicated earlier, two prominent and mutually exclusive classes express either the calcium-binding protein, PV, or the neuropeptide, CCK, in addition to the GABA-synthesizing enzyme, glutamic acid decarboxylase. PV and CCK interneurons are considered basket cells that mainly innervate principal cell somata and proximal dendrites, thus providing perisomatic inhibition.

In the CA1 region, CB1Rs are densely packed on the terminals and preterminal axons of CCK-expressing interneurons. This localization is almost exclusive; few other interneurons express CB1R, and in particular, PV cells do not express any. Although initial immunohistochemical studies reported finding no CB1R on glutamatergic fibers in CA1, this view has been revised as recent pharmacological and immunohistochemical studies demonstrate their presence on excitatory fibers, albeit at a much lower density (perhaps 30-fold lower) than on the inhibitory axons. Interestingly, PV-cells express high levels of MOR, and CCK cells reportedly express none. Unlike the comparatively restricted distribution of CB1Rs, however, the distribution of MORs is broader and encompasses, in addition to the PV cells, a heterogeneous group of interneurons found in all the laminae of CA1. These other interneuron types express vasoactive intestinal peptide, somatostatin, or calretinin in addition to GABA. The PV interneurons target pyramidal cells, but the other

MOR-expressing interneurons generally target other interneurons, suggesting complex circuit effects may result from MOR stimulation. Whether or not the nonPV, MOR-expressing interneurons directly target PV cells is not clear. Given these results, a diffuse form of paracrine signaling from the enkephalin-containing interneurons to PV cells, rather than conventional synaptic transmission, seems possible. Similarly, in dentate gyrus, kappa opioid receptors are present on glutamatergic perforant path fibers. Stimulation in the CA1 region induces a kappa-opioid receptor-dependent enhancement of long-term depression (LTD) of glutamate fibers in s. radiatum, suggesting an association of this opioid receptor subtype on s. radiatum excitatory axons.

Methods

We have made electrophysiological recordings from single neurons in the rat acute brain slice preparation. The slices, which are continuously bathed in an oxygenated, physiological salt-solution, are 400 μm thick and contain microcircuits of excitatory and inhibitory neurons that remain substantially intact. The preparation is especially convenient for pharmacological investigations, as drugs can be applied to bath solution in known concentrations where they affect the entire surface of all cells. We typically study tissue from young adult (5–7 week-old) male rats, to avoid the variability that can be induced by hormonal variations in females. Nevertheless, this introduces the danger that our results may not apply straightforwardly to females. Our recordings are made by impaling neurons with high resistance ('sharp') or with whole cell patch electrodes. Each technique has advantages and disadvantages: sharp electrodes permit stable, long-term recordings (>2 h) to be made routinely, however they lack the ability to control the intracellular membrane potential, or measure transmembrane currents, and cannot be used to detect the smallest synaptic signals. Synaptic neuronal activity was recorded as changes in membrane potential. As we are interested in studying the inhibitory circuits in the hippocampus, we blocked excitatory inputs to pyramidal cells by adding ionotropic glutmatergic receptor blockers into the bath solution. Inhibitory synapses were stimulated by extracellular electrodes placed into the vicinity of the inhibitory interneurons. Inhibitory postsynaptic potentials were recorded from the principal cells of the hippocampus, the pyramidal cells.

Results

Cellular Studies

Opioids suppress inhibitory postsynaptic potentials (IPSPs)

Adding morphine or synthetic MOR agonists to hippocampal brain slices causes large hyperexcitable interictal spike responses (involving numerous pyramidal cells) that spread easily throughout the cell population. This response was at first considered paradoxical, because MORs are present only at a relatively low density in the hippocampus. The apparent discrepancy was reconciled by the finding that hippocampal MORs are strategically located on interneurons, where their activation reduces $GABA_A$-mediated IPSPs. The resulting disinhibition has several major consequences: (1) Excitatory synaptic potentials that are normally shaped and truncated by GABAergic inhibition grow in size and express NMDA-dependent components; (2) Cells within a given circuit communicate readily and directly in ways that are ordinarily suppressed by GABA. This effect is especially significant in regions of high interconnectivity among principal cells, such as the CA3 region, where powerful and self-sustaining recurrent excitatory loops are normally prevented by inhibition. Recurrent activity within the loops is a major factor in spreading epileptic activity to downstream target areas – e.g., the CA1 region; (3) Finally, intrinsic burst potentials are generated. The apparently disproportionate proconvulsant actions of MOR agonists can therefore be attributed to the localization of MORs on interneuron terminals and the disinhibitory effects of their activation on pyramidal cell firing.

Opioids regulate excitatory postsynaptic potentials (EPSPs) indirectly

Recent optical studies, using voltage-sensitive dyes, have revealed that the magnitudes of excitatory synaptic potentials are also under the control of MORs. This influence is a result of opioid modulation of dendritic inhibitory synapses, however, and not a direct influence on the excitatory fibers, since the presence of $GABA_A$ receptor antagonists completely occludes the MOR-mediated effect. While the ability of MORs to regulate $GABA_B$-mediated responses was initially discounted, it now appears that MOR-regulation of $GABA_B$ inhibition of glutamate responses also occurs. Finally, exposure to morphine in vivo can produce persistent loss of GABAergic synapses in the midbrain, and resultant long-term changes in ratio of AMPA–NMDA receptor mediated currents. Since some of these effects were obtained with a single exposure to morphine, it may be that imbalances in the opiate system have deeper and more far-reaching consequences than expected.

Depolarization-induced suppression of inhibition (DSI) by endocannabinoids

A train of action potentials, or a large transient depolarization such as associated with a burst potential, in a postsynaptic pyramidal cell can cause a temporary reduction in the amplitudes of inhibitory postsynaptic potentials experienced by that cell. The decrease in inhibition has been termed DSI. In principle, several mechanisms could account for DSI. One class of mechanisms would be

confined to the postsynaptic cell – for example, a decrease in GABA receptor sensitivity or other alteration that decreased the ability of the GABA receptor to change the cell's membrane potential. Another class of mechanisms would involve presynaptic effects, such as a change in calcium influx into the presynaptic nerve terminals that reduces their ability to release GABA. However, since the decrease in inhibition is triggered by the postsynaptic cell, the presynaptic mechanisms would involve the generation of a signal in the postsynaptic cell that would then travel to the presynaptic receptors – in effect a retrograde signaling process. Although at first retrograde signaling seemed to be the less likely explanation, in fact many experiments done by dozens of laboratories have convincingly shown that DSI is mediated by such a retrograde signal process. Moreover, it is now clear that the retrograde signal is an endocannabinoid.

Short-term activation of presynaptic CB1R is caused when the postsynaptic pyramidal cell experiences a sufficiently large bolus of calcium (generally initiated by depolarization) to synthesize and release endocannabinoids as described earlier. DSI transiently reduces GABA release by blocking the N-type Ca^{2+} channels that serve as the primary stimulators of transmitter release from the CB1R-expressing interneurons. This process reduces the inhibition received by the endocannabinoid-releasing cell for a period that lasts tens of seconds in vitro, and probably on the order of seconds in vivo. An especially important aspect of endocannabinoid signaling is that the endocannabinoid molecules are lipids that cannot diffuse great distances in the mainly aqueous extracellular medium. Moreover, they are rapidly taken up and degraded by efficient mechanisms. It is likely that they can act no more than a micron or so from their source cell, except under extraordinary circumstances in which they are produced in large quantity or the uptake and catabolic systems are inactivated. Thus, endocannabinoids are quintessentially local signals; an endocannabinoid-sourcing cell affects its own inputs, but probably not those of even its closest neighbors. DSI in one cell does not induce DSI in the nearby cell in the hippocampus.

During the resultant transient period of DSI, a cell is free to respond much more vigorously to incoming excitatory synaptic inputs than its still-inhibited neighbors, even if all cells receive the same degree of excitation. A weak train of stimuli, one that is normally subthreshold for induction of long-term potentiation (LTP), becomes capable of inducing LTP if it occurs during the DSI period. Most importantly, simultaneous field potential recordings show that the stimuli that induce LTP in the single cell during DSI do not produce LTP in the population, confirming that LTP induction in the single cell can be attributed to local, endocannabinoid-mediated disinhibition. In this case, while the endocannabinoid effects themselves are short-lasting, the indirect consequence of endocannabinoid action is persistent.

Prolonged CB1R activation can directly induce LTD of inhibitory synapses (iLTD)

If mGluR-dependent endocannabinoid mobilization is prolonged for several minutes, there is a long-lasting form of synaptic depression (iLTD). iLTD underlies the E–S type of long-term potentiation, whereby a given sized EPSP (E) has an increased likelihood of triggering an action potential (spike – S) in a pyramidal cell. Endocannabinoid-mediated iLTD requires minutes-long activation of presynaptic CB1R, subsequent activation of protein kinase A, and the presynaptic protein, RIM 1α, indicating the existence of at least two pathways downstream of CB1R within the presynaptic terminal that inhibit transmitter release. iLTD induction represents a direct effect of long-lasting CB1R activation. Nevertheless, DSI and iLTD heighten neuronal excitability by facilitating LTP induction indirectly.

Interactions Between Opioids and Cannabinoids

Behavioral studies

As noted earlier, the opioid and cannabinoid systems are distinct molecular and cellular entities, with their respective receptors largely located on nonoverlapping cell groups. The output from individual CCK interneurons is not affected by MOR agonists in paired recording. Nevertheless, the two systems clearly interact; cannabinoid- and opiate-mediated actions are intertwined. In behavioral experiments (i.e., place preference and self-administration experiments), opiate antagonists can disrupt a learned behavior induced by cannabinoid agonists or vice versa. Mice genetically altered to lack CB1Rs have a markedly decreased preference for heroin rewards, while normal mice rapidly acquire and maintain strong behavioral responding for heroin. There is ample evidence for cross-talk between the systems in seizure-related phenomena as well. CB1R antagonists can oppose the anticonvulsant actions of morphine, and naltrexone (MOR antagonist) opposes the anticonvulsant effects of CB1R agonists. In contrast, the high-dose proconvulsant MOR effects are also antagonized by CB1R antagonists, underscoring the complexity of the opioid effects on seizures. Since both anticonvulsant and proconvulsant actions of MOR are opposed by CB1R, it appears unlikely that there is a unitary cellular mechanism by which CB1R and MOR actions interact.

Cellular studies

There is some evidence for direct molecular interactions between MORs and CB1Rs. They could heterodimerize, for example, although this seems to contradict immunohistochemical and electrophysiological data indicating that the receptors are on different interneurons. In general, the physiological relevance of heterodimerization has not been well-established.

Assuming that heterodimerization is not a factor in the cannabinoid or opioid responses in the hippocampus, it is a matter of great interest to learn if, or how, the two systems might interact at the cellular or network levels. We have begun to test some predictions (based on a simple model) that opioids and endocannabinoids regulate distinct groups of interneurons that act independently and synapse in parallel on pyramidal cells. This model implies that the pyramidal cell is the junction point for their interactions. Both $GABA_A$- and $GABA_B$-receptor mediated IPSPs shape hippocampal network activity. To rule out network level interactions, an efficient method for activating either class of interneuron in isolation is required. Selective, global elimination of CCK- or PV-cell-mediated inhibition is possible because of their reliance on distinct types of voltage-gated Ca^{2+} channels to release GABA. A great deal of prior evidence shows that the large evoked and spontaneous IPSPs are made by somatic or perisomatic synapses – that is, by basket cells or axo-axonic cells. The PV and CCK cells constitute the overwhelming majority of these cells. Hence, large IPSPs occurring in the presence of agatoxin probably issue mainly from the CCK cells, and large IPSPs occurring in the presence of conotoxin probably issue mainly from PV cells.

$GABA_B$ activation is generally anticonvulsant in animal models, whereas blocking the $GABA_B$ receptors or the $GABA_A$ receptors can exacerbate epileptiform activity in some cases. In view of the roles of $GABA_A$ and $GABA_B$ receptors in epilepsy, it is important to unravel the ability of opioid and cannabinoid receptors to suppress these responses. We wanted especially to test the hypothesis that both receptors would suppress the same GABA responses.

Exogenous MOR and CB1R agonists suppressed $GABA_A$ responses, but only MOR agonists affected both $GABA_A$ and $GABA_B$ responses. We concluded that CB1Rs are not present on the interneurons that produce $GABA_B$ responses. If CB1Rs and MORs interact on the same interneurons, these would have to be the interneurons that produced exclusively $GABA_A$ responses. CB1Rs reduced $GABA_A$ IPSPs in the short and long term (**Figure 1(a)**), but not $GABA_B$ responses (**Figure 1(b)**). Since epileptiform activity that is induced by a variety of convulsants (including $GABA_A$ antagonists) is exacerbated by blocking $GABA_B$ receptors, the aforementioned result offers new insights into how opiates may affect some cases of epilepsy. Knowing which cells are the source of the postsynaptic $GABA_B$ IPSPs, or whether all interneurons are potentially a source, would provide important information about how $GABA_B$ responses can act as anticonvulsants in some animal models.

By using neurotoxins that selectively block GABA release from certain classes of interneurons, we ruled out any substantial overlap between MORs and CB1Rs on the same interneurons. There was no evidence for mutual occlusion between the effects of the selective agonists of the receptors. However, as noted earlier, CB1Rs induce

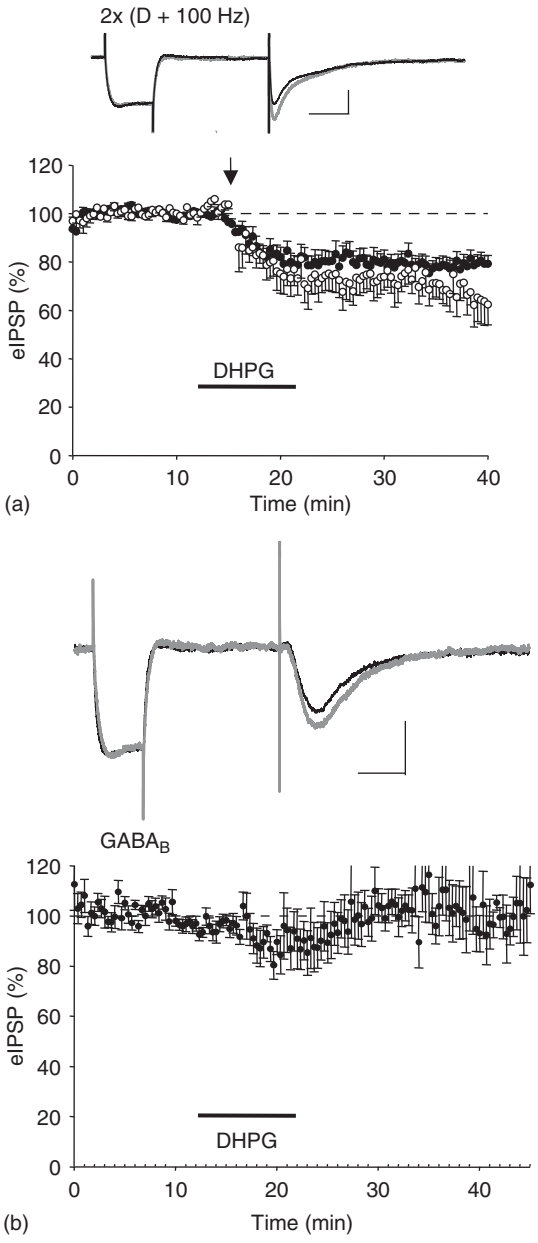


Figure 1 iLTD of $GABA_A$ but not $GABA_B$ IPSPs. (a) Top, Representative trace of an eIPSP before (gray trace) and 20 min after (black trace) synaptic stimulation (2× 100 Hz train, 20 s apart). Calibrations for the traces are: *x*-axis, 2 mV; *y*-axis, 200 ms. Graph: A 10-min DHPG application (black circles, $n = 27$) reduced the amplitude of an eIPSP for more than 30 min (iLTD). Synaptic stimulation (white circles, $n = 6$) also induced robust iLTD. (b) Endogenous cannabinoids mobilized by DHPG do not affect the $GABA_B$ response. A representative $GABA_B$ isolated in bicuculline 20 mM before (black trace) and 20 min after (gray trace) a 10 min application of 50 mM DHPG. Calibrations for the traces are: *x*-axis, 1 mV; *y*-axis, 200 ms. There is no change in the amplitude of the bicuculline (20 μM) – isolated $GABA_B$ response after a 10 min application of DHPG 50 μM ($n = 7$). Modified from Lafourcade CA and Alger BE (2008) Distinctions among GABA(A) and GABA (B) responses revealed by calcium channel antagonists, cannabinoids, opioids, and synaptic plasticity in rat hippocampus. *Psychopharmacology (Berlin)* 198(4): 539–549.

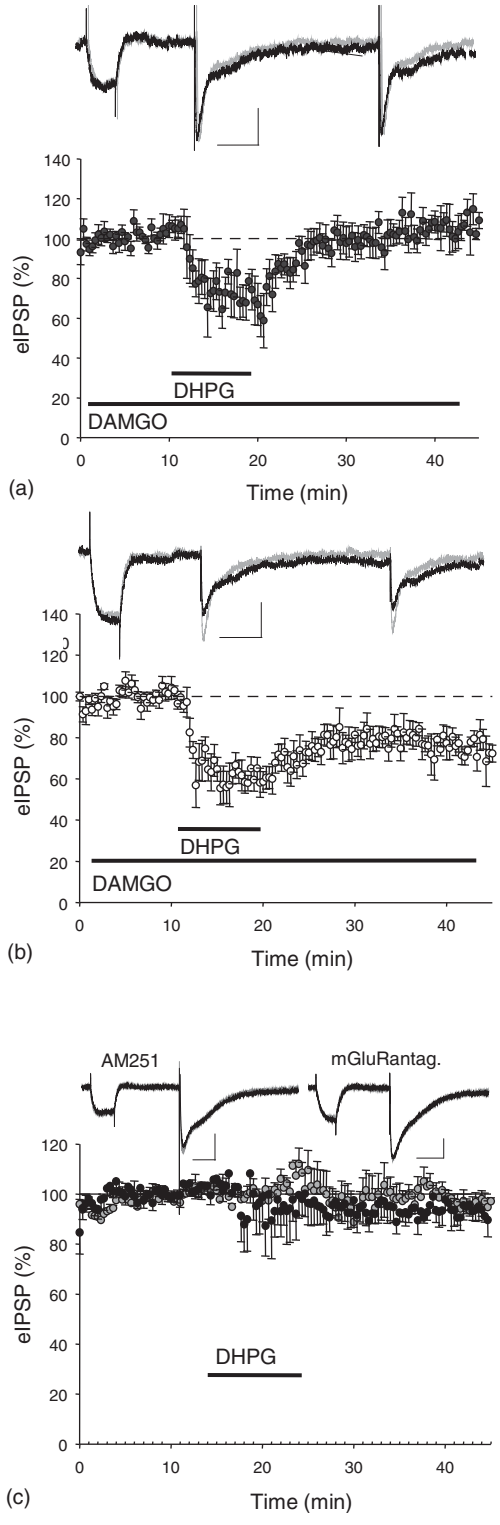


Figure 2 DAMGO prevents or reduces DHPG-induced iLTD induction. (a) Representative trace of a pair of eIPSPs, elicted at a 50 ms interval, before (gray trace) and 20 min after (black trace) DHPG 50 μM application. Pooled data ($n = 6$) of cells that did not show DHPG-induced iLTD in the presence of DAMGO. (b) Representative trace of a pair of eIPSP as in (a), before (gray trace) and 20 min after (black trace) DHPG 50 μM application. Pooled data of cells ($n = 7$) that did show decreased DHPG-induced iLTD in the presence of DAMGO. Dotted lines in (a) and (b) show estimated level of iLTD expected if MOR-expressing and CB1R-expressing cells did not interact. (c) Antagonists of CB1R or mGluR block iLTD. (a) Representative traces of eIPSPs before (gray trace) and 20 min after (black trace) DHPG 50 μM application in the presence of AM251, 3 μM (left), or group I mGluR antagonists (LY 367385, 100 μM, plus MPEP, 5 μM (right). Calib: 2 mV, 200 ms for all traces. iLTD induction by a 10 min DHPG 50 μM application is blocked by AM 251, 3 μM (black circles, $n = 4$) and by LY367385 100 μM plus MPEP 5 μM (gray circles, $n = 4$). Modified from Lafourcade CA and Alger BE (2008) Distinctions among GABA(A) and GABA (B) responses revealed by calcium channel antagonists, cannabinoids, opioids, and synaptic plasticity in rat hippocampus. *Psychopharmacology (Berlin)* 198(4): 539–549.

both short-term (DSI) and long-term (iLTD) responses. Different intracellular cascades mediate the different mechanisms. We did observe that in a sizeable fraction of cases, prior activation of MORs abolished the long-term effects of CB1R activation on inhibitory transmission (Figure 2). Since there was no overt short-term interaction, we infer that intracellular biochemical cascades responsible for the long-term effect could be altered by MOR activation. Unlike the short-term (i.e., DSI) suppression of transmission caused by CB1R activation (mainly transient downregulation of the N-type presynaptic calcium channel), iLTD involves inhibition of the cAMP-dependent protein kinase A pathway that affects vesicle release. The mechanisms of CB1R-induced iLTD are clearly localized in the presynaptic nerve terminals of the affected cells. Accordingly, the data imply a convergence of actions on the same interneurons. Only the CCK cells express CB1Rs, so a subset of these cells must be susceptible to MOR activation. Interference between MOR action and CB1R-induced iLTD is unlikely to take place at the level of the pyramidal cell, because DAMGO did not prevent the pyramidal cell from mobilizing endocannabinoids, as shown by the persistence of the short-term IPSP suppression mediated by mGluRs. Important future tasks will be to determine the identity of the subset of affected interneurons, and the molecular mechanisms of MOR interference with long-lasting CB1R effects. Figure 3 shows a simple summary diagram of these proposed interactions.

Epileptic Seizures and Neuroprotection

Opioids and cannabinoids – convulsant or anticonvulsant?

With regard to epileptic activity, opioids present a confusing picture. In general, they act as anticonvulsants, although both pro and anticonvulsant actions have been reported. Key variables include receptor subtype, agonist dose, and perhaps seizure model. For instance, in the pentylenetrazole model, opiates are anticonvulsant at low doses and proconvulsant at high doses. The

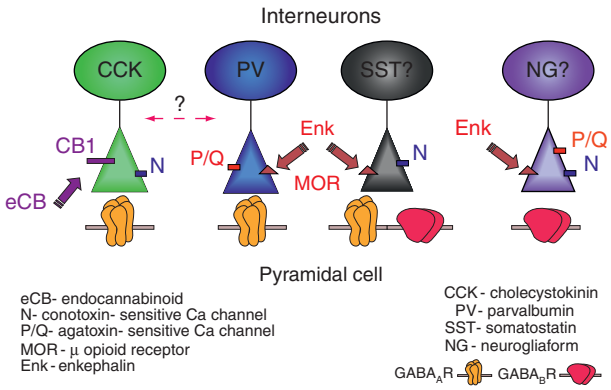


Figure 3 Schematic summary of proposed associations of hippocampal interneurons and MORs and CB1Rs. The diagram is meant to give a flavor for the characteristics of interneurons, and is not intended to be complete. Four major subtypes of interneurons are shown at synapses composed of GABA receptors; the basic properties of CCK and PV cells are widely agreed on, the somatostatin (SST) and neurogliaform (NG) cells less so. There is no direct evidence that the NG cells are the source of $GABA_B$ IPSPs, but they may well be a source. The SST cells express MORs, and may release via N-type channels; there are certainly other cell types with these characteristics. Not shown are electrical junctions: it is thought that CCK cells couple to each other, and PV cells couple to each other, in this way. CB1Rs are shown exclusively on CCK cells, but our recent data points to a minor component on another cell type, perhaps the PV cells. The large question mark and arrow between the PV and CCK cells suggest a possible interaction between them that would explain the unexpected ability of the P/Q channel antagonist, and the MOR agonist, to interfere markedly with CB1R-mediated iLTD.

cerebrospinal fluid of rats exposed to experimentally-induced seizures contains a substance that acts as an anticonvulsant when injected into a separate group of rats, and this anticonvulsant effect is antagonized by naloxone, implying that it is an endogenous opioid of some kind. Several laboratories report changes in the expression of opioid receptors or their endogenous agonists after the occurrence of seizures. These changes could be suppressed by kappa opioid peptides, whereas delta opioid peptides promoted status epilepticus.

Exogenous cannabinoids are generally found to be anticonvulsants, and indeed, this was one of their first documented medicinal uses in humans. Animal studies corroborate this picture, but have provided new insights and puzzles. In a maximal electroshock model of epilepsy, anandamide (administered i.p.) is anticonvulsant, and its effects are abolished by the CB1R antagonist SR141716. In a pilocarpine model, both THC and the synthetic agonist, WIN55212–2, injected i.p., prevented spontaneous seizures, supporting the idea that exogenous CB1R agonists are anticonvulsants. Likewise, CB1R antagonists appear to be proconvulsant. However, endogenous cannabinoids may act differently; as discussed later, some evidence suggests that endogenous cannabinoids have proconvulsant actions.

The precise regulation of the endocannabinoid system may determine whether cannabinoids enhance or diminish the occurrence of seizures. The exogenous application of cannabinoids could, in principle, have proconvulsant effects by inhibiting GABAergic interneurons, causing a concomitant increase in excitatory activity throughout the hippocampus. Nevertheless, cannabis use is not widely associated with epileptogenic effects, despite the prominent localization of CB1R on inhibitory nerve terminals in hippocampus and neocortex. A likely explanation for this observation is that CB1Rs are not indiscriminately located on all inhibitory nerve terminals, but only on those of a subgroup of the basket- and dendrite-targeting cells. This group of cells also expresses CCK. This group comprises a relative minority of all inhibitory synapses – probably ~20% in the hippocampus. Disrupting inhibition from these cells can alter neuronal network activity, but in ways that do not inevitably lead to seizures. As discussed below, CB1Rs present on excitatory nerve terminals, albeit at a low density, can play key roles in epilepsy.

Seizures affect endocannabinoids

Not only do endocannabinoids affect seizure activity, but seizures also directly affect the endocannabinoid system. CB1R receptor expression increases after pilocarpine-induced status epilepticus and kainate-induced seizures, and are accompanied by rapid postsynaptic increases in anandamide production in the hippocampus. Febrile seizures are observed in about 3% of young children, and about one-third of them are of prolonged duration (10–20 min). Prolonged febrile seizures during childhood are associated with an increased risk in developing temporal lobe epilepsy in adulthood. An animal model of febrile seizures has also been developed. In these rats, CA1 pyramidal cells are hyperexcitable and show rhythmic epileptiform discharges. Interestingly, febrile seizures also induce long-term changes in the endocannabinoid system. DSI is enhanced in rat hippocampal CA1 pyramidal cells following a single episode of prolonged febrile seizures during early development. Pharmacological and biochemical experiments have revealed that an increase in CB1R receptor number, rather than increased endocannabinoid mobilization, was the most likely cause. Responses to direct application of a synthetic CB1R agonist were enhanced, and Western blot analysis confirmed that CB1R protein expression was increased. In contrast, measurements of anandamide and 2-AG, revealed that their levels were not altered. As noted, a low density of CB1R does exist on excitatory hippocampal nerve terminals, it is present in high concentrations on CCK interneuron terminals, suggesting that a major effect of increased CB1R expression is a decrease in inhibition.

In a model of the febrile seizures, tetanic stimulation of Schaffer collateral synapses causes an activity-dependent

long-term DSI potentiation. Activation of AMPA/kainate receptors, metabotropic glutamate receptors, and CB1Rs are all required to induce the endocannabinoid plasticity. Mutual occlusion between febrile seizures and the synaptically-induced plasticity of the system suggest that these processes share common mechanisms of induction. The therapeutic implications for epilepsy treatment are complex: activation of CB1Rs is expected to be anticonvulsant, but could cause a long-term upregulation of CB1Rs on GABAergic terminals. Although endocannabinoid-mediated inhibition may not be strongly proconvulsant in some settings (because of the relatively small fraction of inhibitory synapses affected, see earlier sections), it could contribute in others. In contrast, therapeutic antagonism of CB1R during a seizure might transiently increase excitability in the short term, but prevent the upregulation of CB1R, and thus be beneficial in the long term.

Neuroprotection

Cell death may cause, or be caused by, prolonged seizures. A common mechanism of cell death is excitotoxicity resulting from excessive stimulation of glutamate receptors. In an in vivo model, application of the glutamate analog kainic acid to the cortex causes seizures that give rise to large-scale cell death. Although cell death is fairly widespread across cortical areas, the presence of endocannabinoids limits the extent of seizure-induced excitotoxic damage, and thereby acts as a neuroprotectant in this model. This conclusion follows from observations that kainic acid-induced cell death is greatly exacerbated in CB1R KO mice. It seems that interruption of the normal workings of the endocannabinoid system enables the excitotoxic effects to spread to a much wider area than usual. Endocannabinoid-induced neuroprotection is brought about by the ability of this system to limit the further release of glutamate that is triggered by the kainic acid seizure. Release of glutamate initiates a deleterious positive feedback loop, whereby depolarization and calcium-influx triggered by glutamate release causes more glutamate release, etc. Endocannabinoids break the cycle by inhibiting glutamate release. CB1Rs are present on glutamate axons and terminals, although at such a low level their presence was initially overlooked both anatomically and functionally. The levels of endocannabinoids that are released by physiological neuronal activity are insufficient to interfere with normal glutamate transmission. However, the much higher levels of endocannabinoid release associated with massive glutamate release and neuronal depolarization does effectively oppose further glutamate release. Release of glutamate, followed by metabotropic glutamate-induced endocannabinoid release from the pyramidal cells, could be sufficient to activate vast numbers of glutamate-associated CB1Rs. Hence, even though the system is not absolutely effective in preventing all neuronal cell death, it can limit the extent of the damage.

Targeted deletions of CB1R on specific cell populations reveal that CB1Rs on presynaptic glutamatergic nerve terminals in the forebrain and hippocampal hilus are most important for endocannabinoid-mediated neuroprotection. The system-wide, seizure-induced production of endocannabinoids can affect all CB1Rs, on inhibitory as well as excitatory terminals. It might be imagined that by inhibiting GABA release, and thereby limiting inhibition, activation of CB1R on the inhibitory cells would increase the extent of the excitotoxicity. This result does not occur, presumably because CB1R affects only a fraction (perhaps 10%–20%) of all inhibitory terminals – which even though individually powerful, are still in the minority. Continued functioning of the remainder of the inhibitory synapses may contribute some degree of neuroprotection. The distribution of CB1R on glutamatergic terminals is apparently much wider, perhaps encompassing the terminals of all of the major excitatory synaptic inputs to CA1, and accounting for up to half of all of the CB1R in the hippocampus. Thus, the net result of the endocannabinoid inhibition of both GABA and glutamate release would be neuroprotective.

Upregulation of opioid receptors occurs after seizures, and special attention has been given to the protective properties of the endogenous neuropeptide dynorphin in both animal models and human studies. The latter has not been without controversy, however. Initial findings showing correlations between epilepsy and the reported polymorphism of distinctive opiate genes (such as the promoter for the dynorphin gene or the MOR subunit gene) remain controversial. Since dynorphin can activate (with different affinities) the three main opiate receptors (mu, delta, and kappa), and arguably even NMDA receptors, more detailed studies are needed to understand the mechanisms underlying its role in epileptic conditions. New evidence from animal models does support a role for prodynorphin-derived peptides as anticonvulsants, almost exclusively through their activation of kappa opioid receptors. Further work will be needed to determine if such a role has been conserved in humans. MOR mRNA expression is downregulated by global ischemia (an insult that leads to the death of CA1 cells in the hippocampus), whereas intracerebroventricular injection of naloxone in vivo is neuroprotective. Similarly, MOR blockade is protective in models of hypoxia/ischemia, which seems to suggest that the decrease in MOR expression (and therefore the ensuing decrease in excitability) may be an important defense mechanism utilized by neurons.

Conclusions and Future Directions

Functional interactions between the opioid and cannabinoid systems, both of which independently modulate seizures, present a new, albeit challenging, target for pharmacological epilepsy therapies.

Several models have been proposed to explain opioid–cannabinoid interactions throughout the CNS, including shared downstream molecular targets, stimulation of opioid release by cannabinoids, ordopamine-mediated interactions. In hippocampus, immunohistochemical studies have indicated that MORs and CB1Rs are localized on distinct populations of cells. Nevertheless, our studies clearly show interactions between MOR and CB1R effects. These interactions may be subtle, and are centered on long-term regulation of synaptic inhibition. Their potential significance, however, suggests that further investigation of the mutual influence between opioids and cannabinoid systems on inhibition in the hippocampus will pay dividends.

Despite the widespread use of marihuana and heroin, and the increasing amount of research dedicated to understanding these systems, their relevance in some diseases is still controversial and many times confusing. The complexity of the opiate and cannabinoid systems, added to the fact that some diseases (like epilepsy) can have multiple etiologies, contributes to the difficulties researchers face when trying to understand the role that these drugs of abuse play in such diseases. A better understanding of how cannabioids and opioids are regulated, as well as what cells are susceptible to them in control and under epileptic conditions, would help us understand the importance each of these players has in such situations. This goal will certainly not be easily attained, as there are a considerable number of different types of interneurons in the hippocampus, as well as many types of opiate receptors and possibly more than one type of cannabinoid receptor. Understanding how these interneurons maintain normal hippocampal activity, what disrupts that regulation – and what roles are played by opioid or cannabinoid receptors in normal and disease processes – may prove crucial to our developing new approaches to prevent seizures. Such insight may also help us determine to what extent the treatment with exogenous cannabinoids may be useful to alleviate or prevent some of the symptoms associated with these pathologies.

Lastly, the strict regulations scientists face when conducting clinical trials with these drugs of abuse makes it harder to collect sufficient data to reach more robust conclusions. Making it easier for scientists to conduct more clinical trials is of vital importance to reach a faster understanding on the role of commonly-used drugs of abuse on the origin and/or maintenance of epilepsy, as well as on their ability to counteract some of its symptoms.

Acknowledgements

Work in the authors' laboratory is supported by NIH RO1 DA140625, and MH077277 to B.E.A. The material was reproduced with kind permission of Springer Science and Business Media.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; Plasticity of GABAergic Systems during Epileptogenesis; **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; Properties of Interneurons and their Relation to Epilepsy; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Peptides**: Cannabinoids and Epileptiform Activity; Dynorphin and Nociceptin/Orphanin FQ; Participation of Opioid Peptides in Epilepsy; Seizure Susceptibility is Altered by Exposure to Morphine during Mid to Late Gestation Period in the Rat.

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Galanin Modulation of Epileptogenicity

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Introduction

Initial experimental evidence implicating galanin in the regulation of seizure activity came a decade after the discovery of the peptide (by Tatemoto and colleagues in 1983). In 1992, galanin was shown to suppress kindled seizures in rats upon its intrahippocampal administration. A year later, in vitro study suggested that galanin inhibits seizure activity via presynaptic inhibition of glutamate release from hippocampal neurons. Since then, with progress in our understanding of the physiology, biochemistry, and pharmacology of galanin and its receptors, data have accumulated regarding the involvement of galanin in epileptic processes. To further research on this topic, new animal models have been developed, including galanin knockout mice, galanin receptor knockout mice, and animals with focal overexpression of the peptide in limbic structures.

Background

Galanin is a 29 (30 in humans) amino acid neuroendocrine peptide, originally isolated from porcine intestine. Galanin has been identified in many species, including rodents and primates. The peptide modulates a variety of physiological processes and is widely distributed in both central and peripheral nervous systems, as well as in the endocrine system and the gut. Galanin modulates the release of gonadotropic hormone, prolactin, insulin, glucagon, growth hormone, and somatostatin and affects behaviors related to these hormones (for example, appetite, digestion, and sexual behavior).

In the brain, galanin is colocalized with, and modulates both pre and postsynaptic action of other peptides (somatostatin, neuropeptide Y, dynorphin, vasoactive intestinal peptide, substance P) and of classic neurotransmitters (acetylcholine, dopamine, serotonin, norepinephrine, glutamate). The peptide has several central effects, and has been shown to be involved in the regulation of satiety, nociception, mood, learning, and memory.

In the hippocampus, no galanin-containing neurons have been identified; galanin is localized exclusively in the axonal projections originating from the medial septum and locus coeruleus (**Figure 1(a)** and **1(b)**).

Galanin exerts its physiological effects via the activation of one of the three types of receptors, GalR1, GalR2, or GalR3. The first two types are abundantly expressed in the brain, particularly in the hippocampus. Like for many other neuropeptides, intracellular signaling cascades downstream from galanin receptors are multiple, and include inhibition of adenylate cyclase; opening ATP-sensitive K^+ channels (KATP); activation of G-protein coupled inwardly rectifying potassium channels (GIRK); inhibition of L-type and N-type Ca^{2+} channels; stimulation of inositol phospholipids turnover; stimulating or inhibiting Ca^{2+} mobilization; and activating MAP kinase.

Among these multiple effects of galanin, several are likely to be involved in the inhibition of seizure activity. One plausible scenario explaining how galanin might inhibit seizures was described in experiments showing that galanin inhibited depolarization-induced glutamate release from hippocampal slices. This action was prevented by coapplication of glybenclamide, a blocker of K_{ATP}. These results implied that in the hippocampus, galanin opens K_{ATP} channels, which in turn leads to membrane hyperpolarization and eventually to the inhibition of glutamate release from presynaptic terminals.

Methods

Studies of modulatory effects of galanin on experimental seizures have employed several animal models of epilepsy, such as kindling induced by electrical stimulation of the hippocampus; systemic injection of picrotoxin; status epilepticus induced by electrical stimulation of perforant path, by kainic acid, or pilocarpine; and acute chemoconvulsant-induced seizures (e.g., pentylenenetrazole). Biological activity of galanin receptor ligands has been evaluated upon their focal (intrahippocampal) or systemic administration. Functional state of galanin peptide and receptors in the epileptic brain has been studied using immunohistochemistry, quantitative polymerase chain reaction, and tissue binding of radioactive galanin receptor ligands. Manipulations with brain galaninergic signaling involved generation of transgenic mice, including galanin and/or galanin receptor knockouts and animals with upregulation, as well as local delivery of viral vectors carrying galanin gene.

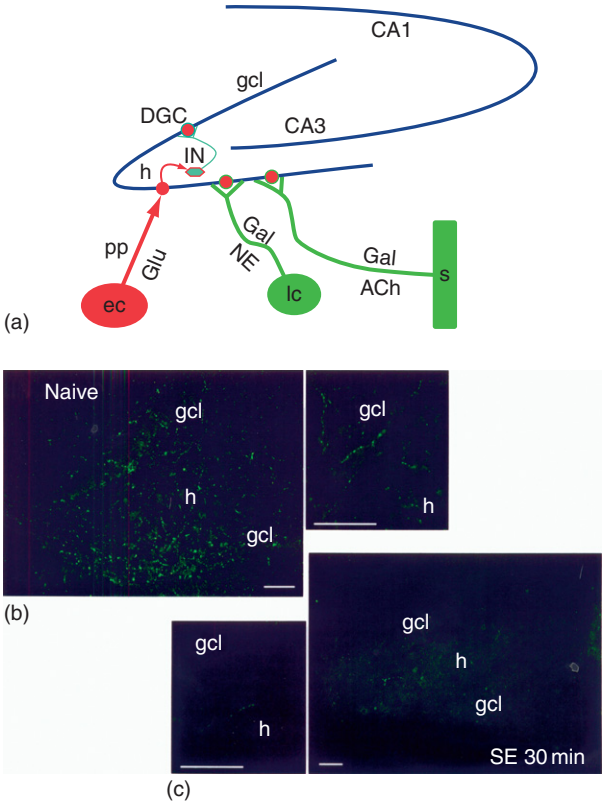


Figure 1 *Galaninergic innervation of the rat hippocampus under normal and seizure conditions*. (a) Anatomical substrate of anticonvulsant effects of galanin in the hippocampus. Dentate granule cells (DGC) (red circles in the granule cell layer) receive excitatory glutamatergic input from entorhinal cortex in a form of perforant path. In hilus DGC excite GABAergic interneurons, which in turn inhibit DGC. At the same time, dentate gyrus receives galaninergic innervation, which comes with noradrenergic fibers from locus coeruleus and cholinergic fibers from septum/diagonal band complex. Abbreviations: ec – entorhinal cortex; pp – perforant path; gcl – granule cell layer; h – hilus; lc – locus coeruleus; s –septum/diagonal band; DGC – dentate granule cells; IN – interneuron; Glu – glutamate; Gal – galanin; NE – norepinephrine; ACh – acetylcholine. Excitatory projections are shown by red and inhibitory-by green lines. (b) Galanin immunoreactivity in naïve rat dentate gyrus. Abbreviations: gcl – granule cell layer; h – hilus. Scale bar: 50 μm. (c) Perforant path stimulation-induced seizures lead to rapid depletion of galanin in the dentate gyrus. Immunohistochemical galanin labeling in the animal 30 min after the end of perforant path stimulation. Note the depletion of the peptide from galanin-positive fibers. Reproduced from Mazarati A (2004) Galanin and galanin receptors in epilepsy. *Neuropeptides* 38:331–343. © Elsevier with permission.

Results

Effects of Exogenous Galanin Receptor Ligands on Seizures

The first report of anticonvulsant effects of galanin came from the experiments involving kindling induced by chemoconvulsant picrotoxin. Investigators showed that central administration of galanin (1–29) (which activated both types of brain galanin receptors) inhibited kindled seizures, and that the hippocampus played the key role in mediating anticonvulsant effects of the peptide. Subsequent studies revealed that intrahippocampal administration of galanin exerted strong antiepileptic effects under conditions of status epilepticus induced (in rats) by electrical stimulation of the perforant path. Similarly, intrahippocampal administration of a GalR2 preferential agonist, galanin fragment (2–11), protected against status epilepticus, particularly during its late phase. This finding was particularly significant, since it is known that the late phase of status epilepticus is resistant to many conventional antiepileptic drugs. At the same time, galanin receptor blockers facilitated the progression of status epilepticus-associated seizure activity.

The role of galanin receptor subtypes, and related downstream signaling cascades, in mediating antiepileptic effects of the peptide was explored in detail using the kindling model of epileptogenesis. In these studies, a preferential GalR1 blocker, synthetic peptide M617, delayed but did not block the acquisition of hippocampal kindling in rats. The anticonvulsant effect of this GalR1 agonist depended on an intracellular pathway involving G_i protein, since the effect was abolished in the presence of G_i protein inhibitor pertussis toxin (PTX); further, GIRK were implicated, since the effect was reversed by a selective GIRK blocker tertiapin Q (**Figure 2**). Interestingly, activation of hippocampal GalR2 may exert either anticonvulsant effects (which are coupled to G_i, but are GIRK-independent) or promote seizures in a manner that depends on $G_{q/11}$ protein (the effect of the peptide was abolished in the presence of $G_{q/11}$ protein blocker [D-Arg1,D-Trp5,7,9,Leu11]-substance P) and further downstream on the mobilization of intracellular Ca^{2+} (as the effect was sensitive to dantrolene, an agent which inhibits the release of intracellular Ca^{2+} from endoplasmic reticulum, **Figure 2**). Additional experiments revealed that a preferential blocker of GalR2, the synthetic peptide M817, abolished anticonvulsant effects of low-frequency electrical stimulation of perforant path on the severity of kindled seizures. These experiments led to the proposal of a sequence of galanin-regulated pathways that may modulate the evolution of the epileptic state (**Figure 2**).

Seizures Themselves Modify the Expression of Galanin and Galanin Receptors in the Brain

Experimental studies involving status epilepticus in rodents have shown changes of galanin expression in the hippocampus following seizures. Profound depletion of galanin in the axonal hippocampal projections, originating both from locus coeruleus and from medial septum, was reported as early as 30 min after the onset of electrically-induced status epilepticus in rats (**Figure 1(b)** and **1(c)**). Reduced expression of galanin was observed as late as 1 week after the insult, and was suggested to be a contributory factor in the progression of both status

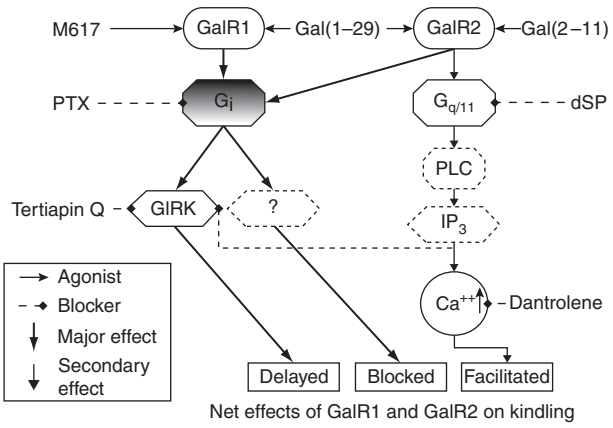


Figure 2 *Proposed mechanisms of antiepileptic action of hippocampal GalR1 and GalR2 under conditions of kindling model of epilepsy, based on the in vivo pharmacological analysis.* Activation of hippocampal GalR1 by both a nonselective agonist galanin (1–29) and by a selective agonist M617 inhibited kindling. These effects were abolished by pretreatment with PTX, an inhibitor of G_i protein, or by pretreatment with Tertiapin Q, a selective blocker of G-protein coupled inwardly rectifying potassium channels (GIRK). Inhibition of Glutamate (Glu) release from hippocampal neurons due to GIRK-induced membrane hyperpolarization is indicated as a likely end-effect of the activation of GalR1. Activation of hippocampal GalR2 using galanin (2–11) (which acts at GalR2) also exerted antiepileptic effect. However, unlike GalR1, the effect of GalR2 activation was not simply abolished by PTX: the inhibition of G_i protein inversed the effect of GalR2 agonist from anticonvulsant to proconvulsant. The latter action of GalR2 was prevented when $G_{q/11}$ protein was blocked by [D-Arg1,D-Trp5,7,9,Leu11]-substance P (dSP). Activation of phospholipase C (PLC), inositol triphosphate accumulation (IP_3) and mobilization of intracellular Ca^{2+} are indicated as plausible downstream signaling. The latter was confirmed experimentally, as the facilitation of seizures by GalR2 agonist was abolished in the presence of dantrolene, which inhibits the release of Ca^{2+} from endoplasmic reticulum. Finally, the fact that inhibition of G_i abolished anticonvulsant effect of GalR2 agonist in Tertiapin Q-independent manner suggests the presence of antiepileptic pathway downstream from GalR which is coupled to G_i, but is independent on GIRK.

epilepticus and of subsequent chronic epilepsy. Concurrently with the depletion of the peptide from the axonal pool, de novo galanin expression was reported in the inhibitory interneurons of hilus of the dentate gyrus after status epilepticus both in rats and in mice. The physiological significance of the latter phenomenon remains unclear, although it has been suggested to represent a compensatory mechanism in response to the fatigue of galaninergic innervation of the hippocampus.

While status epilepticus compromised the expression of galanin in the hippocampus, it did not affect the expression of GalR1. At the same time, the expression of GalR2 decreased by 30% as compared with control values.

In conclusion, seizure activity leads to the depletion of galanin in the hippocampus; given the effects of exogenously applied peptide, this depletion may exacerbate epileptic process. Together with complete (GalR1) or partial (GalR2) preservation of targets for action of galanin in epileptic hippocampus, the discussed findings justify the utility of prospective galanin-based antiepileptic drugs (see later text).

Genetic Manipulations with Galanin and its Receptors Modify Seizure Phenotype

The first direct evidence that endogenous galanin is capable of counteracting seizure activity came from the experiments involving mutant animals. In the status epilepticus model, mice with the functional disruption of the galanin gene showed a greater predisposition to seizures than their wild type littermates (as reflected in a shorter time to induce seizures as well as in greater severity of the convulsions in response to hippocampal stimulation). In contrast, mice that overexpressed galanin in the brain (under control of a dopamine beta hydroxylase promoter, since galanin is colocalized with norepinephrine) showed enhanced resistance to seizure induction. Additional in vitro studies revealed that hippocampal slices obtained from galanin knockout mice exhibited significantly higher glutamate release upon K^+-induced depolarization, while hippocampal slices from galanin-overexpressing animals showed virtually no glutamate release in response to the same procedure. These observations confirmed the earlier suggestion that galanin exerts anticonvulsant effect through presynaptic inhibition of glutamate release.

Overexpression of galanin with the transgene under control of a promoter (platelet-derived growth factor B) which does not confine the overexpression to neuronal populations with a certain neurotransmitter profile, inhibited the progression of kindled seizures, and increased afterdischarge threshold upon hippocampal stimulation. These data, too, are consistent with an antikindling action of galanin.

The development of galanin receptor knockout mice afforded further understanding of the role of galanin receptor subtypes in epileptogenesis. For example, 25% of GalR1 knockout mice exhibited spontaneous seizures. Furthermore, epileptic animals showed a cascade of changes in the expression of other several neuropeptides, which was similar to the pattern of changes observed in models of limbic epilepsy. Furthermore, spontaneously seizing GalR1 knockout mice exhibited reduced frequency of miniature inhibitory postsynaptic currents (mIPSCs) in hippocampal CA1 pyramidal neurons, thus implicating impaired synaptic inhibition in the seizure phenotype. Moreover, the subpopulation of GalR1 knockout animals that did not show spontaneous seizures developed more severe and longerlasting seizure activity, and more profound seizure-induced hippocampal neuronal injury (compared with controls), under conditions of pilocarpine – and perforant path stimulation – induced status epilepticus.

In contrast, GalR2 knockout mice exhibited neither spontaneous seizure phenotype nor enhanced susceptibility in response to convulsions induced by chemoconvulsants. However, given the pharmacological studies discussed earlier, the absence of the enhanced predisposition to seizures in GalR2 knockout animals does not mean that this receptor is not involved in the regulation of seizure activity. Rather, the 'normal' phenotype suggests that the elimination of GalR2 is compensated through the activation of other regulatory factors. This possibility has been supported by the finding that GalR2 mice showed no other physiological deficits (including normal sensory function, feeding behavior, reproduction, mood, learning, and memory) although other lines of research have shown that these activities are modulated by GalR2. Indeed, the lack of phenotypic alterations in transgenic animals – due to the redundancy or compensation – is not uncommon; this is a drawback of the transgenic approach, but can be addressed using an experimental approach discussed later.

Focal Over Expression of Galanin in the Brain Suppresses Seizures

An elegant approach further exploring the anticonvulsant role of galanin was applied in the studies that used an adeno-associated viral (AAV) vector, carrying the galanin gene, to transfect neurons in vivo. A coding sequence of the galanin gene was cloned and inserted into the AAV vector, which also contained fibronectin secretory signal sequence (FIB), to ensure not only expression but also active secretion of galanin into the extracellular space. In vivo injection of AAV-FIB-galanin vector into the inferior colliculus of the rat significantly attenuated seizures induced by focal electrical stimulation of this brain area. In contrast, administration of AAV-galanin vector which led to the overexpression of galanin – but not to the increased secretion of the peptide – had no anticonvulsant effects. Later, investigators showed that the administration of AAV-FIB-galanin into the pirifirm cortex attenuated seizures induced by kainic acid. The necessity of active secretion of galanin from peptide-expressing neurons for achieving the inhibition of seizures was addressed in different set of experiments; these studies showed that simple overexpression of galanin – induced by AAV-Neuron Specific Enolase (NSE)-galanin vector inserted into rat dentate gyrus neurons – was sufficient for mitigating the severity of convulsions induced by intrahippocampal administration of kainate. This latter result suggested that the secretion of galanin, which was presumably induced by seizures from neurons expressing galanin de novo, was sufficient to inhibit focal epileptic activity.

A philosophically similar approach was applied to examining the role of galanin receptors in epilepsy. Peptide nucleic acid (PNA) antisense, was used to downregulate GalR2 in the hippocampus in vivo. PNA is a DNA or RNA mimic, which binds to DNA or RNA in complementary antiparallel fashion, thus inhibiting transcription or translation. PNA which was targeted at mRNA encoding GalR2 reduced the expression of the latter receptor in the hippocampus by 50% after 1 week of focal administration. This downregulation of hippocampal GalR2 expression dramatically increased both the severity and the duration of status epilepticus induced by hippocampal stimulation.

Depending on the Site of Action, Galanin may be Either Anti or ProConvulsant

In addition to its direct action on hippocampal receptors, galanin is capable of modulating limbic seizures through interaction with outside neuronal populations that project to the hippocampus. The interaction between galanin and serotonin (5-HT) in dorsal raphe is a good example of how its external (to hippocampus) modifier function can alter hippocampal activity and limbic seizures. 5-HT possesses antiepileptic properties in the hippocampus via the activation of 5-HT_{1A} receptors. In dorsal raphe, a major source of serotonergic innervation of the hippocampus, local activation of GalR1 augmented the severity of limbic seizures both in rats and in mice, and concurrently reduced serotonin concentration both in dorsal raphe and in the hippocampus. In contrast, local dorsal raphe activation of GalR2 (using a preferential GalR2 agonist), reduced the severity of seizures in both rats and mice, and increased serotonin concentration in both hippocampus and dorsal raphe. Injection of native galanin molecule (which does not discriminate between GalR1 and GalR2) into the dorsal raphe of GalR1 knockout mice produced anticonvulsant effects, thus corroborating the anticonvulsant profile of GalR2 in dorsal raphe.

Finally, systemic depletion of 5-HT in the brain, which was achieved via the administration of a 5-HT-selective neurotoxin (para-chloroamphetamine), eliminated both proconvulsant effects of GalR1 and anticonvulsant effects of GalR2.

These experiments emphasize the importance of the site of action of galanin, and of the neurotransmitter system subject to the modulatory effects of the galanin peptide, in the regulating of seizure activity.

Future Research

Conclusions

Several lines of evidence support the notion that galanin acts as an endogenous anticonvulsant peptide: (1) Exogenous central administration of GalR agonists inhibits seizures, while GalR blockers facilitate epileptic activity;

(2) Compromised galaninergic innervation of the hippocampus parallels the evolution of epileptic state; (3) Transgenic animals which lack either galanin, or its receptor GalR1, exhibit enhance susceptibility to seizures, or even have spontaneous seizure phenotype; (4) Focal overexpression of galanin in limbic structures using virus vector technique mitigates seizure activity, while focal knockdown of galanin receptors (GalR2) in the hippocampus promotes seizures. Antiepileptic properties of galanin, along with the advance of medicinal chemistry, open the opportunity for the development of novel type of antiepileptic drugs, which suppress seizures through the activation of galanin receptors.

A Prospect for Galanin-Based Antiepileptic Drugs

Anticonvulsant effects of galanin, as well as its physiological activity in other neurological disorders (e.g., impairments in memory, mood, and appetite), make it an attractive target for therapeutic interventions. However, native galanin, not unlike other peptides, is a poor candidate for a therapeutic agent owing to its low bioavailability, particularly because of it degradation by peptidases and low permeability through the blood–brain barrier.

The first successful attempt in overcoming this drawback was accomplished using a Combinatorial approach – synthesis of a small nonpeptide molecule with physicochemical, biochemical, and pharmacological properties resembling those of galanin. Screening of the galanin sequence established Trp-2, Asn-5, Tyr-9 as a 'pharmacophore' – that is, an amino acid sequence primarily responsible for the physiological effects of the whole peptide molecule. On the basis of this finding, the first nonpeptide low molecular weight GalR agonist, galnon, was synthesized. This compound exhibited equal affinity towards GalR1 and GalR2, penetrated the blood–brain barrier, and showed potent anticonvulsant effects upon systemic administration in both rats and mice. The next generation of nonpeptide galanin agonists included galmic, with preferred action toward GalR1 over GalR2; this compound was also effective in inhibiting seizures in rodents upon systemic administration.

However, both galnon and galmic suffered the same shortcoming – particularly, they had only moderate to low affinity towards galanin receptors. This drawback prompted the search for alternative strategies for the development of galanin-based antiepileptic drugs. Such efforts led to the synthesis of NAX-5055, a rationally designed analog of truncated galanin neuropeptide that contains lipo-amino acid and basic residues at the C-terminus. The compound was shown to be active after systemic administration and under conditions of several seizure models, including kindling epileptogenesis.

The development of galanin-based antiepileptic drugs is still in its nascent stage. The ongoing research has been focusing on balancing reasonable affinity of such compounds towards galanin receptors with their biological availability (i.e., preservation of anticonvulsant efficacy upon systemic administration), evaluation and mitigation of possible side effects (for example inhibition of the activity of pancreatic beta-cells), and synthesis of ligands with selective affinity towards galanin receptor subtypes.

See also: **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Seizures; **Peptides**: Tachykinins and Epilepsy; **Seizures**: Seizure Termination and the Postseizure Refractory Period; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides; Viral Vector Approaches for Antiepileptic Gene Therapy.

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Participation of Opioid Peptides in Epilepsy

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Introduction

Opioid peptides are endogenous substances that exhibit the pharmacological properties of morphine. Morphine (named after Morpheous, the Greek god of dreams) is an alkaloid analgesic, isolated as one of the components of opium, the infamous product of the poppy, *Papaver somniferum.*

The opioid peptides are involved in physiological actions such as motor behavior, feeding, drinking, analgesia, immune responses, memory, and in the regulation of gastrointestinal, cardiovascular, neuronendocrine, and cognitive functions. They are also implicated in the mechanisms of pain, addiction, tolerance, dependence, stress, depression, and mental illness. Current evidence supports the view that when normal regulatory processes are disrupted, endogenous opioid peptides may play a significant role in the etiology or elaboration of seizure phenomena, and may contribute to neurological deficits encountered in association with epilepsy. Opioid peptides are also involved in neurological disorders such as Alzheimer's disease and Huntington's disease.

Background

Classification and Localization

In the early 1970s, two pentapeptides with opiate-like activity were extracted from porcine brain and characterized. These peptides differed only in the C-terminal amino acid, and named met-enkephalin (Tyr–Gly–Gly–Phe–Met) and leu-enkephalin (Tyr–Gly–Gly–Phe–Leu). At present, three major branches of the opioid peptide greater family are known: the proopiomelanocortin-derived peptides (adrenocorticotrophic hormone, β-lipoprotein, β-endorphin, melanocyte-stimulating hormones, and corticotrophin-like intermediate lobe peptide), the proenkephalin-derived peptides (met-enkephalin, leu-enkephalin, peptide F, amidorphin, met-enkephalin–Arg–Gly–Leu, peptide E, met-enkephalin–Arg–Phe, etc.), and the prodynorphin-derived peptides (neoendorphins and dynorphins). Immunoreactivity corresponding to the different opioid peptides is widely distributed throughout the brain and spinal cord, the highest concentrations being in the hypothalamus, anterior pituitary, reticular formation, caudate, hippocampus, amygdala, periaqueductal gray, substantia nigra, and neocortex.

Although endogenous opioid peptides have been known for many years, initial attempts to label the opioid receptors biochemically were without success. In 1973, three laboratories (Pert and Snyder, Terenius *et al.*, and Simon *et al.*) reported the biochemical demonstration of opioid binding sites using ^{3}H-labeled naloxone, dihydromorphine, or etorphine. For the most part, these initial studies were labeling mu binding sites, since ^{3}H-naloxone and ^{3}H-dihydromorphine are relatively mu selective, whereas ^{3}H-etorphine labels a variety of subtypes in addition to mu. In 1976, Martin and coworkers found different pharmacological profiles for a series of opiate alkaloids and suggested the existence of three types of opiate receptors: 'mu' for morphine, 'kappa' for ketocyclazocine, and 'sigma' for *N*-alylnormetazocine (SKF 10047). Thereafter, in 1977, Kosterlitz's group postulated the presence of a distinct opioid receptor termed 'delta.' The mu receptor is operationally defined as the high-affinity site at which morphine-like opioids produce analgesia and other classical opioid effects. The delta receptor is operationally defined as the receptor (found in peripheral tissues such as vas deferens as well as central nervous system) that exhibits a high affinity for the naturally occurring enkephalins. The kappa receptor is the receptor at which opioids produce analgesia and is highly selective for dynorphins. Opioid concentrations in the low nanomolar range are needed to obtain favorable ratios of specific-to-nonspecific binding.

Phylogenetically, opioid binding sites have been reported in the nervous system of a wide range of species, including insects and mollusks. In the mammalian central nervous system, the existence of opioid receptors is found within neuronal and glial cell membranes, and no significant specific opioid binding is detected in white matter. High levels of opioid receptor binding are detected in the brain areas associated with the limbic system. The importance of the hippocampal formation in learning and memory mechanisms and the role of this region in certain forms of epilepsy have greatly stimulated the analysis of opioid peptides' effects in this structure. Activation of mu and delta opioid receptors induces excitatory effects on the activity of pyramidal neurons via disinhibitory mechanisms involving GABAergic interneurons. In contrast, activation of kappa opioid receptors induces inhibitory effects on perforant path-granule cell excitatory neurotransmission and synaptic plasticity in the hippocampal dentate gyrus.

Effects of Opioid Agonists and Antagonists on Seizure Activity

Reports dating back to the nineteenth century indicate that opium may induce proconvulsant and anticonvulsant

effects. Modern studies indicate that opioid peptides may induce excitation or inhibition, depending on the route of administration and the type of seizure or the experimental model evaluated.

That the endogenous opioids may play a role in epilepsy was considered after it was demonstrated that the intracerebroventricular (i.c.v.) administration of met-enkephalin, leu-enkephalin, morphine, or β-endorphin in normal animals produces a prolonged immobile, stuporous state with intermittent sudden twitches and 'wet-dog shakes'. These behaviors are accompanied by abnormal electroencephalographic activity consisting of brief episodes of rapidly repetitive spikes evolving into slower high-amplitude sharp waves. Both the pathological behavioral signs and the abnormal electrographic patterns are reversed by naloxone, a competitive antagonist for mu, delta, and kappa receptors. Similarly, the injection of morphine facilitates the status epilepticus induced by pilocarpine, an effect blocked by naloxone.

The administration of opioid agonists produces anticonvulsant effects on generalized seizures induced by fluorothyl (pentazocine or meperidine, i.c.v.), estricnine (morphine, i.c.v.), and electroshock (morphine, β-endorphin, DADL, i.c.v.). The opioid peptides modify the seizure activity in natural models of epilepsy. In gerbils, the systemic administration of naloxone enhances, whereas β-endorphin (i.c.v.) or morphine (s.c.) decreases the severity of seizures. The i.c.v. injection of morphine, met-enkephalin, or β-endorphine augments the latency to myoclonus in the photosensitive baboon *Papio papio*. In fully kindled rats, the i.c.v. administration of mu, but not delta, agonists of opioid peptides reduces the intensity of generalized seizures. In contrast, the antagonist naloxone facilitates the development of the kindling. During the postictal period (after generalized seizures induced by kindling), the administration of opioid agonists enhances the refractoriness for subsequent seizures whereas naloxone blocks this effect.

Blockade of delta receptors prevents the establishment of seizure activity in a model of self-sustaining status epilepticus, while their activation facilitates its development. In contrast, activation of mu receptors does not modify seizure activity in that experimental model. Activation of kappa receptors is highly effective in suppressing seizure activity in animal models of epilepsy, and it plays an inhibitory influence in the induction and maintenance of status epilepticus.

Effects of Seizure Activity on Endogenous Opioid Peptides

Evidence from both humans and experimental models supports the view that endogenous opioids are activated as a consequence of the seizure activity. In 1979, Frenk and collaborators suggested that endogenous opioid peptides are released after convulsive seizures, and this induction has inhibitory effects and enhances refractoriness for subsequent seizures during the postictal depression period. In 1981, Vindrola and Fernandez-Guardiola reported that the cerebral tissue levels of enkephalins are modified by electrical kindling model, an effect that depends on the seizure rate and the peptide evaluated; for example, leu-enkephalin increases progressively through the kindling process, whereas met-enkephalin levels augment only after several generalized seizures are produced. At present, several studies support the view that the opioid peptides, their precursors, and their receptors are modified as a consequence of seizure activity in human brain and experimental models, as described below.

Opioid Peptides in Patients with Epilepsy

Opioid peptides are released as a consequence of seizure activity in the human brain. This notion is supported by the increased content of leu-enkephalin, but not of met-enkephalin or β-endorphins, in the cerebrospinal fluid of patients with partial and generalized seizures. This effect is not caused by antiepileptic drugs or because of structural pathological changes. A diffuse release of cerebral opioids, as evidence by displacement of ^{11}C-diprenorphine binding, has been seen in the association cortex after repeated absence seizures and in parieto-temporo-occipital cortex during reading-induced seizures. These findings suggest that opioid-like substances are released as a consequence of seizures.

The opioid receptor distribution is modified in the epileptic focus and the surrounding areas involved in seizure propagation, a situation that depends on the type of epilepsy. In patients with mesial temporal lobe epilepsy (MTLE), positron emission tomography (PET) with specific opiate receptor ligands has revealed a regionally specific increase of opioid receptor availability in the temporal pole and fusiform gyrus ipsilateral to the seizure focus immediately after spontaneous seizures, with a gradual return to basal levels during the interictal period. Interictally, amygdala from patients with MTLE presents reduced mu binding that could be due to epilepsy-induced volume changes of this brain area. In the hippocampus of patients with temporal lobe epilepsy (TLE) induced by tumors or not associated with neuronal loss, the highest density of kappa opioid receptors is found in CA1 and subiculum, whereas hippocampi from patients with MTLE show significant reduction of kappa receptors in CA1. Interictal PET with specific opiate receptor ligands has also revealed increases in both mu and delta opioid receptors in neocortex overlying limbic structures. Augmented mu receptor binding is confined to the middle aspect of the inferior temporal cortex, whereas increased binding of delta receptors is found in the mid-inferior temporal cortex and the anterior aspect of the middle and superior temporal cortex. The interictal enhanced opioid

receptor binding in the neocortex of patients with MTLE has been suggested as a compensatory mechanism that prevents the spread of seizure activity originating in the limbic structures.

In contrast with MTLE, neocortical epileptic tissue surgically obtained from patients with pharmacoresistant TLE show increased cholinergic receptors and no significant changes in opioid receptors, whereas the surrounding cortical areas are characterized by decreased mu and delta opioid receptors. These results indicate an increased excitatory influence in the neocortical epileptic focus (high cholinergic receptor density), but decreased inhibitory neurotransmission in the area of seizure propagation (low opioid receptor binding in the surrounding cortex). In patients with childhood and juvenile absence epilepsy, PET studies revealed an acute reduction in ^{11}C-diprenorphine binding (which binds with similar affinities to the mu, delta and kappa opioid receptors) in association areas of neocortex, but not in other brain areas. This set of results suggests that the reduced opioid receptor binding in the neocortex of patients with TLE and the absence of seizures may facilitate the spreading of epileptic activity (Figure 1).

Recent Results

Kindling and Opioid Peptides

Kindling is an experimental model of epileptogenesis consisting of a progressive development of behavioral and electrographic changes that culminate in generalized tonic–clonic seizures. This process involves functional and structural changes similar to those detected in human TLE. A significant enhancement of leu- and met-enkephalin tissue levels and their precursor proenkephalin in the amygdala complex is detected during electrical kindling. Partial seizures induced by kindling augment met-enkephalin release in the basolateral nucleus, but generalized seizures are associated with a significant decrease in opioid release in this brain area. It is possible that the augmented extracellular levels of opioid peptides after partial seizures prevent seizure propagation from amygdala to other brain structures. The observation that the amygdala fails to respond to electrical stimulation with increased opioid peptide release after the fully kindled state is achieved suggests a facilitation of seizure spread and a reduction in arrest mechanisms in this brain area. It is interesting to note that although the amygdala complex displays significant alterations in opioid tissue levels and release during the kindling process, it does not show mu receptor binding alterations.

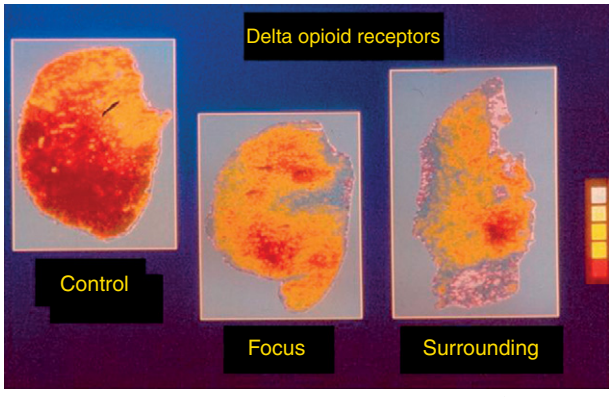


Figure 1 Distribution of mu receptors labeled with [^{3}H]DAMGO in cortex of a control subject, and neocortical epileptic foci and surrounding area obtained from a patient with pharmacoresistant epilepsy. Notice the reduced binding in the surrounding cortex. Structures with high MO receptor binding appear as black and red areas, whereas blue areas delineate structures with low receptor binding.

Striatonigral circuitry has been shown to be involved in the control of seizure activity. In the caudate–putamen, no significant changes have been found in opioid peptide tissue levels, their precursors, or their release during amygdala kindling. However, this region shows an enhanced mu receptor binding after partial and generalized seizures that might be associated with supersensitivity to endogenous peptides released from cells in other brain areas after seizures.

The frontal lobes have an important role in the propagation of complex partial seizures and they participate in the stability of generalized seizures. Cerebral cortex shows elevations of proenkephalin mRNA and met-enkephalin-like immunoreactivity during the early and late kindling stages. Cortex also presents a late (beginning at 2 h) and prolonged (up to 24 h) decrease in both prodynorphin mRNA and dynorphin A immunoreactivity after kindled seizures. Frontal cortex demonstrates increased mu receptor binding after partial and generalized seizures induced by kindling. However, augmentation of opioid peptide release is only evident in this brain area when animals become epileptic and present generalized seizures. In temporal cortex, enhanced release of opioid peptides is only detected after generalized seizures, whereas mu receptor binding is augmented during the early kindling stages and decreased long-term after the last generalized seizure. The enhanced opioid peptide release in frontal and temporal cortices detected immediately after generalized seizures in the epileptic brain may underlie postictal seizure suppression as well as some behavioral disturbances noticed during the postictal and interictal periods.

In hippocampus, expression of proenkephalin mRNA and tissue levels of derived peptides are enhanced throughout the course of amygdala kindling development, persisting in the fully kindled state. Prodynorphin mRNA levels transiently increase (1 h) after kindled seizures, and return to baseline by 2 h, whereas dynorphin A levels are slightly decreased at 1 h, but increase twofold at 2 h and return to baseline by 6 h. Hippocampus presents increased

leu-enkephalin and dynorphin release immediately after partial and generalized seizures induced by amygdala kindling stimulation. This brain area presents a rapid and sustained increase of met-enkephalin-like immunoreactivity, but there is no evidence for met-enkephalin release after kindling seizures – although release of this peptide, in addition to leu-enkephalin and dynorphins, is evoked by high-potassium stimulation. This result can be interpreted in two ways: First, if the leu-enkephalin released originates in proenkephalin-containing neurons, then it is possible that met- and leu-enkephalins derived from the proenkephalin precursor are differentially released by amygdala kindling stimulation. Second, it is possible that the leu-enkephalin released after kindling stimulation is derived from prodynorphin localized in mossy fibers (production of leu-enkephalin by dynorphinergic neurons has been demonstrated).

Brain structures in which no significant receptor binding alterations are observed during early kindling stages (entorhinal cortex) and 24 h after the last generalized kindling seizure (substantia nigra) show enhanced tissue levels of opioid peptides and their precursors during the kindling process. These regional discordances suggest that the regulation of opioid peptide synthesis and release, and regulation of opioid receptor binding, depend on independent processes. The lack of correspondence of these events in restricted anatomical structures suggest that opioid peptides released in one brain region may induce changes in distant receptor (e.g., via transport in cerebrospinal fluid).

These data (see Table 1) demonstrate a constellation of alterations in several opioid peptide systems with distinct spatiotemporal patterns, particularly in regions known to be important in kindling and epilepsy. The mismatch between changes in the tissue content and release is probably because they are regulated by separate mechanisms.

Table 1 Changes in opioid peptides during the kindling process

	Partial seizures	*Generalized seizures*
Amygdala		
Proenkephalin	↑	↑
Enkephalins tissue levels	↑	↑
Enkephalins release	↑	↓
Mu receptors	n.c.	n.c.
Hippocampus		
Proenkephalin	↑	↑
Prodynorphin	n.a.	↑
Enkephalins tissue levels	↑	↑
Dynorphins tissue levels	n.c.	↑
Leu-enk release	↑	↑
Met-enk release	n.c.	n.c.
Dynorphins release	↑	↑
Mu receptors	n.c.	n.c.
Caudate–Putamen		
Proenkephalin	n.c.	n.c.
Enkephalins tissue levels	n.c.	n.c.
Enkephalins release	n.c.	n.c.
Mu receptors	↑	↑
Cerebral cortex		
Proenkephalin	↑	↑
Prodynorphin	n.a.	↓
Enkephalins tissue levels	↑	↑
Dynorphins tissue levels	n.a.	↓
Enkephalins release	n.c.	↑
Mu receptors	↑	↑ (Frontal) ↓ (Temporal)

n.c., no changes; n.a., no data available. Partial seizures, stage 2–3 kindling seizures; generalized seizures, fully kindled seizures.

Kainic Acid and Opioid Peptides

Kainic acid (KA) is an analog of the excitatory amino acid transmitter glutamate. It is a potent excitatory compound that produces important neurotoxic actions. The systemic or intracerebral administration of KA initially induces status epilepticus, which is followed by long-term changes such as spontaneous seizures and progressive neuronal damage, primarily in the hippocampus. These changes closely resemble the pathology associated with TLE in humans. Particularly because of these long-term effects, KA injections in animals have been considered an experimental model of MTLE.

Hippocampal proenkephalin and prodynorphin mRNA levels are elevated during the KA-induced status epilepticus and return to basal levels by 48 h after treatment. In contrast, KA causes a transient decrease in hippocampal tissue content of met-enkephalin and dynorphins, both during and following status epilepticus (3–6 h after KA administration). Subsequently (1–4 days after KA), increased immunoreactive met- and leu-enkephalin levels in the hippocampus are detected, sometimes proceeded by an initial decrease a few hours after seizure onset.

During the KA-induced status epilepticus, there is a strong but transient, increase of leu-enkephalin, met-enkephalin, and dynorphin release in the hippocampus. These changes are prominent only during status epilepticus. Several days after status epilepticus, the hippocampal opioid peptide release is decreased (relative to control animals) in basal conditions but evoked by K^+ stimulation. Interestingly, tissue content in basal conditions is augmented, suggesting that the mechanisms of release are altered. It is probable that the depleted tissue content and enhanced biosynthesis of prodynorphin- and proenkephalin-derived peptides, observed short-term after KA, result from their enhanced release during status epilepticus. In contrast, the reduced extracellular opioid peptide levels detected long-term after KA could be associated with status epilepticus-induced hippocampal damage or high enzymatic activity involved in degrading opioid peptides.

In contrast to amygdala kindling, during which met-enkephalin is not released in hippocampus (see earlier), KA-induced status epilepticus augments the extracellular levels of this peptide in this brain area. This observation suggests that hippocampal proenkephalin- and prodynorphin-derived peptides may be differentially released in the two experimental models of epilepsy. However, several days after the ictal event both KA and kindling model share similar effects – i.e., decreased hippocampal opioid peptide release.

Concerning opioid receptors, the autoradiography approach does not reveal mu receptor changes 24 h after KA-induced status epilepticus in hippocampus, amygdala, or other regions of limbic cortex – structures that present intense seizure activity and neuronal damage. In contrast, some brain areas such as neocortex and substantia nigra – which do not show significant metabolic alterations during status epilepticus, but do present with edema, perivascular hemorrhages, and progressive necrosis – demonstrate enhanced mu receptors 24 h after KA. The increment of mu receptors in these specific brain areas may be involved in effects produced by short periods of hypoxia or ischemia.

Pharmacological experiments indicate that mu but not delta opioid receptor stimulation intensifies KA-induced neurotoxicity in rat hippocampus. It is possible that the enhanced mu receptor binding detected in amygdala and hippocampus 40 days after status epilepticus is involved in the neuronal necrosis detected in these brain areas of adult rats surviving long-term after KA (**Figure 2**).

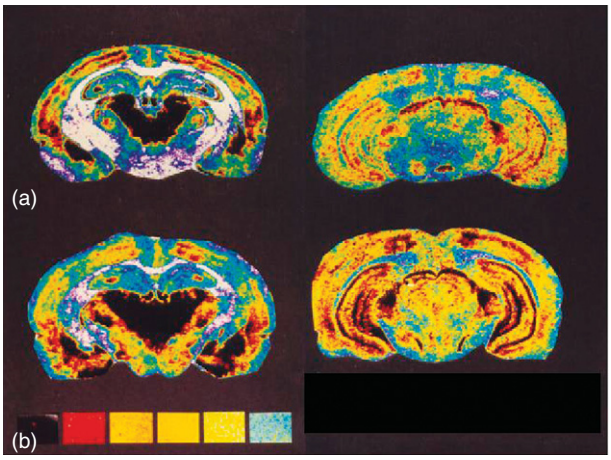


Figure 2 Distribution of mu receptors labeled with [^{3}H]DAMGO in coronal sections of adult rats at the level of amygdala and dorsal hippocampus (left) and substantia nigra, central gray and ventral hippocampus (right). In comparison with the sections of a control rat (a), the sections of an animal sacrificed 40 days after KA-induced status epilepticus (b) shows high receptor binding in amygdala, ventral hippocampus, and substantia nigra. Notations as in **Figure 1**.

Does a Single Seizure Activate Opioid Peptide System?

An important issue to be considered is whether a single subconvulsant stimulation is able to modify the opioid peptides in a normal brain. The application of a single kindling electrical stimulation in amygdala of naive animals enhances opioid release in amygdala and hippocampus and increases proenkephalin gene expression in olfactory tubercle, ventral striatum, arcuate nucleus, and ventral hippocampus. In normal rats, a subconvulsant amount of pentylenetetrazol (PTZ, 30 mg/kg, i.p.), an antagonist of $GABA_A$ receptors, induces significant changes in opioid peptide system. Thirty minutes after its administration, animals show an enhanced analgesic response, an effect associated with increased opioid peptide release in amygdala. Twenty-four hours after PTZ injection, there is reduced mu and delta receptor number in cortex and amygdalae, whereas the steady-state proenkephalin mRNA levels in hippocampus, amygdala, and piriform cortex is augmented. If PTZ enhances the extracellular levels of opioid peptides, they may lead to opioid receptor internalization and their subsequent degradation. This idea is supported by data indicating that the administration of opiates induces rapid internalization of opioid receptors. The possible functional effect of reduced opioid receptors following the administration of subconvulsant amounts of PTZ may be associated with subsequent decreased sensitivity to the inhibitory effects of opioid peptides, leading to progressive enhanced seizure susceptibility, thus facilitating the epileptogenesis (**Figure 3**).

It is not clear whether the increase in the steady-state proenkephalin mRNA levels is due to an accelerated transcription or due to reduced degradation. However, the elevated biosynthetic capacity reflected by the increased proenkephalin mRNA level 24 h after PTZ administration would tend to replenish losses from the enkephalin pool due to their significant release following PTZ.

From this set of data, it is clear that a single subconvulsant stimulation is able to modify the opioid peptide system, a situation that may result relevant to the epileptogenic process.

Antiepileptogenesis and Opioid Receptors

Spiny rats of the genus *Proechimys* (Family, Echimydae; Suborder, *Hystricomorpha*) are mammals inhabiting the ground of moist neotropical forests in Central and South America. These nocturnal terrestrial rodents have a high population density in their habitat; they do not dig burrows, and they move around rather sluggishly within a small home range. *Proechimys guyannensis*, the common Brazilian name of which is casiragua, has received some attention as natural host of infectious agents. This animal has also been of interest because of its remarkable genome size, which is the highest recorded so far in mammals and which harbors several repetitive DNA families.

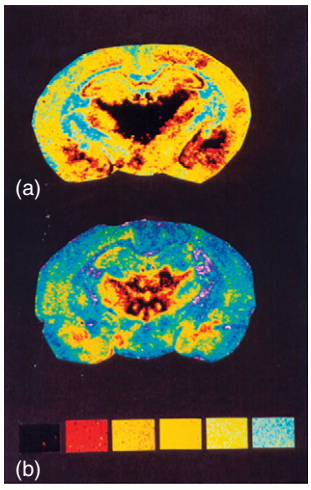


Figure 3 Distribution of mu receptors labeled with [^{3}H]DAMGO in coronal sections of adult rats at the level of amygdala and dorsal hippocampus. In comparison with the section of a control rat (a), the section of an animal sacrificed 24 h after the systemic administration of a subconvulsant amount of pentylenetetrazol (b) shows low receptor binding in several brain areas. Notations as in Figure 1.

Proechimys guyannensis shows a striking resistance to several different paradigms of experimental epilepsy. Indeed, these animals present a high sensitivity to the acute effects of pilocarpine and KA injections, but they do not develop long-lasting status epilepticus or spontaneous seizures (up to 120 days postinjection). When compared with Wistar rats, most of *P. guyannensis* animals show a higher mean afterdischarge threshold and their afterdischarge duration is longer from stages 1–4 of kindling, but not statistically different in stage 5. These observations have led to the view that there must be some factors preventing the afterdischarges from gaining access to structures related to the motor organization of the epileptic seizure.

An interesting aspect demonstrated by *P. guyannensis* is that during kindling stages 4 or 5, these animals present an initial behavior similar to that observed in catalepsy syndrome induced by opiates. Under normal conditions (and compared with Wistar rats), the *P. guyannensis* brain shows lower delta receptor binding in cingulated cortex, olfactory tubercle, thalamus, and substantia nigra, and greater binding in CA1 field of hippocampus. In contrast, mu binding is enhanced (frontal, piriform and temporal cortices, medial amygdala, dentate gyrus, and periacueductal gray) or reduced (cingulated cortex, olfactory tubercle, thalamus, basolateral amygdala, CA1-3, and substantia nigra). This pattern of mu receptor binding is similar to that detected in the brain of kindled rats during the interictal period. It is possible that the high mu receptor binding detected in specific brain areas of the *Proechimys* rodents may underlie the catalepsy-like behavior and lower kindling progression, as well as the extreme sensitivity of these animals to the acute effects of pilocarpine and KA injections.

The different organization of inhibitory and modulatory systems that *Proechimys* brain presents may represent a useful tool for the investigation of the role of opioid peptides on postictal and interictal seizure refractoriness and protection against epileptogenesis.

Postictal and Interictal Effects of Endogenous Opioid Peptides

Endogenous opiates are among the neurotransmitters and neuromodulators that have been implicated in ictal termination and in the enhanced refractoriness to subsequent seizures during the postictal (after seizure) and interictal (between seizures) periods. Indeed, the activation of endogenous opioid peptides by electroconvulsive seizures is able to curtail the epileptogenesis process induced by kindling. Opioid peptides have also been implicated in some of the behavioral disturbances – such as rigidity, analgesia, and immobility – observed during the postictal period, as well as interictal enhanced locomotion. In hippocampus, opioid peptides are involved in the memory disturbances and reduced responses to pain detected during the postictal period.

Cerebral metabolic alterations after kindled seizures match with postictal and interictal behavioral disturbances. Immediately following the end of a fully kindled seizure (0 time postictal), decreased metabolism is detected in caudate–putamen and pyriform cortex, whereas enhanced metabolism is found in the hippocampus and substantia nigra. During the interictal period (28 days after the last kindled seizures) rats with bilateral epileptic foci present forebrain metabolic rates significantly lower than those in controls or kindled rats with unilateral focus. All of these metabolic disturbances are reversed by naloxone administered immediately before seizure induction, suggesting that the postictal and interictal metabolic alterations are opioid mediated. It is possible that once kindled seizure activity is established, specific brain areas may respond to electrical stimulation with enhanced activation of opioid peptides followed by metabolic changes in areas with high opioid receptor binding.

Disruption in 'emotional tone' associated with anger, aggression, and depression are characteristics often attributed to interictal behavior in TLE. Endogenous opioids represent natural 'euphoragens' and their release may explain the beneficial effect of electroconvulsive shock treatment on clinical depression, whereas their reduction during the postictal and interictal periods is associated in certain behavioral deficits. Postictal explosive motor behaviors in epileptic animals have been suggested as a result of an opioid withdrawal effect. Indeed, these behaviors are enhanced by pretreatment with naloxone.

One hypothesis to explain some interictal disturbances is that recurrent seizures cause a chronic exposure to atypically high extracellular levels of the endogenous opioids, with a subsequent physiological dependence on

these neuropeptides. Interictal behaviors may therefore be viewed as 'withdrawal responses' – i.e., when seizures do not occur. Other explanation is that during the interictal state, basal extracellular opioid peptide levels in hippocampus are decreased, rather than increased, suggesting that kindling induces a progressive hypoactivity of opioid peptide systems in this brain area that may be a causal factor in producing an increase in local excitability and epileptogenesis (**Figure 4**).

Future Goals

Epilepsy results of a shift in the excitation and inhibition balance and opioid peptides may have an important moderating influence. Knowledge of the pro- or anticonvulsant effects of the opioid peptides has potentially important implications for the progressive nature of epilepsy, with respect to both epileptogenesis and the development of postictal and interictal behaviors. Much work is needed to develop and test new opiate drugs that might be effective in treating epilepsy and its consequences. Studies using selective agonists and antagonists, antisense oligonucleotides, or animals lacking opioid receptors could be useful to investigate future strategies for the treatment of epilepsy. Careful screening of opiate agonists and antagonists will be therefore necessary before the antiepileptic efficacy is pursued in man.

Importantly, epilepsy appears to induce a progressive hypoactivity of opioid peptide systems that may lead to higher susceptibility to subsequent seizures and postictal and interictal behavioral disturbances. We therefore should perhaps pursue new strategies to activate these depressed endogenous opioids. Deep brain stimulation, used as a therapy for intractable chronic pain and Parkinson's disease, has been associated with enhanced opioid peptide release. Thus, we might expect deep brain stimulation to restrict epileptic activity by the activation of endogenous opioid peptides. However, additional studies are needed to support the idea that the activation of endogenous opioids might be appropriate for the therapy of certain forms of epilepsy.

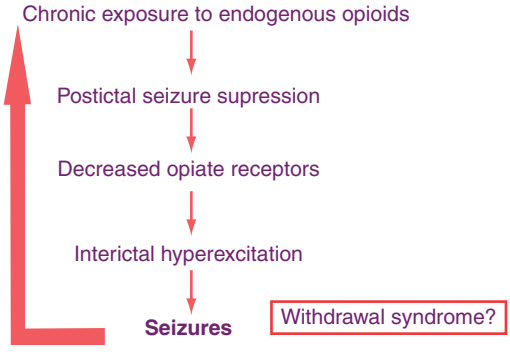


Figure 4 Diagram showing the hypothesis of a withdrawal syndrome that may result from chronic exposure to endogenous opioids released as a consequence of repetitive seizures.

Finally, the analysis of the brains of models of resistance to epilepsy, such as *P. guyannensis*, is a useful approach for the investigation of the substrates underlying the protection against seizure genesis and epileptogenesis, and to determine how the opioid peptides are involved in the circuits associated with a natural seizure resistance condition.

Acknowledgements

The author thanks Ms. Leticia Neri Bazán and Mr. Héctor Vázquez Espinoza for their excellent technical assistance. This study was supported by the National Council for Sciences and Technology of Mexico (grant 45943-M). This article is dedicated to the memory of Augusto Fernandez-Guardiola, who was a pioneer in the evaluation of opioid peptides during the kindling process.

See also: **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; **Models**: Characteristics and Mechanisms of Kindling; **Peptides**: Dynorphin and Nociceptin/Orphanin FQ; Endocannabinoids, Opioids and the Regulation of Hippocampal Inhibition; Seizure Susceptibility is Altered by Exposure to Morphine during Mid to Late Gestation Period in the Rat.

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Seizure Susceptibility is Altered by Exposure to Morphine during Mid to Late Gestation Period in the Rat

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Introduction

Prenatal opiate exposure can be damaging to humans, leading to deficits in cognition and increased seizure susceptibility in exposed children. Animal models have shed light on the wide array of neurobehavioral systems that may be affected in prenatally opiate-exposed offsprings. Maternal opiate abuse may affect the systems in the central nervous system (CNS) that are developing in the fetus during drug exposure.

Morphine is an opioid receptor agonist that binds with varying affinity to all three major opioid receptors: μ, δ and κ. Receptors for opioids, gonadal steroids, catecholamines, γ-aminobutyric acid (GABA), and excitatory amino acids (EAA) are present during the second half of gestation in the rat. Morphine exposure during this phase of development has several long-term effects on adult progeny, including alterations in the expression of opioid peptides and their receptors as well as alterations in normal synaptic transmission in opioid peptide-containing afferents. Studies of these changes suggest that limited exposure to opiates during fetal development may produce long-lasting, perhaps permanent alterations in normal opioidergic function in the brain. One such alteration in normal opioidergic function may be an increase in seizure susceptibility.

Seizures are defined as sudden alterations of electrical activity in the brain, sufficient in magnitude to alter motor or sensory function, behavior, or consciousness. The incidence of seizures is age-related, and influenced by gonadal steroids in both humans and animals. In humans, the incidence of seizure is highest during the first few years of life. In rats, the occurrence of seizures in response to convulsant agents increases during the second and third postnatal weeks and then decreases – between postnatal days (PND) 30–35 – just prior to puberty.

Background

Opioid Systems Involved in Seizures

Acute or chronic administration of opiate drugs either potentiates seizures or induces seizure protection, depending on the dose and route of drug administration and the type of seizure. In humans, high doses of opiates potentiate focal as well as generalized seizures. Intravenous administration of morphine in newborns elicits neonatal seizures. Infusion of morphine into the cerebrospinal fluid induces seizures in adults. A sudden cessation of opiate intake, after an extended period of abuse may also induce seizure-like activity, manifesting in symptoms such as tremors, hyperactivity, twitching, and myoclonic jerks.

In rats, systemic administration of low doses of opioid receptor agonists such as morphine, fentanyl, pentazocine, and meperidine generally induces seizure protection. However, morphine and fentanyl seem to have biphasic effects on seizures: low doses increase seizure thresholds and thus reduce seizure susceptibility, while high doses reduce seizure thresholds (increase seizure susceptibility) in several seizure models. Morphine administered in high doses increases the number of focal seizure episodes, the duration of post-seizure akinesis, and the incidence of generalized clonic seizures in the picrotoxin model of epileptic seizures. Additionally, high morphine doses given intrathecally elicit myoclonic seizures, and these seizures are not reversed by the opiate antagonist

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naloxone. Repeated administration of low doses of morphine decreases seizure thresholds, thereby potentiating seizures. Intracerebral administration of endorphin, met-enkephalin and morphine potentiates kindling seizures in adult rats. Moreover, both μ- and δ-opioid receptor agonists elicit seizures in rats as young as 1 day old.

Brain Structures Involved in Seizures

The limbic system, and specifically the hippocampus, is one of the brain regions involved in the effects of opiates on seizure susceptibility. The endogenous opioid peptides, enkephalins and dynorphins, have differential effects on hippocampal excitation. The proenkephalin-derived opioid peptides, which preferentially bind μ- and δ-opioid receptors, generally have excitatory effects in the hippocampus that may result in seizure activity. Enkephalins block GABA release from inhibitory interneurons, resulting in disinhibition. Enkephalins may also excite neurons directly by closing potassium (K^+) channels or by augmenting *N*-methyl-D-aspartate (NMDA) receptor-mediated responses.

The prodynorphin-derived opioid peptides, which bind to κ-opioid receptors, primarily have inhibitory actions in the dentate gyrus (DG) and CA3 subfield of the hippocampus. They inhibit glutamate release from presynaptic terminals. However, they also potentiate a voltage-dependent K^+ current (the M-current) via κ receptors, and may have non-opioid receptor-mediated effects on the NMDA receptor.

Aside from the hippocampus (and limbic system in general) playing a significant role in seizure initiation, several other brain structures may also influence seizure genesis and spread, including substantia nigra (SN), superior colliculus (SC), and subthalamic nucleus (STN) – which inhibits the spread of epileptic seizures in the rat brain and therefore control seizure propagation. All of these seizure-controlling structures are functionally connected via neuronal pathways, influenced by the inhibitory GABA and EAA neurotransmitter systems.

Because opioid peptides and receptors are widely distributed in the brain, exogenous opiates such as morphine may affect not only the hippocampus but also other seizure-controlling brain structures including SN, SC, and STN. There are studies showing that intrathecal administration of morphine induces convulsive activity in rats. Microinfusion of morphine into the SN pars reticulata, for example, increases seizure susceptibility to pilocarpine-induced seizures, and this effect can be blocked by the opiate antagonist, naloxone. In contrast, in maximal electroshock-induced seizures, intra-nigral application of the μ-opioid agonist [d-Ala^2]-Met-enkephalin decreases seizure susceptibility, while κ-opioid agonist, dynorphin A 1–17 amide, has no effects. The finding that μ but not κ-opioid receptor agonists administered into the SN alters seizure susceptibility suggests that the μ-opioid receptor activation may be responsible for the opioid effects in the SN. The differential effects of μ-opioid receptor agonists in the SN, as described earlier, suggest that the effects of μ receptor agonists might be seizure model-dependent.

Methodological Approaches

Seizure Models

Many experimental seizure models have been developed in animals. Most models currently employed are based on the acute administration of convulsant drugs to living animals or in vitro preparations, and therefore involve mostly nonspontaneous seizures. Animal seizures are classified as partial or generalized. Partial seizures are limited to a part of one cerebral hemisphere, and generalized seizures involve both hemispheres. These models can be further subdivided into electrical, chemical, and genetic seizures, depending on how the seizure is induced. Electrical and chemical seizure models have the advantage of providing full control over seizure precipitation, but, in most cases they induce nonspontaneous seizures.

Epileptic seizures usually originate from an imbalance between neural excitation and inhibition. To resolve such imbalance, a suppression of neural excitation or potentiation of neural inhibition is necessary. Neural excitation and neural inhibition are mediated in large part by EAAs such as glutamate and aspartate, and by inhibitory amino acids such as GABA. Interestingly, both EAA and GABA systems can be affected by morphine. As indicated previously, morphine administration induces either anticonvulsant or proconvulsant effects depending on its dose. Whether exposure of the developing brain to morphine during gestation, as a result of maternal drug abuse, has long-term effects on seizure susceptibility or generation is yet to be established. Both opioid receptor agonists and antagonists administered systemically affect seizure susceptibility in several seizure models.

Seizure Models in our Studies

In all our studies, the injection protocol for prenatal morphine exposure followed the procedures developed in our original work in 1985. Pregnant dams were injected with 5–10 mg per kg morphine or physiological saline twice daily on gestation days 11–18, and were then left undisturbed until delivery of pups on gestation day 22. The timing of morphine injection was chosen to evaluate whether this drug affects the development of μ-opioid receptors in the offspring; the critical period for the appearance of μ-opioid receptors is gestation day 14–15 in rats.

After birth, on PND 1, pups were cross-fostered, and left undisturbed until weaning, at which time they were

housed individually. They were tested for seizure susceptibility with different convulsant agents before and after puberty (PND 25 and PND 38, respectively), and in young adulthood (PND 60–80). The effect of prenatal morphine exposure on seizure susceptibility was examined in both male and female rats. Some males and all females were gonadectomized (GNX) and the effects of gonadal hormones were assessed. Specifically, the involvement of ovarian hormones in females was examined in most of our experiments; estrogen and progesterone were replaced, alone or in combination, to elucidate their effects on seizure susceptibility.

Table 1 Seizure susceptibility in adult prenatally morphine-exposed male rats

Chemoconvulsants	*Morphine effect*
EAA	
NMDA	↑
Low Mg^{2+}	↑
Kainic acid	↔
GABA	
Flurothyl	↓
Bicuculline	↓

Arrows signify a decrease (↓), an increase (↑), or no alteration (↔) induced by prenatal morphine exposure in seizure susceptibility compared with prenatally saline-exposed controls.

Recent Results

Effects of Prenatal Morphine Exposure on EAA- and GABA-mediated Seizure Susceptibility

EAA system

Low magnesium-induced epileptiform discharges. Epileptiform activities were evoked in entorhinal cortex (EC)-hippocampal slices of male and female rats, by removing magnesium from the perfusion solution, which unblocks the NMDA subtype of glutamate receptors. The latency to onset of seizure-like events and recurrent status were assessed to investigate whether prenatal morphine exposure alters NMDA-dependent epileptiform discharges in morphine-exposed male and female rats.

NMDA-induced seizures. NMDA-induced seizures were examined by administering several doses of NMDA (150, 175, 200, 225, and 250 mg per kg) to adult male and female rats, exposed prenatally to morphine. The incidence and latency to onset of stereotypy and clonic-tonic seizures were noted. The stereotypy involved sigmoidal tail movements (tail-twisting), caudal biting, and enhanced rearing activity associated with orientation, jumping, and running. The NMDA administration was lethal in some cases.

Kainic acid-induced seizures. Kainic acid-induced seizures were assessed in adult male and female rats to several doses (10, 15 or 20 mg per kg) of KA, in order to examine non-NMDA receptor mediated EAA-induced seizures. Kainic acid-induced wet-dog shakes (WDS), scratching, washing, and clonic seizures were recorded during one hour after kainic acid administration. The incidence and latency to onset of WDS and clonic seizures were evaluated.

The results of the EAA-mediated seizure studies demonstrate (Table 1) that prenatal morphine exposure increases seizure susceptibility in seizure models associated with NMDA receptor activation, but do not affect seizure susceptibility in non-NMDA receptor-associated models. Specifically, in slices in which low magnesium induces epileptiform discharges, morphine-exposed males have reduced latencies to onset of initial seizure activity and recurrent status; male rats also show a decreased latency to NMDA-induced seizures (at 200 mg per kg). In contrast, prenatal morphine exposure has no effects on kainic acid-induced clonic seizures in adult male rats. These data suggest that there are differences in the sensitivity of NMDA and non-NMDA receptors to prenatal morphine exposure in different brain regions, especially in different subdivisions of the hippocampus. While the highest concentration of the NMDA receptors is in the CA1 region of the hippocampus, the highest concentration of the KA receptors is in the CA3 region. The results of our experiments suggest that prenatal morphine exposure affects differently the cross-talk between opioids and different subtypes of glutamate receptors, or has different influences on the CA1 and CA3 region of the hippocampus.

GABA system

Flurothyl-induced seizures. Flurothyl is a convulsant ether that is thought to affect GABA receptors. The effects of prenatal morphine exposure on flurothyl-induced seizures were tested in male rats prior to puberty on PND 25, after puberty on PND 38, and in young adulthood at PND 60–70. Animals were exposed to flurothyl at a constant flow rate ($30\,\mu l\,min^{-1}$) in an air-tight chamber until tonic–clonic seizures occurred. Flurothyl produces two types of seizures that occur sequentially: clonic seizures occur first, and tonic–clonic seizures follow.

Bicuculline-induced seizures. Bicuculline is a $GABA_A$ receptor antagonist, which induces clonic and tonic-clonic seizures after systemic administration. The effect of prenatal morphine exposure was examined in bicuculline-induced seizures at PND 25, 38 and 60–70 in male rats. Bicuculline seizures have two distinct phases, clonic and tonic-clonic components, each of which originates from separate morphological substrates in the brain. Clonic seizures are characterized by a rapid contraction and relaxation of the muscles of the head and forelimbs. Tonic-clonic seizures usually begin with wild running followed by a tensing of the muscles (tonus) and then a long-lasting clonus, which may end in death. Loss of the righting reflex is observed during tonic-clonic seizures. The latencies to

onset of both clonic and tonic-clonic seizures, as well as the incidences of each, were examined.

Our work on GABA-mediated seizures demonstrates that prenatal morphine exposure alters susceptibility to flurothyl and bicuculline in an age-dependent manner. Prenatal morphine exposure increases seizure susceptibility in males at PND 25, whereas decreases it at PND 38 in both seizure models. In gonadally intact adult male rats (Table 1), seizure susceptibility is decreased in prenatally morphine-exposed animals after administration of flurothyl and bicuculline, while this decrease is not apparent in adult GNX male rats. It seems, therefore that the presence or absence of male gonadal hormones plays a role in modulating the effects of prenatal morphine exposure on seizures induced by chemicals (flurothyl and bicuculline) affecting the GABA system. The effect of prenatal morphine exposure on bicuculline seizure susceptibility switches from proconvulsant at prepubertal ages to anticonvulsant at older ages. This switch in the effect of prenatal morphine exposure supports the clinical data showing that puberty is a critical period for seizure susceptibility. Seizures may start, end, or change their character during puberty. Our data suggest that the effect of prenatal morphine exposure on seizures may be affected by the level of androgens in young male rats. Further, the opioid receptor antagonist naloxone is able to block the bicuculline-induced seizures in prenatally morphine-exposed, gonadally intact male rats; that is, prenatal morphine exposure increases the latency to onset of tonic-clonic seizures in saline-but not in naloxone-injected males. These results demonstrate that prenatal morphine exposure induces long-term, perhaps permanent alterations in the opioid system interacting with GABA-mediated seizures.

Effects of Gonadal Hormones on EAA- and GABA-mediated Seizures in Prenatally Morphine-exposed Animals

Studies show that changes in ovarian hormone levels (especially estrogens) play an important role in increasing seizure susceptibility in women. Susceptibility to seizures in human females varies at times of hormonal changes, such as throughout the menstrual cycle and during puberty and menopause. In several basic research studies, the effects of ovarian hormones on seizure susceptibility have also been investigated in GNX female rats. While estrogen generally increases seizure susceptibility in GNX female rats, progesterone decreases it. There are, however, other studies showing 'protective' effects of estrogen against seizures. The mechanism(s) of ovarian hormone actions on seizure susceptibility is not yet established.

We investigated the effects of ovarian hormones on seizures induced by pharmacological agents influencing both NMDA and non-NMDA systems in prenatally morphine-exposed adult female rats. Our work demonstrates that in the low magnesium slice model, the development of epileptiform activities or seizure-like events are not affected by hormonal conditions in brain slices from control females, while they are suppressed in brain slices from prenatally morphine-exposed, estrogen-replaced GNX females. Additionally, in NMDA-induced seizures, estrogen replacement in GNX females has mostly anticonvulsant effects in both control and prenatally morphine-exposed females. In contrast, estrogen has proconvulsant effects in both prenatally morphine-exposed and control females in kainic acid-induced seizures. Thus, it is likely that the effect of estrogen on seizures is differently associated with NMDA and non-NMDA systems of female rats.

Our hormone studies on GABA-mediated seizures show that prenatal morphine exposure alters seizure susceptibility of female rats in age- and ovarian hormone-specific manner. In adult female rats, prenatal morphine exposure has anticonvulsant effects in estrogen-treated GNX females in both flurothyl- and bicuculline-induced seizures. However, prenatal morphine exposure increases seizure susceptibility to flurothyl-induced seizures at PND 15, decreases seizure susceptibility tested just prior to puberty at PND 25, and has no effects on seizures at PND 38 (after puberty). In bicuculline-induced seizures, prenatal morphine exposure does not affect seizure susceptibility at PND 25 or 38. These results suggest that there is an estrogen-opiate interaction in GABA-mediated seizures.

Other Changes in the Opioid Systems of Morphine-exposed Animals that Might Account for Changes in Seizure Susceptibility

Proenkephalin and prodynorphin mRNA

Using the *in situ* hybridization technique, we explored the alterations in proenkephalin and prodynorphin mRNA in the hippocampus of prenatally morphine-exposed males (Table 2). The results demonstrate that prenatal morphine exposure down-regulates proenkephalin mRNA and met-enkephalin peptide levels in the DG of the

Table 2 Levels of opioid peptides mRNA in adult prenatally morphine-exposed male rats

Brain areas	*pENK*	*pDYN*
CA1	↔	↔
CA2	↔	↔
CA3	↓	↓
DG	↓	↑

Arrows signify a decrease (↓), increase (↑), or no alteration (↔) induced by prenatal morphine exposure in the measured parameter compared with prenatally saline-exposed controls.
pENK = proenkephalin, pDYN = prodynorphin, DG = dentate gyrus.

hippocampus. In the DG, the changes in opioid peptides could reflect levels within the DG cells themselves or in the afferent terminals from other structures since: (1) Proenkephalin-but not prodynorphin-derived peptides are present in the axons of the lateral perforant path, which project from the lateral entorhinal cortex (EC) and innervate the DG. (2) Interneurons in the dentate hilus contain proenkephalin-but not prodynorphin-derived peptides. (3) Opioid peptides (both proenkephalin- and prodynorphin-derived) in the DG are found in the granule cells themselves. Granule cells may release opioid peptides in the DG from dendrites or mossy fiber collaterals. The changes in opioid peptides seen after prenatal morphine exposure is certainly due (at least in part) to alterations in the granule cells, since that explanation is consistent with the *in situ* hybridization findings showing changes in proenkephalin and prodynorphin mRNA levels in granule cells. To conclusively address the source of changes in opioid peptides, the cell bodies in EC should also be examine with *in situ* hybridization.

Proenkephalin- and prodynorphin-derived peptides are present and detectable in DG, CA3, and CA1 subfields of the hippocampus. In the CA3, a decrease is found following prenatal morphine exposure in the levels of three of the prodynorphin-derived peptides dynorphin A (1–8), dynorphin β, and α-neoendorphin. The low levels of these peptides may be due to chronic changes in opioid processing, which could cause increased peptide degradation. An alternative explanation is that there may be increased peptide release in the CA3 of prenatally morphine-exposed males.

It should be noted that alterations in opioid peptide mRNA in some brain regions correlate with changes in only some but not all of the peptide products of that mRNA. It is known that post-translational processing within a particular cell can vary with conditions over time, depending on a wide variety of regulatory events. There is evidence that there are regional and species differences in processing which yield varied products. The purpose of these variations may be to alter receptor subtype selectivity (different peptide end products have varying selectivity for opioid receptors). Peptide processing can be modified by cell activity.

Opioid receptors

Mu-opioid receptor binding is upregulated in dendritic strata of the DG (Table 3) in morphine-exposed males, possibly to compensate for the decreased level of met-enkephalin in this region. An increase in μ-opioid receptor binding has been found in the inner molecular layer of the upper blade of the DG, which receives afferents from Layers IV-VI of the medial EC. Most μ-opioid receptors in the molecular layers of the DG are localized in interneurons that contain GABA immunoreactivity. Therefore, it is likely that the increased μ-opioid receptor levels in the inner molecular layer of the DG of prenatally morphine-exposed males have a net disinhibitory effect.

Table 3 Mu-opioid receptor binding density in adult prenatally morphine exposed male rats

Brain areas	*Morphine effect*
CA1	↑
CA2	↑
CA3	↑
DG	↑

Arrows signify an increase (↑) induced by prenatal morphine exposure in the measured parameter compared with prenatally saline-exposed controls.
DG = dentate gyrus.

The increase in μ-opioid receptor binding in stratum lucidum of CA3, the area of mossy fibers projection, is another interesting finding. Although prodynorphin-derived peptides are the best known endogenous ligands for the κ-opioid receptor, they also bind to μ-opioid receptors with reasonable affinity. Therefore, it is possible that the μ-opioid receptors are upregulated to compensate for the decreased availability of prodynorphin-derived peptides, which can serve as ligands for μ-opioid receptors.

In CA1 and CA2, as in CA3 (Table 3), the increase in μ-opioid receptor binding indicates that the hippocampal circuit may be more excitable in prenatally morphine-exposed males. Endogenous enkephalin-derived peptides, which may serve as ligands for the μ-opioid receptors in that region, are present in these subfields in the temporoammonic tract from the EC, as well as in interneurons. Mu-opioid receptors are disinhibitory in CA1, and therefore higher μ-opioid receptor levels in morphine-exposed animals may indicate a more excitable CA1.

Conclusion and Future Directions

Prenatal exposure to morphine alters susceptibility to seizures. Decreases in the excitatory proenkephalin-derived peptides and increases in the inhibitory prodynorphin-derived peptides would be expected to decrease hippocampal excitation. These findings are consistent with the results of our seizure study using flurothyl and bicuculline as chemoconvulsants, which showed an increased latency to seizure onset. Although these seizures are not likely to begin in the hippocampal formation, the clonic component of seizures propagates through the limbic system and may be modulated by the hippocampal formation.

The opioid peptide findings in the DG are also consistent with our studies showing plasticity changes in the DG of prenatally morphine-exposed male rats. At perforant

path-DG synapses, prenatally morphine-exposed males show long-term depression (LTD) while controls do not. It is possible that the decreased levels of proenkephalin-derived peptides do not allow for sufficient activation of μ-opioid receptors to induce long-term potentiation, and therefore result in the expression of LTD. Similarly, if the increased levels of prodynorphin-derived peptides are released and neurotransmitter release from perforant path terminals are inhibited, they may induce LTD.

Together, our seizure studies show that prenatal morphine exposure has long-term effects on the endogenous opioid system in exposed offsprings, and these effects endure through adulthood. While the exact nature of changes underlying the modification in seizure susceptibility following prenatal morphine exposure remains unclear, our work raises many important questions about the role of opioids in brain development, and as such may have far reaching implications.

Prenatal exposure to morphine alters susceptibility to seizures that emerge from different morphological neural substrates in the brain. Clonic seizures originate from the forebrain, and tonic-clonic seizures arise from the brainstem. How does prenatal morphine exposure target different brain regions and different systems within the CNS during development? As noted earlier, gestational drug exposure affects those neurotransmitter and neuromodulator systems that are developing during drug exposure. Some of the CNS systems that might be affected include catecholamines, opioids, GABA, and EAAs. During CNS development, neurotransmitters are thought to act as signals to facilitate or inhibit neural growth and synaptogenesis. Because opiates inhibit neural growth and development, fetal exposure to exogenous opiates could interfere with neuronal growth such that these neurons may respond inappropriately to neurotrophic signals.

Prenatal morphine exposure clearly induces long-term, perhaps permanent alterations in several CNS systems. These alterations are evident long after the opiate exposure is discontinued, and therefore cannot be attributed to tremor, hyperactivity, twitching, myoclonic jerks, or seizure-like activities associated with acute opiate withdrawal. Thus, our studies are the first to show long-term effects of prenatal morphine exposure on adult male and female seizure susceptibility. Although, similar consequences are reported in clinical studies of children born to women who abuse opiates during pregnancy, no studies have been conducted in adolescents or in young adults.

If morphine exposure in early life alters developmental events, morphine may somehow be mistaken for endogenous opioids in the regulatory pathways, thereby leading to alterations in the biological profile of the organism. An excess of opioids such as heroin, morphine, or methadone, as a result of maternal abuse, may alter growth by acting like the overexpression of trophic factors controlling growth. This possibility could well provide valuable insight into the mechanisms of how opioids can exert detrimental influences on the developing organism, and may hold a key for prophylactic measures or provide insight for treatment.

See also: **Hormones and Gender**: Sex and Seizure Sensitivity; Sex Differences in Seizure Sensitivity; **Models**: Model Characterization in Relationship to Human Disorders; **Peptides**: Endocannabinoids, Opioids and the Regulation of Hippocampal Inhibition; Participation of Opioid Peptides in Epilepsy.

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Tachykinins and Epilepsy

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Introduction

Status epilepticus (SE) is a condition defined as epileptic seizures that last more than 30 min, without full recovery of consciousness between seizures. SE frequently causes neuronal damage and has a 27% mortality in adults. It is estimated to affect 150 000 people per year in the United States.

Animal models of SE have shown several features that may explain the poor outcome of that acute syndrome. Seizures tend to become self-sustaining and independent of their original cause. They also become pharmacoresistant to standard anticonvulsant drugs. Associated with SE, there is a great increase in glycolytic rates in limbic system structures such as the hippocampus, amygdala, and pyriform cortex. In an animal model of SE, the neuronal damage follows this metabolic pattern, and is associated with hippocampal damage and subsequent chronic epilepsy. Within seconds to minutes of SE onset, the ratio of excitatory to inhibitory receptors increases via receptor trafficking. Internalization into clathrin-coated pits leads to a reduction in the number of available synaptic $GABA_A$ receptors, which causes strong (but partial) pharmacoresistance to GABAergic anticonvulsants. At the same time, subunits of glutamate ionotropic receptors move in the opposite direction, and the number of synaptic NMDA and AMPA receptors increases significantly.

Within minutes to hours, many plastic changes occur to neuropeptides, including tachykinins. Some of those changes are maladaptive, with an increase in proconvulsant neuropeptides and a decrease in inhibitory neuropeptides, thereby raising the excitability of the limbic system. In the hippocampus of rats with SSSE, there is a reduction of inhibitory peptides like dynorphin, galanin, somatostatin, and neuropeptide Y. In the same models, there is an increase in the expression of proconvulsant tachykinins, substance P and neurokinin B. These changes may be a factor in causing and/or maintaining the shift from stimulus-dependent seizures to self-sustaining seizures.

Background

Tachykinins and their receptors

Tachykinins are one of the largest known peptide families. From low invertebrates to prochordates and vertebrates, many tachykinins are highly conserved. Classically thought of as a type of neuropeptide, tachykinins are located throughout the peripheral and central nervous system and even in nonneural tissue. Most tachykinins end at the C-terminal with the sequence Phe–Tyr/Ile–Gly–Leu–Met–NH_2. Though current research has suggested many novel human tachykinins and receptors, only seven have thus far been identified (**Table 1**), including substance P (SP), neurokinin A (NKA), neurokinin B (NKB), endokinin A, endokinin B, endokinin C, and endokinin D (EKA-D).

The three genes responsible for encoding the mammalian tachykinins are named preprotachykinin 1 (TAC1), preprotachykinin 3 (TAC3), and preprotachykinin 4 (TAC4). TAC1 contains transcripts for both SP and NKA. Its expression occurs in all human tissues except the placenta. Alternate splicing allows for α, β, γ, and δTAC1 variants. The α and δTAC1 variants code for SP only, whereas β and γTAC1 code for SP along with NKA. While the expression of α and δTAC1 is widespread in

Table 1 Amino acid sequences of mammalian tachykinins

Species	*Tachykinin*	*Sequence*
Mammals	Substance P	Arg-Pro-Lys-Pro-Gln-Gln-***Phe***-Phe-***Gly-Leu***-Met.NH2
Mammals	Neurokinin A	His-Lys-Thr-Asp-Ser-***Phe***-Val-***Gly-Leu***-Met.NH2
Mammals	Neurokinin B	Asp-Met-His-Asp-Phe-***Phe***-Val-***Gly-Leu***-Met.NH2
Rat/mouse	Hemokinin-1	Arg-Ser-Arg-Thr-Arg-Gln-***Phe***-Tyr-***Gly-Leu***-Met.NH2
Human	Hemokinin-1	Thr-Gly-Lys-Ala-Ser-Gln-***Phe***-Phe-***Gly-Leu***-Met.NH2
Human	Endokinin C	Lys-Lys-Ala-Tyr-Gln-Leu-Glu-His-Thr-***Phe***-Gln-***Gly-Leu***-Leu.NH2
Human	Endokinin D	Val-Gly-Ala-Tyr-Gln-Leu-Glu-His-Thr-***Phe***-Gln-***Gly-Leu***-Leu.NH2

The three residues common to all tachykinins are shown in bold and italic.
The N-terminally extended forms of NKA (neuropeptide K and neuropeptide g) and of human HK-1 (endokinins A and B) are not shown.
Reproduced from Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM and Maggi CA (2004) Tachykinins and tachykinin receptors: A growing family. *Life Sciences* 74(2): 1445–1463.

the central nervous system, β and γTAC1 expression is more common peripherally.

TAC3, which encodes NKB, is expressed in the central nervous system and some peripheral tissues, including the placenta. Although there are two splice variants (α and βTAC3), they only differ in an untranslated region and both express NKB.

TAC4 is unique in that its gene product varies across species. In mice and rats, TAC4 encodes for hemokinin-1 (HK1), whereas in humans it encodes for endokinins A-D. In humans, TAC4 has four splice variants – α, β, γ, and δTAC4. EKA and C are encoded by αTAC4, EKB is encoded by all four splice variants, and EKD is encoded by βTAC4. EKC and EKD do not contain the classical tachykinin C-terminal motif. Instead of ending in FXGLM-NH_2, EKC/D have the sequence FQGLL-NH_2. EK peptides are expressed in all human peripheral tissues including the placenta, but are not strongly expressed in the brain.

There are currently three identified mammalian tachykinin receptors called NK_1, NK_2, and NK_3. Encoded by TACR1, TACR2, and TACR3, respectively, they are structurally homologous G-protein coupled receptors and have a common mechanism of action through the breakdown of phosphoinositol. Although all tachykinins are agonists for all three receptors, they have different affinities for each receptor (**Table 2**). For example, SP and the EK peptides have the strongest affinity for NK_1. It was previously thought that SP was the strongest activator of NK_1, but SP-like endokinins (EKA/B) have a very similar potency for NK_1. Also this receptor is found in several tissues that express only EKA/B. Therefore, EKA/B are thought of as peripheral endocrine/paracrine agonists of the NK_1 receptor. NKA has the highest affinity for NK_2, and NKB has the highest affinity for NK_3.

Tachykinins are located in a multitude of sites (**Figures 1** and **2**). In the CNS, these peptides are found in posterior pituitary gland, thalamic nuclei, substancia nigra, periaqueductal gray, locus coeruleus, the caudate nucleus, globus pallidus, septum, hypothalamus, medulla oblongata, nucleus accumbens, olfactory bulb, cerebral cortex, superior colliculus, reticular formation, and both dorsal and ventral horns of the spinal cord.

In the PNS, tachykinins are located in enteric neurons of the gut, respiratory tract, blood vessels, urinary system, skin, and immune system. In nonneuronal tissue, these proteins are in blood and carcinoid tumors.

Physiological Roles of Tachykinins

Because tachykinins and their receptors are so widespread, they play a role in a spectrum of activities. These versatile peptides are involved in hypotension, tachycardia, intestinal motility, biliary secretions, pancreatic secretions, tone of airway musculature, bladder tone and motility, pain, and inflammation when injected peripherally.

Cardiovascular effects

Tachykinins cause a reduction in blood pressure in humans. For example, when a multireceptor tachykinin agonist eledoisin is rapidly injected intravenously, blood pressure drops, spinal fluid pressure rises, respiration rate increases, and skin vasodilates. Also, after SP injection, diastolic blood pressure falls significantly. Tachykinins, like SP and physalaemin (an NK_1 agonist), cause tachycardia when injected intravenously. They also cause increased cardiac output and myocardial contractile force.

Gastrointestinal effects

SP, eledoisin, and physalaemin all cause stimulation of intestinal motility. These contractile, excitatory responses are detected in all parts of the gut and all layers of muscle. When eledoisin is injected subcutaneously in dogs, it causes vomiting, abundant salivation, ejection of watery stools containing mucus and blood, and violent tenesmus. When dogs are injected with physalaemin, bile flow increases only because of gall bladder contraction. In fact, this NK_1 agonist causes decreased bile flow in the hepatic duct. Also, physalaemin causes increased exocrine secretions of the pancreas in dogs.

Table 2 Agonists and Antagonists at tachykinin receptors

	NK_1	NK_2	NK_3
Amino acid residues	407	398	465
Natural agonist potency	SP > NKA > NKB	NKA > NKB > SP	NKB > NKA > SP
Synthetic, selective agonists	[Sar^9, $Met(O_2)^{11}$]SP [Sar^9]SP sulfone	[βAla^8] NKA_{4-10} [Nle^{10}] NKA_{4-10}	[$MePhe^7$] NKB Senktide
Synthetic antagonists	FK888 CP 99,994[a] CGP 49823[a] RP 67580[a] GR 203040[a]	L 659,877 MEN 10,627 SR 48968[a]	R487 GR 138,676 SR 142801[a]
Monoreceptor test system	Dog carotid artery	Rabbit pulmonary artery	Rat portal vein

[a]Nonpeptide.

Modified from Maggi CA, Patacchini R, Roverd P and Giachetti A (1993) Tachykinin receptors and tachykinin receptor antagonists. *Journal of Autonomic Pharmacology* 13: 23–93.

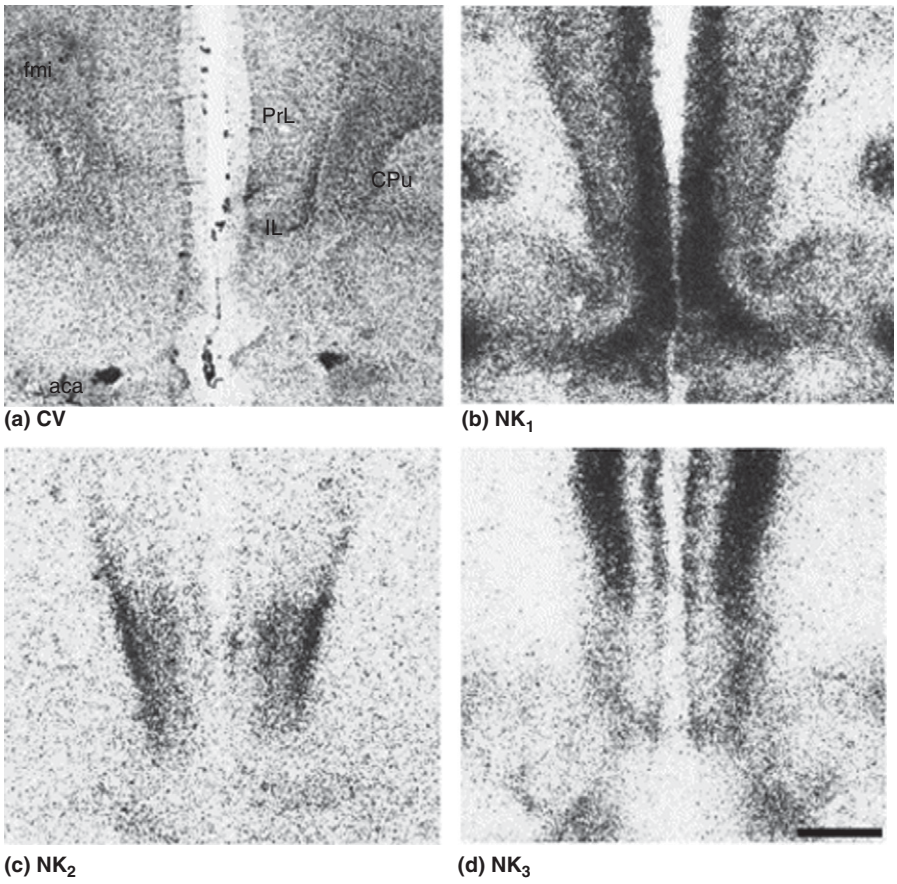


Figure 1 Comparison of the localization of NK_1, NK_2 and NK_3 binding sites in the rat prefrontal cortex. Adjacent coronal sections through the prefrontal cortex were incubated with either Bolton and Hunter substance P (65 pM, 30 min), [^{125}I] NKA (50 pM, 90 min in the presence of 1 μM senktide and 0.1 μM SR 140333) or Bolton and Hunter Eledoisin (65 pM, 45 min). Cresyl violet staining (CV). Scale bar: 1 mm. Adapted from Saffroy M, Torrens Y, Glowinski J, Beaujouan JC (2003) Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. *Neuroscience* 116(3): 761–773, with permission from Neuroscience Journal.

Respiratory system effects

Tachykinins also play a role in the airway systems, namely the muscular tone of the trachea and bronchi. In humans, SP causes increased airway resistance at low doses and bronchodilation at high doses.

Pain and inflammatory effects

The tachykinin substance P is highly linked to nociception. Once the skin is lesioned, high levels of potassium are released by vessels, thereby depolarizing free nerve endings and increasing their rate of action potentials. These activated nerves release substance P, which triggers an inflammatory response by dilating neighboring blood vessels. Dilated vessels induce edema, mast cell release, and histamine activation. Histamines then sensitize the free nerve endings to cause increased nociception. SP is involved in inflammatory disease as well; in diseased tissue, there are abnormal levels of SP, SP-nerve fibers, and NK_1 receptors. NK_1 receptor antagonists have been shown to be beneficial in animal models of inflammatory disease. In rodent models, SP is produced not only by neuronal tissue, but also by inflammatory cells like macrophages, lymphocytes, eosinophils, and dendridic cells. SP causes enhanced release of cytokines, histamine, and arachidonic acid derivatives. These inflammatory mediators promote leukocyte proliferation, thereby magnifying the inflammatory response.

Other effects

Bladder contraction is augmented significantly with exogenous endokinins at extraphysiological levels.

CNS effects

When injected into various regions of the CNS, tachykinins are associated with hypotension, bradycardia, increased gastric acid secretion, increase of respiratory volume, inhibition of colonic propulsion, increased sexual behavior, aggressive behavior, increased learning, improved memory, and anxiety. It has even been shown that SP can cross the blood–brain barrier (BBB) (in both the apical-to-basolateral (AP-BL)

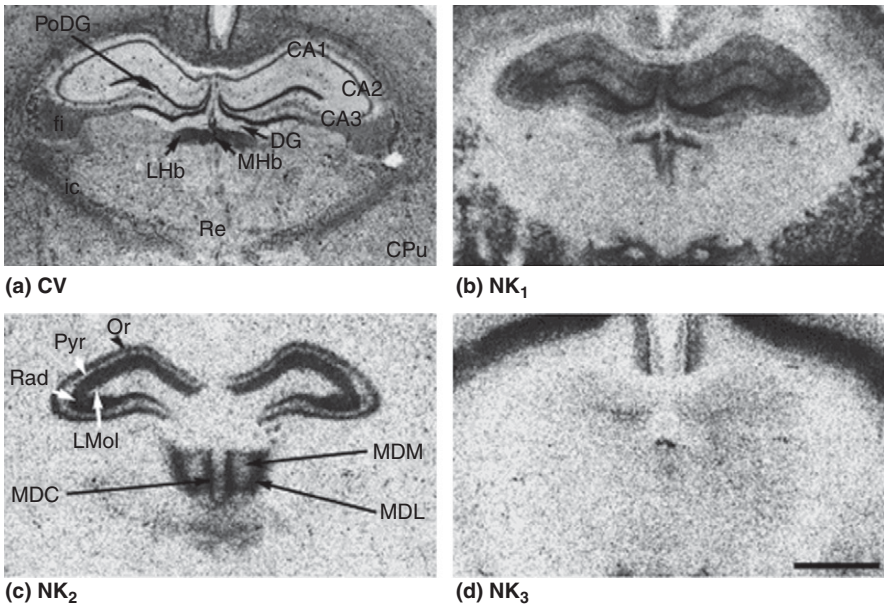


Figure 2 Comparison of the localization of NK_1, NK_2 and NK_3 binding sites at the dorsal hippocampal and thalamic level (incubation conditions are reported in **Figure 1**). Scale bar: 2 mm. Adapted from Saffroy M, Torrens Y, Glowinski J, Beaujouan JC (2003) Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. *Neuroscience* 116(3): 761–773, with permission from Neuroscience Journal.

and basolateral-to-apical (BL-AP) directions) altering both brain function and behavior. The evidence of an active transport pathway, the decreased permeation with endocytic inhibitors, and the 10–20 min lag time suggest a transcytotic mechanism of transport. The pathway involves endocytosis at the apical end of the BBB and exocytosis at the basolateral side. Pro9 SP, a high-affinity NK_1 substrate, inhibits the transport of SP, indicated that the NK_1 receptor is involved in the transport of SP across the BBB. Knowledge regarding the mechanism of transport of tachykinins across the BBB can be used to design future pharmacological agents.

Pathological Roles of Tachykinins

At abnormal levels, tachykinins are linked with diseases like schizophrenia, Alzheimer's disease, Huntington's disease, and epilepsy. When levels of TAC1 mRNA in the amygdala were examined in schizophrenic subjects, it was found that the SP system of the amygdala was altered; in the basal, lateral, and accessory basal amygdaloid nuclei, there was a significant reduction of TAC1 mRNA. In Alzheimer's disease, SP immunoreactivity is diminished; levels of TAC1 mRNA are lower in the basal nuclei of the stria terminalis and medial preoptic area than in controls. And in Huntington's chorea, SP fibers in the substantia nigra are greatly affected, perhaps contributing to many of the symptoms of this disease such as choreiform movements and dementia.

Altered levels of tachykinins are also associated with epilepsy – the focus of the rest of this review.

Methods

A description of the methods used in most of our studies can be found in the article by Liu and collaborators in the *Proceedings of the National Academy of Sciences* (1999) (see Further Reading section).

Results

Substance P and SE

Substance P is normally present in low concentrations in the rat hippocampus, specifically in the CA2–CA3 junction and in the subiculum; there are no SP fibers in the dentate gyrus in control rats. In contrast, in rats with SSSE, the levels of SP-immunoreactivity (ir) showed a dramatic increase (**Figure 3**). In the dentate gyrus, SP-ir was found to increase 30-fold, in CA1 13-fold, and in CA3 35-fold. In situ hybridization of TAC1 mRNA revealed a significant increase in TAC1 mRNA in CA1, CA3, dentate gyrus, and hippocampal mossy fibers.

The effect of SP has also been examined on the induction of SSSE. In control rats, it took 30 min of perforant path stimulation (PPS) to induce stage 4–5 seizures and SSSE; the seizure activity continued for 24 h (i.e., past the end of the PPS period). Seven minutes of PPS caused only stimulus-dependent seizures that stopped as soon as the PPS was stopped. When SP was injected into the hippocampus, seven minutes of PPS was sufficient to trigger

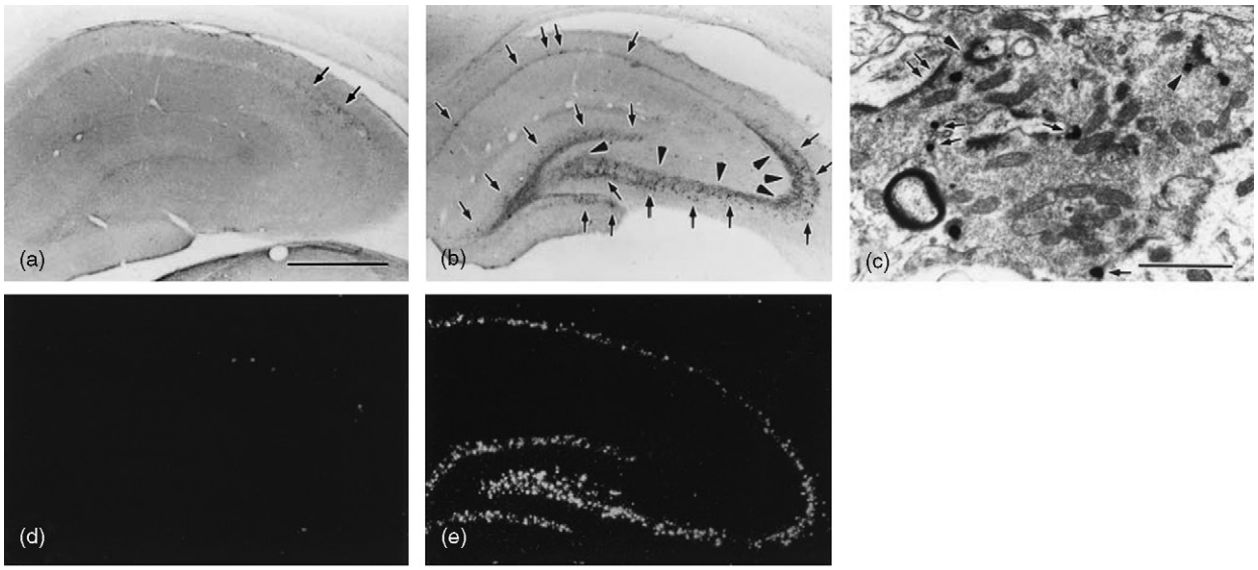


Figure 3 Seizure-induced neurochemical changes in the hippocampus. In the control hippocampus (a), some SP-ir fibers are seen at the junction of CA2 and CA3, and a few SP-ir cells are present in the stratum oriens of CA1 andCA2 (arrows). Twenty-four hours after 30-min PPS (b), there is a dramatic increase in SP-ir cell bodies in CA3, CA1, in the granule cell layer (arrows), and in mossy fibers (arrowheads). (c) Electron micrograph of a typical mossy terminal in CA3 was obtained from a rat killed 24 h after 30-min PPS. The SP-immunoreactive immunogold particles in the mossy fiber terminal are located at a presynaptic density (arrowheads) and in extrasynaptic areas along the internal face (arrows) of the terminal membrane. Asymmetric synapses on the dendrites of CA3 pyramidal neurons (double arrows) are typical features of excitatory connections. (d, e) PPT-A mRNA expression in the hippocampus. In the control hippocampus (d), a few PPT-A mRNA-positive neurons are scattered in the stratum oriens of CA1, CA2, and CA3. Very few PPT-A mRNA-positive cells are present in the pyramids of Ammon's horn. Three hours after 30-min PPS (e), a dramatic increase in PPT-A mRNA is observed in the dentate granule cell layer and in all pyramidal cell layers. (Bars 5 200 mm in a, b, d, and e, 1 mm in c). Adapted from Liu *et al*. (1999) Substance P is expressed in hippocampal principal neurons during status epilepticus plays a critical role in the maintainance of status epilepticus. *Proceedings of the National Academy of Sciences* 96: 5286–5291.

SSSE. The SSSE in the control rats (after 30 min PPS) and in the SP-injected rats (after 7 min PPS) led to severe neuronal loss throughout CA1 and CA3 pyramidal cell layers, a distribution of damage resembles neuronal loss seen in human epilepsy. In both control rats and SP-injected rats, the SP antagonists spantide II and RP-67 580 blocked initiation of SE and SE-induced hippocampal damage, and terminated anticonvulsant resistant SSSE. These antagonists prevented the development of SE when injected before PPS and reduced seizure duration when administered after the end of PPS.

Another study revealed that TAC1 knockout (KO) mice show a resistance in developing SE when injected with kainic acid. When control rats were compared with KO rats, the KO rats exhibited shorter, less intense seizures. It took longer for seizures to commence and recovery was more rapid. Also, in TAC1$^{-/-}$ rats, there was less hippocampal damage. This result suggests a strong proconvulsant role of SP and the TAC1 gene.

SP's proconvulsant nature is thought to be a result of its ability to cause increased presynaptic glutamate release. In hippocampal slices perfused with SP for 5 min, there was a threefold increase in glutamate concentration, an effect that may increase the limbic system's excitability and therefore increases seizure susceptibility. This finding in hippocampus is consistent with the demonstration that SP causes glutamate release in spinal cord.

Neurokinin B and SE

Normally NKB is present throughout all interneurons of the hippocampal formation, especially in large hilar neurons of the dentate gyrus. There are also many NKB-positive cells in CA1 pyramidal layer and faint staining of NKB immunoreactivity in CA3 terminal field of mossy fibers. Our preliminary results show that NKB injection into hippocampus causes severe seizures, and that NKB antagonists block SSSE induced by perforant path stimulation.

Kainic acid-induced (KA) seizures caused a significant cell loss in CA3 and hilus, and extrahippocampal damage in the amygdala, pyriform cortex, and thalamus. KA-induced seizures were also associated with an increase of NKB-ir in mossy fibers, CA3, and the dentate gyrus at days 10–60 after injection. During this same period, there was an enhanced expression of TAC3 mRNA in granule cells and hilar interneurons. Two to ten days after the intraperitoneal injection of KA, the CA1 pyramidal cell layer showed an increase of TAC3 mRNA that dropped to control levels by 60 days. Interestingly, pentylenetetrazole-kindled (PTZ) seizures caused no cell loss, but also were

associated with increases in NKB-ir and TAC3 mRNA hybridization.

NKB works through similar mechanisms as SP. Not only does it enhance glutamate release presynaptically, it also potentiates glutamate actions postsynaptically. These actions can boost excitability in the limbic system, which (like the effects of SP) may contribute to increased seizure susceptibility.

SP in Human Epilepsy

SP facilitates epileptic activity of the principal cells of the hippocampus, but can also reduce seizure activity, presumably by activating SP receptor (NK1R)-expressing interneurons. A study of the CA1 region in autopsied human brains in which no pathology was found showed, in a subpopulation, colocalization of NK1R-immunoreactivity with calbindin (but little or no colocalization with parvalbumin, calretinin, cholecystokinin or somatostatin) in multipolar interneurons with smooth dendrites, that these interneurons may participate in dendritic inhibition. In hippocampi which were surgically ablated for intractable epilepsy, these cells survived but were morphologically altered. In sclerotic hippocampi from epileptic patients, the number of NK1R-immunoreactive interneurons was reduced, but their dendritic arborization was greatly increased, with intense branching and frequent beading, suggesting intense reactive changes (**Figure 4**). Other studies have shown a loss of SP-IR interneurons in the hilus, but an increase in stratum moleculare, and sprouting of SP-IR projections from the supramamillary nucleus. The functional significance of those changes remains open to various interpretations.

Conclusions and Directions for Future Research

Tachykinins are important excitatory neuropeptides that not only have physiological functions, but also play key roles in disease states of the gut, respiratory system, and the brain. Expressed in all human tissues, tachykinins are involved in a plethora of mechanisms. From the treatment of schizophrenia to inflammatory disease, the pharmacologic potential of tachykinin receptor agonists and antagonists is increasing. The pharmacology of the older tachykinins (substance P and the neurokinins) and their receptors are already fairly well developed. However, the characterization of the newer agonists and antagonists is in its infancy, and the future may bring a host of novel applications. For example, tachykinin antagonists should be considered for the treatment of pharmacoresistant epilepsy and SE, in the treatment of inflammation and blood – brain barrier breakdown, and in pain syndromes. Through a better understanding of the roles of tachykinins and their receptors in epilepsy (and other illnesses), more effective therapies can be designed.

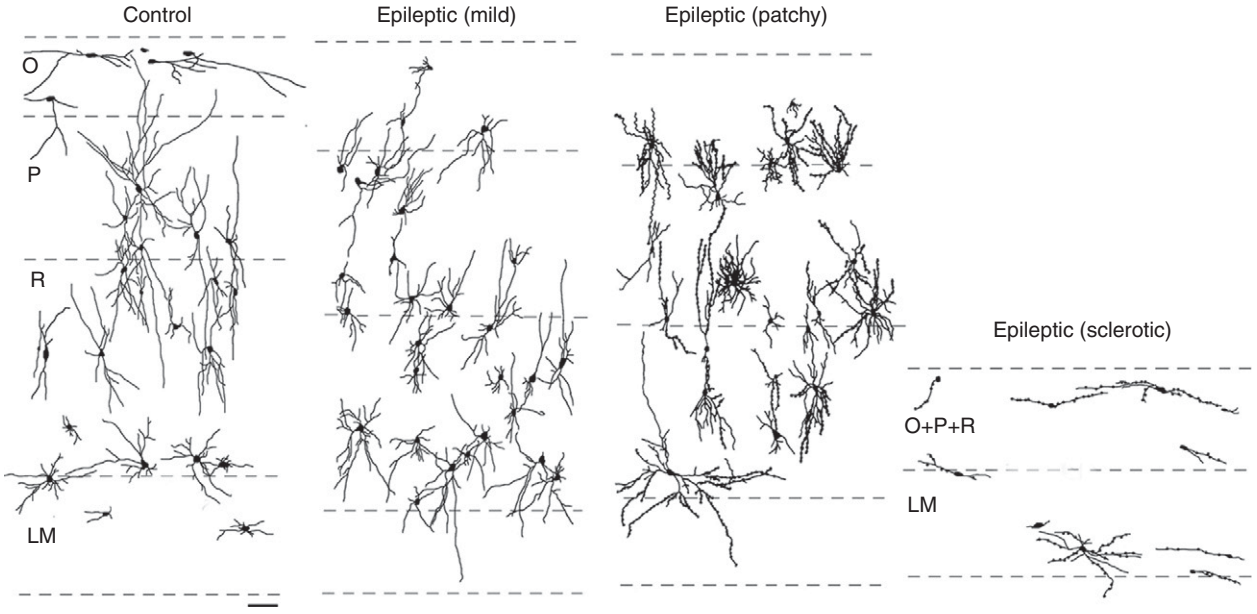


Figure 4 Camera lucida drawings from the CA1 region show changes in the distribution and morphology of the SPR-immunoreactive interneurons. In the non-sclerotic cases (mild and patchy) the number and distribution of positive cells are unchanged, but their morphology is considerably altered, the dendrites have more branches and become beaded. In the sclerotic samples the number of cells is decreased and most of them have only a few distorted dendrites. Scale bar_0.1 mm. Reproduced from Toth K, Wittner L, Urban Z, *et al*. (2007) Morphology and synaptic input of substance P receptor-immunoreactive interneurons in control and epileptic human hippocampus. *Neuroscience* 144: 495–508, with permission from Neuroscience Journal.

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W Löscher, University of Veterinary Medicine Hannover, Hannover, Germany; Center for Systems Neuroscience, Hannover, Germany

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Introduction

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Introduction

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AEDs. Animal models of epilepsy that simulate this multidrug type of resistance are important to uncovering the specific mechanisms of AED resistance in epilepsy, and to developing new and more effective therapies. Thus, the identification and characterization of one or more model systems that would predict efficacy in the AED-resistant patient population would be a valuable asset for any AED discovery program.

Pharmacoresistance in animal models, based on human experience, can be minimally defined as persistent seizure activity that does not respond, or responds poorly, to monotherapy at tolerable doses with at least two current AEDs. An animal model for pharmacoresistant epilepsy should have good parallels to human forms of AED-resistant epilepsy. The goal should be to develop animal models that reflect the most common forms of pharmacoresistance in humans. Thus, most researches involved in model development concentrated on animal models for a common form of human drug-resistant epilepsy, the temporal lobe epilepsy (TLE) or limbic epilepsy syndrome.

Background and Methodologies

There are different approaches to developing animal models of drug-resistant epilepsy (**Figure 1**). One is to use models of seizures or epilepsy that per se are resistant to antiepileptic effects of AEDs. An example is the 6-Hz psychomotor seizure model in mice, which can be viewed as a potential screen for therapy-resistant epilepsy. In this model, which is used in the early drug identification studies by the Antiepileptic Drug Development (ADD) program at the University of Utah, electrical stimulation by low-frequency (6 Hz) rectangular pulses of 0.2-ms duration, delivered through corneal electrodes for 3 s, induces seizures that are reminiscent of 'psychomotor seizures' occurring in human limbic epilepsy. At 22 mA, the convulsant current in 97% of the mice tested (CC_{97}), all AEDs (phenytoin, lamotrigine, ethosuximide, levetiracetam, valproate) examined in this model block the seizures, demonstrating that the model does not discriminate between clinical classes of AEDs at this current. However, when the current is increased to twice the CC_{97} (i.e., 44 mA), most AEDs loose their efficacy, and only levetiracetam and valproate provide complete protection against the 6-Hz seizures (**Figure 1**). Based on these observations, it was suggested that the 6-Hz stimulation may provide a useful and rather inexpensive model of therapy-resistant limbic seizures.

Another, much more labor-intensive and expensive approach is the use of chronic epilepsy models such as kindling (**Figure 1**). In the kindling model of TLE, repeated excitatory stimuli induce partial and, later, secondarily generalized seizures that increase in length and severity with continued stimulation. In the amygdala-kindling model in rats, in which kindling is produced by repeated electrical stimulation of the amygdala, exposure to low doses of lamotrigine during kindling development leads to a reduced subsequent anticonvulsant response to the drug in fully kindled animals. The same observation was reported for kindling with pentylenetetrazole. Lamotrigine-refractory kindled rats are also resistant to

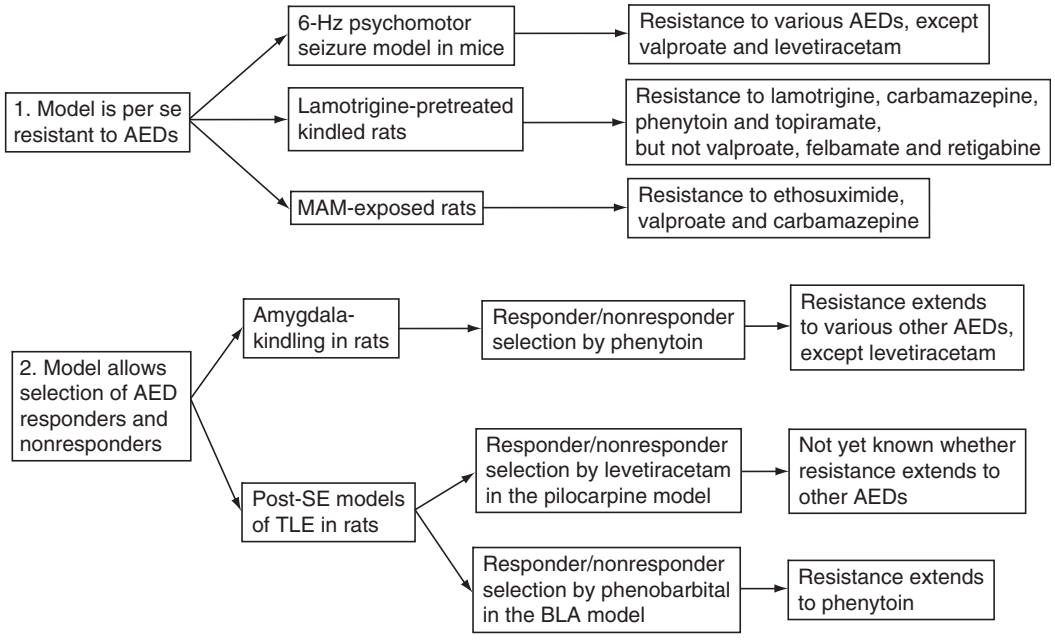


Figure 1 Experimental approaches used in the development of animal models of drug-resistant epilepsy.

carbamazepine, phenytoin, and topiramate, but not valproate, felbamate, and retigabine. It was suggested that lamotrigine-resistant kindled rats may serve as a model of drug-refractory epilepsy. In view of the fact that loss of efficacy (tolerance) develops upon prolonged exposure to several AEDs, including lamotrigine, the loss of efficacy of several AEDs in lamotrigine-resistant rats could be a consequence of cross-tolerance between lamotrigine and AEDs that act, at least in part, by the same mechanism – i.e., modulation of voltage-dependent sodium channels. Whereas cross-tolerance between AEDs acting by similar mechanisms has been demonstrated previously in animal models such as kindling, it is not clear whether cross-tolerance plays a role in AED resistance in patients with epilepsy.

In human patients, seizure activity associated with cortical dysplasia is often resistant to commonly used AEDs. In rats, exposure to treatment with the antimitotic agent methylazoxymethanol acetate (MAM) in utero produces a neuronal migration disorder similar to the cortical dysplasias seen in human brain. In such MAM-exposed rats, seizures induced by either kainate or the cholesterol biosynthesis inhibitor AY-9944 are refractory to valproate, ethosuximide, or carbamazepine (**Figure 1**). Thus, these rats seem to provide a two-hit model of refractory seizures.

An alternative approach to develop animal models of drug-resistant epilepsy is the treatment of large groups of kindled or epileptic rats with AEDs, with subsequent selection of subgroups of animals that either respond or do not respond to this treatment (**Figure 1**). An animal model of epilepsy allowing selection of subgroups of animals with drug-refractory and drug-responsive seizures could be a valuable tool to study why and how seizures become intractable, and to develop more effective treatment strategies. Two models that allow such subgroup selection are described in the following paragraphs.

Results

Phenytoin-Resistant Kindled Rats

We became interested in developing such an animal model almost 20 years ago, leading to the discovery of phenytoin-resistant subgroups of amygdala-kindled Wistar rats (**Figure 1**). By repeated testing of the anticonvulsant effect of phenytoin in large groups of rats of the Wistar outbred strain, we found that the individual response of fully kindled rats to phenytoin differs; i.e, that kindled seizures in some animals consistently respond whereas kindled seizures in others never respond to phenytoin. In recent years, we have repeated the selection of phenytoin responders and nonresponders in kindled Wistar rats several times, using either phenytoin or its prodrug fosphenytoin for selection. Average data from more than 200 rats show a consistent anticonvulsant response to phenytoin in only about 20% of the animals, no anticonvulsant response in another 20%, and a variable response in the remaining 60%. Based on our data, we suggest that the three subgroups of amygdala-kindled rats model three different clinical scenarios. The nonresponder subgroup models drug-refractory patients with TLE in whom AED treatment does not significantly reduce seizure frequency. The variable responder group models patients in whom AED treatment reduces seizure frequency but does not achieve complete control of seizures. The responder subgroup models patients who achieve complete control of seizures during AED treatment.

Following the identification of phenytoin-resistant kindled Wistar rats, most clinically available AEDs were tested in such animals. Except the novel drug levetiracetam, all examined AEDs were significantly less efficacious or not efficacious at all in phenytoin-nonresponders compared to phenytoin responders, demonstrating that the phenytoin resistance of a subgroup of kindled Wistar rats extends to various other old and new AEDs. This result reflects the clinical situation in patients with TLE, because most patients who are refractory to one AED are also resistant to other AEDs, including newly developed drugs.

Subsequent studies showed that the different response to phenytoin of the responder and nonresponder subgroups is not due to differences in the location of the kindling electrode in the amygdala, difference in kindling rate or severity of kindled seizures, drug pharmacokinetics, seasonal variations in drug response, or the sex of the animals. Furthermore, adverse effects of phenytoin were the same in responders and nonresponders, indicating that resistance is restricted to the anticonvulsant effect of this drug.

In the search for mechanisms of resistance in phenytoin-nonresponders, several differences between responders and nonresponders were found (**Figure 2**). Breeding studies with phenytoin responders and nonresponders suggested that the ability to respond or not to respond to phenytoin is genetically determined, although it does not follow a simple scheme of inheritance. The involvement of genetic factors in the different drug sensitivity in responders and nonresponders was substantiated by a study in which we tested phenytoin in various outbred and inbred rat strains. Besides genetics, another possible explanation for development of different pharmacosensitivity in kindled rats would be the kindling process itself. Epileptic patients may initially respond to an AED, but this effect may be lost with increasing duration of the disease – i.e., when epilepsy becomes chronic. To address the influence of kindling on the anticonvulsant response to phenytoin, we tested phenytoin's anticonvulsant effect before and after kindling in the same rats. Following kindling, rats were repeatedly tested with phenytoin to

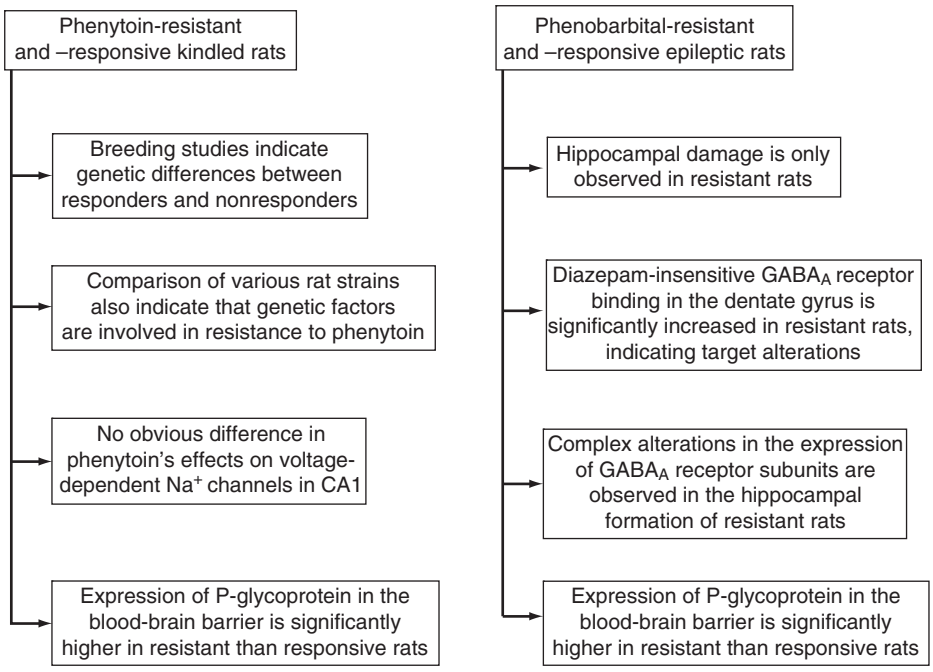


Figure 2 Differences between AED-responders and AED-nonresponders in animal models of drug-resistant epilepsy.

allow subgroup selection. This study indicated that kindled phenytoin-nonresponders become nonresponders, at least in part, through the kindling process – i.e., through the kindling-induced brain alterations. In view of the results of our breeding studies, the genetic background of an individual rat seems to determine whether it becomes a responder or nonresponder by kindling-induced limbic epileptogenesis.

With respect to kindling-induced brain alterations that could determine whether a rat becomes a responder or nonresponder to an AED such as phenytoin, we concentrated on three possibilities. One is that the brain target of phenytoin, i.e., voltage-dependent sodium channels, exhibits a reduced sensitivity to phenytoin in nonresponders. However, in kindled rats with in vivo resistance to the anticonvulsant effect of phenytoin (phenytoin-nonresponders), in vitro modulation of sodium currents by phenytoin in hippocampal CA1 neurons did not differ from respective data obtained in phenytoin responders; that is, phenytoin resistance was not associated with any obvious change in the modulation of sodium currents by this drug.

Apart from target changes, an alternative explanation of the AED resistance in phenytoin-nonresponders is upregulation of drug efflux transporters such as P-glycoprotein (Pgp) in the blood–brain barrier. Such upregulation of Pgp has been determined in resected brain tissue of patients with intractable epilepsy. Because phenytoin and several other AEDs are substrates of Pgp, regionally restricted overexpression of Pgp in the blood–brain barrier is likely to reduce concentrations of AEDs at their brain targets, leading to AED-resistance. In phenytoin-nonresponders, we found significantly increased expression of Pgp in brain capillary endothelial cells in the kindled focus (the amygdala) compared to phenytoin-responders, strongly indicating that Pgp may be involved in pharmacoresistance in this model.

The breeding studies and experiments in various rat strains described above strongly indicated that genetic factors are involved in the inter-individual differences in the efficacy of AEDs in the amygdala-kindling model. Such genetic factors could also explain the increased expression of Pgp in the kindled amygdala of phenytoin-nonresponders – e.g., by assuming a polymorphism affecting the promoter region of the Pgp gene (*Mdr1a*), which governs the expression of Pgp in the blood–brain barrier in rats. However, sequencing of the *Mdr1a* gene coding sequence revealed no general differences in phenytoin responders and nonresponders.

As a further approach for identification of genes that may encode key proteins involved in molecular mechanisms underlying phenytoin resistance in the kindling model, gene expression profiling by microarrays was used. Using the Affymetrix oligonucleotide microarray, we have surveyed the expression levels of more than 26 000 genes and ESTs in responders and nonresponders. About 50 genes were differentially expressed in the two groups of kindled rats. Currently, real-time quantitative

PCR (QPCR) is being used to confirm the data from the microarrays.

Compared to models in which seizures are resistant to AEDs in all animals, a major advantage of the phenytoin-resistant kindled rat model is that it allows the investigator to directly compare AED-refractory and AED-responsive rats from the same strain, which is an important benefit for studies on mechanisms of pharmacoresistance. Other groups have started to use this model in the search for further differences between AED-resistant and AED-responsive rats. Even though the model is very time- and labor-intensive, it does not require any continuous EEG/video monitoring of seizures, because seizures are induced at will. However, animal models of chronic epilepsy with spontaneous recurrent seizures have, of course, greater parallels with human epilepsies than the kindling model.

Post-Status Epilepticus Models of TLE

Stimulated by the findings in the kindling model, we started studying whether pharmacoresistant rats can also be selected from TLE models with spontaneous recurrent seizures. In post-status epilepticus (post-SE) models of TLE, a chemically or electrically induced SE is followed, after a latent period, by spontaneous recurrent seizures. This group of models is often considered to have the greatest parallels with human TLE, but until recently only few studies examined the pharmacological responsiveness of the spontaneous seizures in these models. One obvious reason is that drug efficacy testing in rats with spontaneous recurrent seizures is technically difficult, expensive, and time-consuming, because AEDs have to be administered over a prolonged period and rats have to be recorded continuously by video/EEG monitoring during this period for the occurrence of seizures, to allow comparison of seizure frequency during treatment with seizure frequency in control periods.

In the pilocarpine model of TLE in Wistar rats, prolonged administration of levetiracetam via osmotic minipumps resulted in a large inter-individual variation in drug response. About 40% of the epileptic rats were responders with complete or almost complete control of spontaneous seizures, another 40% were nonresponders, and the remaining rats could not clearly be included in either group because of variation between pre- and post-drug control seizure frequency.

Prompted by these promising data from the pilocarpine model, we investigated whether responders and non-responders also occur in another post-SE model of TLE. In this model, SE is induced by prolonged electrical stimulation of the basolateral amygdala (BLA), which leads to development of spontaneous recurrent seizures in >90% of the animals. Prolonged treatment of epileptic Sprague–Dawley rats with phenobarbital, at maximal tolerated doses, results in two subgroups – responders and nonresponders. In two independent studies in 11 and 15 epileptic rats, 36% and 40% of the rats were resistant to treatment with phenobarbital, demonstrating the reproducibility of this model. When the phenobarbital-resistant rats were subsequently treated with phenytoin, 83% of these rats were also resistant to the latter drug (**Figure 1**), thus fulfilling the minimum requirements for a model of drug-resistant epilepsy described above. Plasma drug levels and adverse effects of phenobarbital and phenytoin were comparable in responders and nonresponders, demonstrating that the resistance is restricted to the anticonvulsant effect of these AEDs. The severity or duration of the initial brain insult (the SE) did not differ between responders and nonresponders, indicating that the different AED response in the two subgroups is genetically determined.

In the search for mechanisms of resistance in phenobarbital-nonresponders, several differences between responders and nonresponders were found (**Figure 2**). Although most rats develop epilepsy with spontaneous recurrent seizures after SE induced by sustained electrical stimulation of the BLA, hippocampal damage with neurodegeneration in CA1, CA3, and dentate hilus occurs in only a subgroup of these epileptic rats, strongly indicating that such hippocampal damage is not critically involved in epileptogenesis. When hippocampal damage was compared in phenobarbital-responders and-nonresponders, only the nonresponders exhibited such damage, supporting the clinical notion that hippocampal sclerosis is often associated with AED resistance. Autoradiographic imaging of diazepam-sensitive and diazepam-insensitive $GABA_A$ receptor binding in the hippocampal formation showed a significant shift to enhanced diazepam-insensitive binding in the dentate gyrus of nonresponders, suggesting functional alterations in $GABA_A$ receptors. This finding was supported by recent immunohistochemical analyses of $GABA_A$ receptor subunit expression in responders and nonresponders. Phenobarbital-resistant epileptic rats exhibited complex alterations in $GABA_A$ receptor subunits in hippocampus and dentate gyrus, which certainly alter the efficacy of AEDs that act via modulation of $GABA_A$ receptors.

In addition to studying target alterations that could underlie the AED resistance observed in our model, we determined the expression of Pgp in brain capillary endothelial cells that form the blood–brain barrier. Phenobarbital-nonresponders exhibited significantly higher expression of Pgp in piriform cortex and hippocampus than responders, indicating that reduced brain uptake and increased brain efflux of phenobarbitone could be involved in resistance to this drug. For direct proof of this hypothesis, we coadministered the selective

Pgp inhibitor, tariquidar, in phenobarbital-resistant rats, showing that resistance can be counteracted, at least in part, by such treatment.

The variety of changes in phenobarbital-resistant epileptic rats (**Figure 2**) makes it particularly challenging to determine the functional significance of each of these alterations. Nevertheless, consideration of the individual changes could provide clues to the cellular basis of drug-resistant epilepsy and may lead to novel therapeutic strategies.

In conclusion, as suggested by our recent studies in post-SE models of TLE in rats, it is possible to identify rats with drug-refractory spontaneous seizures by prolonged AED administration and continuous EEG/video monitoring of spontaneous recurrent seizures. Such drug-refractory rats seem to be ideal models for pharmacoresistant epilepsy and could be used for evaluating the therapeutic potential of novel compounds for the treatment of epilepsy. Furthermore, as shown by our recent data in phenobarbital-resistant rats, such AED-refractory rats are extremely valuable to study the mechanisms underlying pharmacoresistance.

Future Goals

Drug-resistant epilepsy represents a challenge for both experimental and clinical research to gain new insights and perspectives of its mechanisms in order to improve future rational management approaches. As shown in this article, there are a number of potentially useful chronic animal models of drug-refractory epilepsy which can be used to study mechanisms of drug resistance. Such models should be added to the late preclinical phases of AED development in order to prove at a relatively early stage if a new drug exhibits advantages over standard AEDs in drug-resistant seizure types. There should be a continuing effort to validate existing models and identify new models of different types of refractory epilepsy. It is hoped that further investigation and exploitation of such models may contribute to our understanding of the processes through which epilepsy becomes intractable. Investigation of the basis of drug resistance in epilepsy should offer new rational approaches to treatment, for instance by design of novel AEDs that are not targets for brain-expressed resistance mechanisms. Furthermore, animal models of AED-resistant epilepsy can be used to study prognostic factors of resistance, and to investigate whether resistance can be prevented by pharmacological treatment after a brain insult.

Acknowledgements

The author's own studies have been supported by grants (Lo 274/9) from the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the National Institutes of Health (R21 NS049592).

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Brain Imaging of P-glycoprotein Function in Drug-Resistant Epilepsy

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Introduction

Drug efflux transporters constitutively expressed at the blood-brain barrier (BBB) limit the ability of many drugs to access the brain. The prototypic drug efflux transporter, P-glycoprotein (Pgp), was discovered in 1970s as a transporter involved in multidrug resistance of cancer cells, but was also the first drug efflux transporter detected in endothelial cells of the human BBB. Subsequently, Pgp was located in brain capillary endothelial cells of several species, including humans and other primates, rats, mice, cattle, and pigs, suggesting that Pgps may constitute a general defense mechanism in the mammalian BBB, protecting the brain from being intoxicated by potentially harmful lipophilic compounds from natural sources and other lipophilic xenobiotics that otherwise could penetrate the BBB by simple diffusion without any limitation.

Epilepsy was the first CNS disorder for which drug resistance was associated with enhanced expression of Pgp in the brain. Pathologically elevated expression of Pgp has been found in resected brain tissue of patients with refractory temporal lobe epilepsy (TLE) undergoing epilepsy surgery, as well as in limbic brain regions of mouse and rat models of TLE. In such animal models, increased expression of Pgp was associated with decreased brain concentration of antiepileptic drugs (AEDs) such as phenytoin. These observations have led to the multidrug transporter hypothesis, which proposes that refractory epilepsy may be the consequence of a localized overexpression of transport proteins that prevents AEDs from penetrating the BBB in sufficient concentration and would explain why patients are resistant to multiple AEDs with distinct mechanisms of action.

However, species differences in substrate recognition by Pgp have been reported, and there is as yet no direct evidence that human Pgp transports any AED. Furthermore, whether overexpression of Pgp is exclusive to patients with pharmacoresistant seizure disorders remains to be resolved. Even if differences in Pgp expression between AED-resistant and responsive patients exist, overexpression of Pgp in pharmacoresistant epilepsy patients may be an epiphenomenon related to the frequency of seizures or underlying pathology, without any functional consequences for brain levels and efficacy of AEDs. Noninvasive brain imaging of Pgp function in AED-resistant and responsive epilepsy patients is the only strategy to evaluate whether overexpression of Pgp has any functional consequences that may be relevant for the mechanisms underlying pharmacoresistance in epilepsy.

Background and Methods

Modern brain imaging methods now provide unprecedented opportunities for the in vivo study of CNS function. There are several methods for imaging compounds in the brain, including positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). PET and SPECT are imaging techniques in which a radionuclide is introduced into a molecule of potential biological relevance and administered to a patient or an experimental animal. Both PET and SPECT imaging are high sensitivity methods in which radioactive-labeled drugs are used for measuring local neuronal activity, in vivo neurochemistry, or in vivo pharmacology. These methods are particularly attractive to clinical researchers, because they can be applied to the living human brain. PET typically makes use of the isotopes carbon 11, nitrogen 13, oxygen 15, or fluorine 18. These isotopes decay by emitting a positron, with radioactive half-lives of 20, 10, 2, and 110 min, respectively. The emitted positron annihilates with an electron and emits two annihilation photons (gamma rays) that can be detected outside the body. Typically, the isotopes are made in a cyclotron and, because of their short half-lives, need to be rapidly incorporated into the molecule of interest immediately prior to administration. In SPECT, isotopes that decay by electron capture and/or γ emission are used, and include both 123-iodine (^{123}I) and the long-lived meta-stable nuclide 99 m-technetium (^{99m}TC).

Both PET and SPECT, together with radiolabeled substrates and inhibitors of Pgp, have been used for quantitative assessment of Pgp function in the BBB. For PET studies on Pgp function, radiotracers based on known Pgp substrates have been developed and characterized in vitro and in vivo in laboratory animals, including [^{11}C]-daunorubicin, [^{11}C]-colchicine, [^{18}F]-paclitaxel, [^{11}C]-carvedilol, [^{11}C]-loperamide, and [^{11}C]-verapamil. [^{11}C]-verapamil is

the best validated of these PET tracers that has already been used in humans. In Pgp-knockout mice, a 10-fold increase in [^{11}C]-verapamil uptake in brain was found compared with the wild-type mice. A similar effect has been described in normal mice by administration of the Pgp inhibitor cyclosporin A (CsA). In humans, a 70% increase of [^{11}C]-verapamil concentrations in brain was determined following CsA infusion as compared with baseline scans, which confirmed that [^{11}C]-verapamil is transported by Pgp at the human BBB. However, none of the radiotracers available now is ideal for PET imaging owing to their low uptake in brain and complex pharmacokinetics, so there is a need to develop novel imaging probes. The same is true for SPECT. The radiotracer most widely used as a transport substrate for Pgp in SPECT studies is [^{99m}Tc]-sestamibi, a cationic radiotracer originally developed for myocardial scintigraphy. Because of its unique role as a Pgp-specific substrate, this compound can be used to examine Pgp functional activity in vitro and in vivo under pathologic conditions. [^{99m}Tc]-sestamibi is widely used to detect in vivo Pgp activity in tumors, and may be useful for monitoring the development of Pgp-mediated multidrug resistance. In rats, inflammation-mediated downregulation of brain *mdr1a* mRNA, the gene encoding Pgp in brain capillary endothelial cells in rodents, is associated with higher accumulation of [^{99m}TC]-sestamibi in the brain, further demonstrating the potential utility of [^{99m}Tc]-sestamibi for noninvasively assessing Pgp function in vivo. However, to a lesser extent, [^{99m}Tc]-sestamibi is also transported by multidrug resistance-associated protein 1 (MRP1), which may form a bias for results obtained with [^{99m}Tc]-sestamibi. Alternative Pgp substrates for SPECT analysis are [^{99m}Tc]-tetrofosmin and a 99mTc(I)-tricarbonyl complex of sestamibi. However, because all these compounds are cationic radiotracers, cellular functions other than Pgp and MRP1 function, most likely cationic ion transporters, may affect their distribution. Furthermore, in contrast to PET, SPECT does not enable quantitative measurements of uptake and efflux kinetics of Pgp substrates and thus has only limited use in studying Pgp function in the BBB.

Recent Results

The use of PET or SPECT to determine Pgp function in patients with refractory epilepsy has started only recently. In seven patients with drug-resistant unilateral TLE, the Pgp substrate R-[^{11}C]verapamil and PET were used to test for differences in Pgp activity between epileptogenic and nonepileptogenic brain regions. Unfortunately, the most interesting epileptogenic brain region, i.e., the hippocampus, could not be analyzed owing to spill-in of radioactivity from the adjacent choroid plexus. However, in five of the seven patients, increased efflux of R-[^{11}C] verapamil was determined in ipsilateral parahippocampal regions, indicating increased Pgp expression in the epileptogenic hemisphere. These pilot data in a small sample size of patients may indicate that regionally enhanced Pgp activity contributes to drug resistance in some patients with TLE.

Differences in Pgp expression in patients with epilepsy may be due to polymorphisms in drug transporter genes. One of these, a single nucleotide polymorphism (SNP) in exon 26 (C3435T) of the *Multidrug Resistance 1* (*MDR1*) gene, is associated with altered expression, functionality, and substrate specificity of the *MDR1* product Pgp. Because the efflux drug transporter Pgp is highly expressed at the BBB, genetic variation in its expression or functionality could directly affect brain uptake and extrusion of AEDs. Indeed, several recent genetic association studies have indicated an association of the 3435CC genotype, which is associated with increased Pgp expression, with AED-resistant epilepsy. However, other studies did not support such an association. In healthy volunteers differing in *MDR1* haplotypes, no difference in the brain distribution of [^{11}C]verapamil could be detected by PET analyses. However, in a recent SPECT study in patients with epilepsy, the 3435CC genotype was associated with reduced brain uptake of [^{99m}TC]-sestamibi.

Of course, the ideal PET or SPECT radiotracer for studies in epilepsy patients would be a radiolabeled AED. About 30 years ago, [^{11}C]-phenobarbital and [^{11}C]-phenytoin were synthesized and the latter radioligand has been used to study in vivo distribution of phenytoin in monkeys and humans. In a study of eight patients with medically resistant partial epilepsy and two patients without epilepsy, [^{11}C]-labeled phenytoin was used to evaluate the brain kinetics of this AED. At steady-state, brain:blood [^{11}C]-concentration ratios were determined for the visual cortex, resulting in an average value of 1.32 (range 1.05–1.66) in epilepsy patients compared to 1.61 (1.34–1.87) in nonepileptic patients. Whether phenytoin steady-state concentration is lower within the epileptic focus was not addressed in this study. Preliminary data indicated that [^{11}C]-phenytoin kinetics within the estimated epileptogenic area were not significantly different when compared with the contralateral homologous brain region in the same epilepsy patients, but additional data with high-resolution PET are needed for such an analysis. Furthermore, as indicated by the study on [^{11}C]-verapamil in epilepsy patients, the uptake and efflux kinetics rather than steady-state levels of Pgp substrates should be examined in epileptogenic brain regions.

Future Goals

In the past few years, methods for assessing the role of Pgp expression in cerebral kinetics of drugs in humans have

become more accessible. By using imaging techniques such as PET or SPECT, a future goal will be the comparison of brain kinetics of Pgp substrates in AED-resistant and-responsive patients with epilepsy. Furthermore, if such studies support a role of Pgp in AED-resistant epilepsy, PET or SPECT may be used to identify AED-resistant patients – with regionally enhanced Pgp activity – who would most likely benefit from coadministration of Pgp inhibitors. Recent studies from our laboratory using AED-resistant epileptic rats with increased Pgp expression in epileptogenic brain regions have shown that AED resistance could be counteracted by coadministration of the selective Pgp inhibitor, tariquidar. Thus, animal models may be useful to develop principles of how to identify and treat individuals in whom Pgp overexpression is involved in pharmacoresistance.

Recent advances in small animal imaging have provided new ways to perform biologic research. These nondestructive, in vivo imaging techniques permit longitudinal studies of the same animals over an extended period. Both SPECT and small animal PET ('microPET') have been extensively used to study brain kinetics of Pgp substrates in mice or rats. We plan to use a high-resolution small animal PET system for studying the potential role of Pgp in AED resistance by two strategies in rats (**Figures 1** and **2**). In the first strategy, a pilocarpine-induced status epilepticus (SE) is used. We have shown recently that such SE is followed within 48 h by a regionally restricted, significant overexpression of Pgp in brain capillary endothelial cells forming the BBB. The overexpression of Pgp in the hippocampus is associated with a significantly reduced brain:plasma ratio of phenytoin. Thus, such rats are ideal models to study whether the regionally restricted increase in Pgp expression and function can be detected by PET using radiolabeled Pgp substrates such as [^{11}C]verapamil or [^{11}C]phenytoin (**Figure 1**).

The second approach, illustrated in **Figure 2**, is much more time- and labor-extensive. In this approach, AED-resistant and responsive epileptic rats are selected by prolonged treatment with phenobarbital. Epilepsy is induced by prolonged electrical stimulation of the basolateral amygdala (BLA), resulting in spontaneous recurrent seizures after about 4–6 weeks following the SE. About 30–40% of the epileptic rats are resistant to both phenobarbital and phenytoin, whereas the remaining rats respond to these AEDs. We have shown recently that AED nonresponders exhibit a significant overexpression of Pgp in brain capillary endothelial cells of the hippocampus, dentate gyrus and parahippocampal areas. Inhibition of Pgp by tariquidar counteracts the AED resistance. We now plan to use small animal PET to study the differences in brain kinetics of Pgp substrates between AED responders and nonresponders. Because

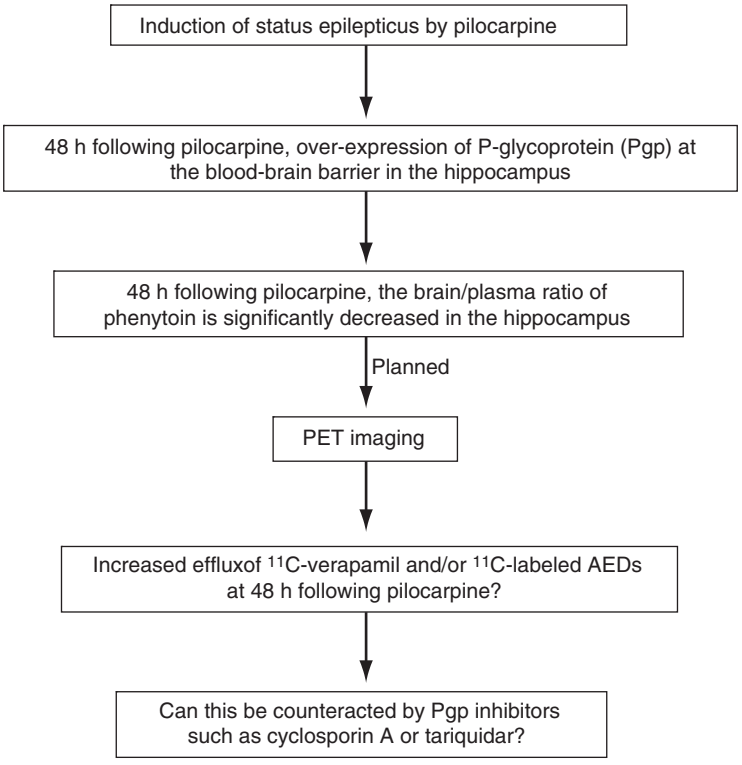


Figure 1 Schematic illustration of a relatively simple experimental approach to obtain rats with regionally restricted overexpression of Pgp in the brain for brain imaging studies with PET or SPECT. We have recently shown by immunohistochemistry that overexpression of Pgp in brain capillary endothelial cells develops within 24–48 h after a pilocarpine-induced status epilepticus.

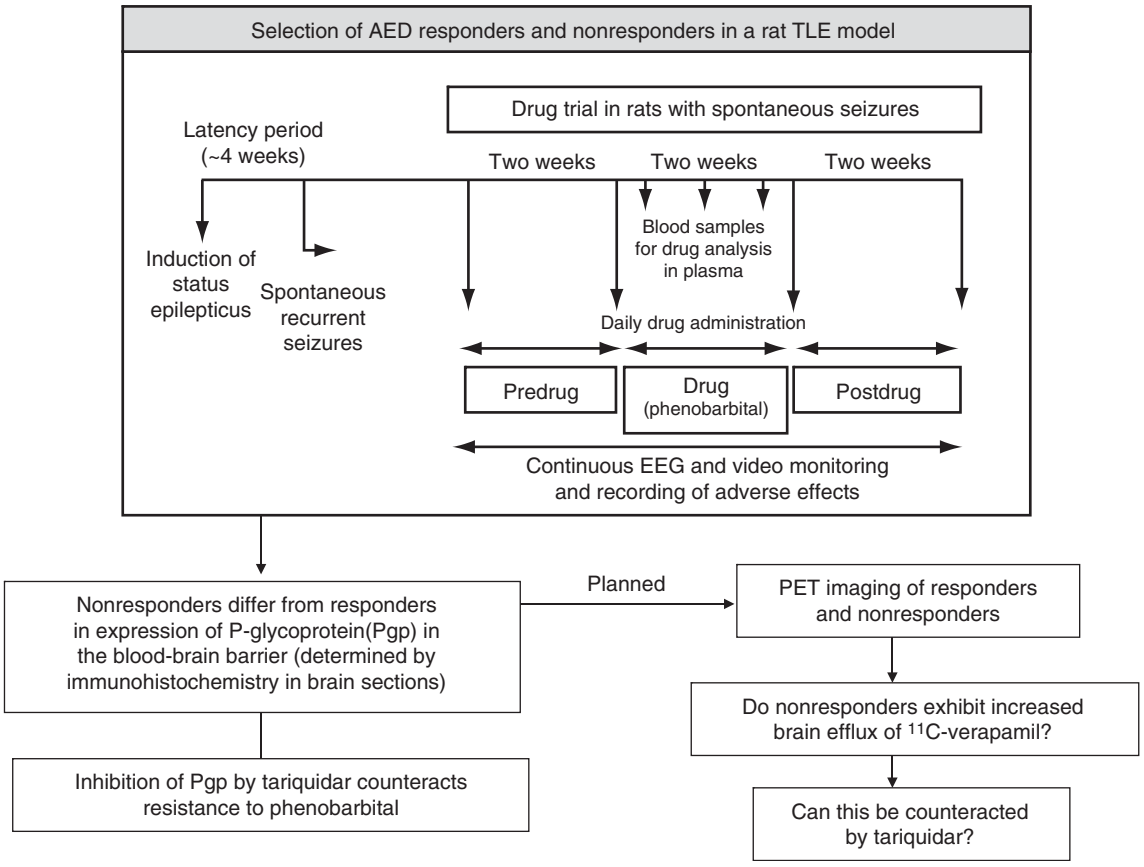


Figure 2 Schematic illustration of a rat model allowing selection of AED responders and nonresponders for brain imaging studies with PET or SPECT. We have recently shown by immunohistochemistry that nonresponders exhibit significantly higher expression of Pgp than responders in brain capillary endothelial cells of hippocampus, dentate gyrus, and piriform cortex. This overexpression of Pgp in epileptogenic brain regions seems to be involved in AED resistance, because inhibition of Pgp by tariquidar counteracts resistance to phenobarbital.

our rat model has strong parallels with human TLE, we hope that the results from these studies will help to develop strategies for evaluating the multidrug transporter hypothesis in patients with refractory TLE.

In conclusion, the recent development of radiolabeled Pgp substrates for human use now allows investigators to directly evaluate the role, if any, of Pgp in medically refractory epilepsy. Furthermore, PET and SPECT are useful to explore the relationship between polymorphisms of *MDR1* and the pharmacokinetics of Pgp substrates within the brain of patients with epilepsy. Information about the transport function of Pgp and the effect of modulating this function may improve the therapeutic treatment of patients with pharmacoresistant epilepsy.

Acknowledgements

This study has been supported by grants (Lo 274/9) from the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the National Institutes of Health (R21 NS049592).

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Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue

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Introduction

A majority of epilepsy patients respond to available anticonvulsant or antiepileptic drugs (AEDs) with an acceptable degree of seizure control. However, a considerable fraction of epilepsy patients – about 30% – do not respond adequately to available drugs. Patients are considered to be 'pharmacoresistant' if they still suffer seizures, even though their compliance has been ascertained and the drugs of choice for a given clinical syndrome have been maintained within the therapeutic range. This phenomenon of pharmacoresistance is a major challenge for pharmaceutical industry, clinicians, and basic researchers alike.

Two hypotheses have emerged to account for an insufficient seizure control – the transporter hypothesis and the target hypothesis. The 'transporter hypothesis' is based on numerous observations that transporter proteins in the endothelium of the blood–brain barrier limit the brain parenchymal concentration of substances by actively extruding a large variety of chemically unrelated drugs, including various AEDs. The observed upregulation of these transporters in both tissues from human epilepsy surgery and in animal models of epilepsy lend support to this concept (which has been the subject of several reviews). The 'target hypothesis' stems from investigations demonstrating a reduced efficacy of drugs in exerting established effects at various channels or receptors in epileptogenic tissues.

Voltage-activated sodium channels are the hallmark of excitable cells, and particularly of neurons. Modulation of sodium currents is the common denominator of various AEDs, including: phenytoin, carbamazepine, lamotrigine, and valproic acid. Molecular changes in these channels have been implicated in both an increased excitability of neurons and a reduced effectiveness of some of the AEDs. For instance, recent evidence suggests that alterations in the β1 subunit of sodium channels may be crucial for both the phenotype of generalized epilepsy with febrile seizures and a reduced responsiveness to modulation by phenytoin. In addition to the fast sodium current, the persistent sodium current also plays a crucial role in hyperexcitability. Mutation of $Na_v1.2$ causes a slowed inactivation and an increased persistent current when expressed in *Xenopus* oocytes. Transgenic mice expressing the mutated channel exhibited focal seizure activity in the hippocampus, generalizing to the neocortex. Since the persistent sodium channel is inhibited by some AEDs – e.g., lamotrigine, further studies may reveal whether an altered modulation of the persistent sodium current contributes to a reduced efficacy of lamotrigine in treating seizures.

Among the voltage-gated potassium channels, the hyperpolarization-activated cation current (I_H) has recently attracted considerable interest partly due to the fact that it represents a novel target for AEDs (lamotrigine, gabapentin). This current has several functions in cortical neurons, including stabilization of membrane potential and modulation of temporal summation of synaptic input. The impact of this current on neuronal excitability has long been underestimated. Judging from the about 100% increase in resting membrane resistance in the presence of the selective I_H antagonist ZD7288, the conductance mediated by I_H channels represents about 50% of the resting conductance of cortical neurons.

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A majority of epilepsy patients respond to available anticonvulsant or antiepileptic drugs (AEDs) with an acceptable degree of seizure control. However, a considerable fraction of epilepsy patients – about 30% – do not respond adequately to available drugs. Patients are considered to be 'pharmacoresistant' if they still suffer seizures, even though their compliance has been ascertained and the drugs of choice for a given clinical syndrome have been maintained within the therapeutic range. This phenomenon of pharmacoresistance is a major challenge for pharmaceutical industry, clinicians, and basic researchers alike.

Two hypotheses have emerged to account for an insufficient seizure control – the transporter hypothesis and the target hypothesis. The 'transporter hypothesis' is based on numerous observations that transporter proteins in the endothelium of the blood–brain barrier limit the brain parenchymal concentration of substances by actively extruding a large variety of chemically unrelated drugs, including various AEDs. The observed upregulation of these transporters in both tissues from human epilepsy surgery and in animal models of epilepsy lend support to this concept (which has been the subject of several reviews). The 'target hypothesis' stems from investigations demonstrating a reduced efficacy of drugs in exerting established effects at various channels or receptors in epileptogenic tissues.

Voltage-activated sodium channels are the hallmark of excitable cells, and particularly of neurons. Modulation of sodium currents is the common denominator of various AEDs, including: phenytoin, carbamazepine, lamotrigine, and valproic acid. Molecular changes in these channels have been implicated in both an increased excitability of neurons and a reduced effectiveness of some of the AEDs. For instance, recent evidence suggests that alterations in the β1 subunit of sodium channels may be crucial for both the phenotype of generalized epilepsy with febrile seizures and a reduced responsiveness to modulation by phenytoin. In addition to the fast sodium current, the persistent sodium current also plays a crucial role in hyperexcitability. Mutation of $Na_v1.2$ causes a slowed inactivation and an increased persistent current when expressed in *Xenopus* oocytes. Transgenic mice expressing the mutated channel exhibited focal seizure activity in the hippocampus, generalizing to the neocortex. Since the persistent sodium channel is inhibited by some AEDs – e.g., lamotrigine, further studies may reveal whether an altered modulation of the persistent sodium current contributes to a reduced efficacy of lamotrigine in treating seizures.

Among the voltage-gated potassium channels, the hyperpolarization-activated cation current (I_H) has recently attracted considerable interest partly due to the fact that it represents a novel target for AEDs (lamotrigine, gabapentin). This current has several functions in cortical neurons, including stabilization of membrane potential and modulation of temporal summation of synaptic input. The impact of this current on neuronal excitability has long been underestimated. Judging from the about 100% increase in resting membrane resistance in the presence of the selective I_H antagonist ZD7288, the conductance mediated by I_H channels represents about 50% of the resting conductance of cortical neurons.

This marked conductance would tend to shunt potential changes generated by other conductances, in fact blockade of I_H reveals intrinsic burst firing in certain previously regularly firing pyramidal neurons. The properties and pharmacology of I_H are discussed in another article.

Remarkably little is known about the role of glutamatergic receptors in pharmacoresistance. Three types of ionotropic receptors are activated by physiologically released glutamate, and are named according to the most potent agonists: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazol-propionic acid (AMPA), and kainate. These receptors have been cloned and exhibit fascinating molecular biology. Each receptor consists of a multimer of subunits. The AMPA and NMDA receptors are tetrameric, while kainate receptors are pentameric arrays of subunits. Splice variants of subunits and mRNA editing cause important changes in voltage-dependence and in the relative permeability of the channel for Na^+ and Ca^{2+}. These posttranscriptional modifications, which may be affected to some extent by the cellular activity, not only provide a rich diversity of functional channels, but also put genetic attempts to understand epilepsy into perspective. Several lines of evidence suggest an interaction of AEDs with glutamatergic transmission. Valproate for instance decreases AMPA binding and reduces neuronal responses to iontophoretic application of kainate and quisqualate. Both AMPA and kainate receptor-mediated currents are also decreased to a different extent by topiramate. Interestingly, topiramate reduces the field potentials by only about 10% but the build-up of intracellular Ca^{2+}, induced by four consecutive stimuli, is markedly reduced. Whether this modulation is altered in epileptogenic tissue is unknown.

The NMDA receptors differ from AMPA and kainate receptors in their unique voltage-dependent block by magnesium ions and by a variety of modulatory sites (e.g., the glycine site). Augmentation of NMDA receptor-mediated synaptic transmission by removal of magnesium is a well-established model of acute hyperexcitability in slices from rodents. NMDA receptors have also been implicated in the mediation of the large synaptic responses recorded from human epileptogenic tissue. However, detailed patch-clamp analysis has revealed no correlation between the magnitude of NMDA-receptor mediated currents and the severity of epilepsy in human tissue samples. In any case, a modulation of NMDA receptors as a therapeutic option is probably seriously constrained; direct attenuation may cause adverse effects on learning and memory, and targeting NMDA modulatory sites may cause psychotropic side effects. Few data are available on the possible modulation of NMDA receptors by clinically established AEDs (except for felbamate), let alone an altered efficacy relating to pharmacoresistance.

$GABA_A$ receptors are the target of several drugs in clinical use, and molecular changes of these receptors have been linked to reduced drug efficacy. In previous studies, acute hyperexcitability (in the zero magnesium model) was seen to cause a decrease in the level of mRNA encoding the $GABA_A$-receptor 2-subunit. Moreover, in the pilocarpine model of epilepsy, a decrease in the $GABA_A$-receptor 1-subunit was demonstrated, causing a reduced augmentation of $GABA_A$ responses by zolpidem, a selective agonist for the type 1 benzodiazepine site on $GABA_A$ receptors. Data from human temporal cortical pyramidal neurons when compared with those from adult rat cortical neurons revealed similar findings regarding the dose–response characteristics toward GABA and clonazepam modulation.

We began our investigation by focusing on two modern AEDs, tiagabine and lamotrigine. Tiagabine was chosen for its well-defined action in blocking GABA uptake, while lamotrigine represented potential due to its diverse cellular effects. In our setting, about 25% of patients operated on had received lamotrigine and were not responding adequately. A comparison of the cellular effects of lamotrigine in tissues from nonresponding patients and those in tissues from patients not treated with lamotrigine (potential responders) may, in the long run, reveal differences contributing to pharmacoresistance.

Background

Tiagabine, the derivative of nipecotic acid, a well-known blocker of GABA uptake, is a useful AED in animal models of epilepsy and in focal and complex partial epilepsy in humans. The established effect of tiagabine is the reduction of GABA uptake through blockade of GABA transporter 1 (GAT 1) at low concentrations, whereas other GAT subtypes are only blocked at much higher concentrations. The block of uptake elevates extracellular GABA levels and prolongs the inhibitory action of GABA at $GABA_A$ receptors. Furthermore, it facilitates the recruitment of previously inaccessible $GABA_B$ receptors. This would augment the inhibitory potency of GABA (in addition to a prolongation of $GABA_A$ receptor activation alone). Such an effect, for instance, has been suggested as contributing to an antinociceptive action of tiagabine in vivo. The well-defined action of tiagabine, whereby it selectively augments GABAergic inhibition, initially suggests that it is a potent anticonvulsant in cases where inhibition is impaired by aberrant GABA receptors or insufficient GABA release.

However, several reports have indicated considerable side effects of tiagabine in some patients, particularly the induction of absence status epilepticus (SE). This result corresponds to in vivo experimental results showing that tiagabine augments spike-wave activity in epilepsy-prone rats. In healthy Wistar rats, tiagabine induces hypersynchronous slow EEG activity during wakefulness and enhances delta activity during non-REM sleep. These

effects may be related to an altered negative feedback of GABA on its own release via presynaptic $GABA_B$ receptors. Although single inhibitory postsynaptic potentials (IPSPs) are augmented by tiagabine, subsequent IPSPs may be abnormally attenuated because of prolonged presence of GABA in the extracellular cleft. These tiagabine-mediated changes in the temporal pattern of inhibition during repetitive activation would help to facilitate cortical synchronization and may contribute significantly to the mechanisms of spike-wave activity.

Lamotrigine belongs to the last generation of AEDs. The spectrum of reported effects includes the 'usual suspects': a use-dependent depression of sodium-dependent action potentials (APs), decrease of glutamate release, and increase in GABA release. Especially, given that contrasting effects have been reported, several important questions about lamotrigine mechanisms of action remain unanswered. For instance, decreased GABA release was inferred from experiments on slices from the basolateral amygdala, whereas increased GABA release has been suggested on the basis of studies in the entorhinal cortex. Interestingly, lamotrigine was shown to augment I_H in the hippocampus, and several reports provide a link between reduced I_H and epileptic disorders: decreased I_H in entorhinal cortex neurons following kainate-induced seizures; smaller I_H in a genetically epilepsy-prone rat (WAG/Rij); and absence epilepsy in a knockout mouse for the HCN2 subunit. The augmentation of I_H is not confined to lamotrigine; gabapentin also increases the I_H in hippocampal neurons (albeit through a different mechanism). It remains to be seen whether alterations of I_H-subunit composition render the heteromeric channel less responsive toward either drug. Nevertheless, the 50% reduction of I_H in WAG/Rij animals (compared with nonepileptic rat strains) suggests a considerable change in this potential target of drug therapy.

Methods

The methods for tissue handling and some of the electrophysiological techniques have been described in another article. We additionally employed patch-clamp recordings with infrared video-microscopy. These methods have been described previously. In brief, individual slices were placed in a recording chamber attached to an upright microscope equipped with an infrared video camera and contrast enhancement to visualize individual neurons. Whole-cell recordings were carried out in the voltage-clamp and current-clamp modes to investigate the possible effects of lamotrigine on neuronal firing behavior. In some of the experiments, the standard pipette filling solution also contained biocytin, to allow for subsequent morphological identification of the recorded neuron.

Recent Results

Effects of Tiagabine on Neurons in Human Epileptogenic Tissue

To characterize the cellular effects of tiagabine, we first evaluated the possible actions on neuronal properties. Application of tiagabine (10 μM) induced slight and variable effects on membrane potential. Some neurons depolarized and a considerable number of neurons hyperpolarized; however, on average, membrane potential remained unaltered (control, -72.9 ± 2.8 mV; tiagabine, -74.5 ± 3.4 mV; $p > 0.5$). Neuronal input resistance decreased slightly in some neurons, in others the input resistance increased, and on average input resistance decreased by less than 10%. Firing of APs during constant current injection was reduced in some neurons, but in most neurons, AP firing was essentially unaltered. The lack of consistent effects of tiagabine on membrane potential might be anticipated from the small difference between resting membrane potential and the $GABA_A$ reversal potential usually present in the neocortex and the reduced function of $GABA_B$ receptors. Interestingly, the neuronal input resistance was also only marginally reduced by tiagabine, implying a low steady-state concentration of GABA in the vicinity of the recorded neuron, and thus only marginal increases after the reduction of GABA uptake by tiagabine.

We also evaluated the effects of tiagabine on synaptic potentials. In human epileptogenic cortex, synaptic responses usually consist of a large depolarization without discernible inhibitory component. Unlike bicuculline-induced paroxysmal depolarization shifts (PDSs) in the rodent neocortex, the synaptic responses in the human tissue were devoid of the typical barrage of APs and are therefore referred to as depolarization shifts (DSs). To avoid threshold phenomena, the effects of tiagabine on DSs were evaluated at different stimulus intensities (2–20 V, 2 V increment). At low stimulus intensities, a marked depression of the amplitude (by 75% and 70%, at 6 and 8 V stimulus intensity, respectively) was recorded. At high intensities, the depression was much smaller (20% and 15% at 18 and 20 V, respectively). Consequently, the input–output relation of synaptic responses was considerably altered by tiagabine application, yielding a more shallow increase. To further evaluate the effects of tiagabine, the voltage-dependence of DSs was investigated. To this end, DSs were elicited at various membrane potentials induced by families of current injections (from about -0.5 to $+0.7$ nA, 600 ms). Hyperpolarizations increased the amplitude of DSs in control and in the presence of tiagabine. Depolarizing the neurons did not reveal a clear-cut $IPSP_A$ component, neither in control conditions nor in the presence of tiagabine. The excitability of neurons was apparently unaltered in the brief period between the onset of current injection and stimulation, but the repetitive firing generated at higher current injections after the stimulus was abolished by tiagabine. During this period, AP generation is usually precluded in the healthy

neocortex by the temporally distinct $IPSP_A$ and $IPSP_B$ components. Since $GABA_B$ receptors are impaired in human epileptogenic tissue, this observation suggests an augmented $GABA_A$ shunting effect following the stimulus, despite the lack of a clear $IPSP_A$ component.

To further evaluate this result, we tested the excitability of neurons by brief (300 ms) current injections applied at various intervals (up to 1500 ms) after eliciting a DS. Comparing the firing evoked by current injection with and without preceding synaptic response at different intervals provided an estimate of the time course of possible inhibition, despite the absence of any obvious inhibition (i.e., hyperpolarizing component) in the DS. In control conditions, this paradigm revealed a marked depression in the number of APs (50%) at 100 ms poststimulus. This depression gradually declined; at delays near 500 ms, the firing was the same as without orthodromic stimulation. We then evaluated the effects of tiagabine with this paradigm, and found a marked increase in the magnitude and duration of AP depression. The APs evoked by current pulses with a delay of 100 ms after the synaptic response were depressed by 90%, and at 1 s delay, by about 70%. Application of the $GABA_B$-receptor antagonist CGP55845A in the presence of tiagabine did not change the magnitude and time course of AP depression, indicating that this depression was mediated by $GABA_A$ receptors.

Two DSs evoked with pairs of stimuli (interstimulus intervals, 100 ms to 1.5 s, increment: 100 ms) are virtually constant in human epileptogenic neurons. Bicuculline-induced PDs in the rodent neocortex, however, exhibit a marked transient depression. We considered the possibility that this constancy of DSs during paired stimulation in human tissue – compared with the marked transient depression of bicuculline-induced PDSs in rat cortex – is due to insufficient release of GABA in the human tissue. We therefore evaluated the effects of tiagabine on synaptic responses elicited with paired stimulation of incrementing intervals. The second DSs of the pairs were slightly reduced in amplitude and duration by tiagabine, but a marked time-dependent depression was not observed.

Together, these data indicate that inhibition of neuronal firing through $GABA_A$ receptors can be augmented by tiagabine. The failure to obtain a marked time-dependent depression of DSs during paired-pulse stimulation in either control conditions or in the presence of tiagabine is consistent with the reduced function or density of $GABA_B$ receptors. Therefore, the marked depression of bicuculline-induced PDSs in rat neuocortex underscores the role of presynaptic $GABA_B$ receptors in the maintenance of small synaptic responses and temporal coding of synaptic processing in the healthy neocortex.

The obtained data also have important implications for pharmacoresistence. Tiagabine certainly dampens excitability of neurons in the epileptogenic human cortex by augmenting $GABA_A$ receptor-mediated inhibition; this effect can be inferred from tiagabine's marked depression of AP firing. However, the net effect of augmented $GABA_A$ inhibition may be rather limited, as only half the neurons have an inhibition with a normal ionic gradient, while the other half have a depolarizing $GABA_A$ response (see article, **Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue**). Therefore, tiagabine – and probably also direct modulators of $GABA_A$ receptors (e.g., benzodiazepines) – would have little net effect on single $GABA_A$ receptor-mediated events in arrays of neurons. Additionally, the potentially beneficial effect of tiagabine – a reduction in the magnitude and frequency of excess synaptic activity through presynaptic $GABA_B$ receptors – is not possible, despite the increase in ambient GABA due to the reduced function of $GABA_B$ receptors. Together, the lack of 'net effect' on single $GABA_A$ responses (due to heterogeneity of reversal potential across cells) and the reduced $GABA_B$-receptor function would render all possible sites for increased GABA concentration by tiagabine rather ineffective. Moreover, in perifocal tissue, the likely normal $GABA_B$-receptor function would make inhibition more vulnerable to frequency-dependent depression when the availability of GABA is prolonged by tiagabine. This effect might, paradoxically, facilitate recruitment of perifocal tissue and aggravate seizure activity.

Effects of Lamotrigine on Neurons in Human Epileptogenic Tissue

We started investigating the effects of lamotrigine on human neocortical neurons with both sharp microelectrodes and patch-clamp recordings. Depolarizing current injections of incrementing amplitudes elicited, above a given threshold, trains of APs of constant amplitudes (on average about 90 mV). Some neurons exhibited an initial doublet of APs, which may have been due to the presence of a T-current giving rise to an 'extra' AP. Fast-spiking or bursting neurons were only rarely encountered and have not yet been included in the analysis. Application of lamotrigine (100 μM) had no consistent effects on membrane potential and neuronal input resistance. However, it caused a marked and significant reduction in the number of APs during a given current injection with both recording techniques. Also the amplitude progressively declined during a train of APs. These effects were reversible upon washout and are probably related to findings indicating an altered sodium-dependent inactivation in the presence of lamotrigine.

We also evaluated the effects of lamotrigine on synaptic responses with sharp microelectrode recordings. To this end, the same paradigms described elsewhere in this encyclopedia were employed. Input–output curves were slightly altered by lamotrigine, the slope was slightly shallower, and the half-maximal stimulation shifted toward higher stimulus intensities. Also the amplitude progressively declined

during a train of APs. These alterations are probably beneficial, since they shift these parameters of the input–output curve toward those obtained in the healthy rat cortex. The maximal amplitudes obtained were not significantly altered by lamotrigine. In any case, these effects would be compatible with a reduced release of glutamate at low to intermediate stimulus intensities. We also evaluated the synaptic responses at near maximal stimulus intensities, which effectively recruit inhibition in the rat cortex. Families of current injections of incrementing amplitudes, with concommitant stimulation, were used to quantify synaptic responses (see earlier). A small $GABA_B$ receptor-mediated IPSP was revealed with the larger amplitudes of injected current. Application of lamotrigine caused a significant reduction of this component, and estimates of the conductance indicate a decrease by about 60%.

With the patch-clamp method, we specifically investigated the properties of I_H and its modulation by lamotrigine. These data are also presented elsewhere in this encyclopedia. Briefly, application of lamotrigine had no consistent effect on the magnitude and time course of I_H.

Future Goals

The data concerning the effects of tiagabine support our conclusion in another chapter – namely, that $GABA_B$ receptors are greatly reduced in the human epileptogenic neocortex. Although tiagabine augments the availability of GABA (as the decreased firing probability after a stimulus clearly demonstrates), the synaptic responses are only slightly affected. The constant, time-invariant occurrence of DSs in the presence of tiagabine, compared with the marked transient failure of bicuculline-induced PDSs, emphasizes the pivotal role of $GABA_B$ receptors in controlling synaptic processes. Concerning the implications for pharmacoresistance, the augmentation of $GABA_A$ inhibition by tiagabine may augment some tonic inhibition, but would have little net effect on the average GABAergic function in an array of neurons. The augmentation of hyperpolarizing inhibition is likely to be counterbalanced by the augmentation of inhibition in those neurons exhibiting a depolarizing $GABA_A$ response. This very phenomenon would of course also render other drugs ineffective that directly modulate the receptor kinetics.

Future experiments will focus on pharmacologically isolated IPSPs for quantifying synaptic currents to further evaluate the role of GABA uptake during the time-course of inhibition. Using the whole-cell patch-clamp technique will allow us to set the ionic gradient of $GABA_A$ inhibition, thus greatly facilitating the characterization of the pharmacological properties of $GABA_A$ receptors in the human epileptogenic cortex. Ultimately, we would like to establish a correlation between $GABA_A$ receptor function and the clinical history of a given patient (resected tissue sample). The most effective method to achieve this goal would be molecular analysis of the expression pattern of $GABA_A$ receptors and transporters involved in the maintenance of the chloride gradient in human tissue. Immunohistochemical characterization of relevant proteins ($GABA_A$ and $GABA_B$ receptor subunits, together with transporters, e.g., KCl cotransporter and channels, e.g., voltage-activated chloride channels) would provide a more representative view of the cellular alterations of a given tissue compared with the recordings from individual neurons. In addition, for such studies the inevitable lack of controls could be avoided by using postmortem material.

Lamotrigine attenuation of APs in human pyramidal neurons was also identified. For some of the tissues, the lamotrigine levels in blood plasma and tissue had been determined and were within the therapeutic range. Therefore, a reduced parenchym level of lamotrigine (due to multidrug transporters) appears very unlikely, and the effects observed in the slices are probably also present in vivo. Yet, these patients are pharmacoresistant, suggesting that use-dependent depression of APs, common to several AEDs, may not be sufficient to control seizure activity. A more quantitative analysis of the alterations of sodium-current properties by lamotrigine and other AEDs is needed, and should be compared with properties of rat cortical neurons, both under control conditions and in epileptic rats (e.g., after SE). Using procedures for acute isolation of neurons, and perhaps a short-term culture preparation to prolong the use of these unique human tissue samples, would further this goal greatly.

Our data indicate a marked depression of inhibition by lamotrigine, in line with a published observation on amygdala neurons of rats. From the data obtained to date, we tentatively propose that pharmacoresistance to lamotrigine may be due to a combination of the two opposing effects. On the one hand, lamotrigine reduces the excitability of pyramidal cells. On the other hand, lamotrigine appears to reduce inhibition, presumably by a reduction of GABA release. This decrease may be brought about by reduced excitability of GABAergic interneurons. Choosing non-pyramidal cells for patch-clamp recordings would provide evidence of whether lamotrigine affects sodium currents of fast-spiking interneurons. In this context, the synaptic responses of interneurons would be of particular interest. If synaptic activation normally elicits several APs in interneurons, a use-dependent depression of sodium currents may affect the output quite markedly. This phenomenon may also be an issue with other drugs that modulate sodium currents. The resulting depression of GABA release might be useful for reducing focal activity – since GABA has a depolarizing effect in human epileptogenic neurons. However, a depression of GABA release in perifocal tissue would facilitate spread of focal activity and increase the probability of seizure activity.

In conclusion, the triad of deficits we observed in the human epileptogenic cortex – depolarizing $GABA_A$ responses, decreased $GABA_B$-receptor effects, and decreased density of I_H – may combine to render various pharmacological strategies ineffective. The pivotal role of this triad in epilepsy is obvious from observations on knockout mice, where impairment of each mechanism in isolation is sufficient to induce seizures.

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See also: **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; Preclinical Drug Development in Epilepsy; **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; **Pharmacoresistance**: Animal Models of Drug-Resistant Epilepsy; Brain Imaging of P-glycoprotein Function in Drug-Resistant Epilepsy; Mechanisms of Pharmacoresistance; **Pyramidal Cells**: Intrinsic Mechanisms Contributing to Hyperexcitability of Human Epileptogenic Cortex.

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Mechanisms of Pharmcoresistance

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Introduction

Drug resistance is a feature of different forms of epilepsy or epileptic syndromes. It is defined as the persistence of unacceptable seizures, in spite of reasonable pharmacotherapy and drug use to the highest tolerable dosage. It affects between 20 and 30% of patients with epilepsy overall. Interestingly, many of the childhood epilepsy syndromes do not respond to clinically employed anticonvulsants. For example, infantile spasms, neonatal seizures, and atonic seizures are frequently pharmacoresistant. Further, Ohtahara syndrome, Dravets syndrome, West syndrome, Rasmussen encephalitis, Lennox Gastaut syndrome, and the migrating focal seizures during infancy are frequently associated with pharmacoresistance. In such conditions, drug resistance may exist from the onset of disease. Interestingly, seizurelike events induced in the intact hippocampus from young rodents are also refractory to antiepileptic drugs, suggesting that immature neuronal circuits do not respond to currently used antiepileptic drugs (AEDs).

In adolescent and adult epilepsy patients, a particularly high incidence of drug resistance is encountered in patients with focal or partial epilepsies. In addition, drug resistance can also be a feature of status epilepticus, where the resistance may evolve over time. Prolonged drug resistant status

In conclusion, the triad of deficits we observed in the human epileptogenic cortex – depolarizing $GABA_A$ responses, decreased $GABA_B$-receptor effects, and decreased density of I_H – may combine to render various pharmacological strategies ineffective. The pivotal role of this triad in epilepsy is obvious from observations on knockout mice, where impairment of each mechanism in isolation is sufficient to induce seizures.

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In adolescent and adult epilepsy patients, a particularly high incidence of drug resistance is encountered in patients with focal or partial epilepsies. In addition, drug resistance can also be a feature of status epilepticus, where the resistance may evolve over time. Prolonged drug resistant status

epilepticus is a rare condition, but is extremely dangerous because of its dramatic consequence with respect to cell death and subsequent reorganization of the nervous system. These consequences, in turn, are believed to aggravate the epileptic condition and cause cognitive impairment as well as other malfunction of the CNS.

Pathological mechanisms involved in pharmacoresistance are currently classified into three general categories: disease-related mechanisms, genetics (e.g., polymorphisms in drug transporters), and drug-related mechanisms (e.g., tolerance). Within the category of disease-related mechanisms, two hypotheses have been proposed: The transporter hypothesis postulates that overexpression of multidrug transporters could reduce the concentration of AEDs in the epileptogenic brain regions; this view has been developed in the other article of this volume. The target hypothesis postulates alterations in drug target; this approach is developed in more detail in the following sections.

Background

Models of Drug Resistance

There are no good predictive in vivo animal models for drug resistance. Different types of spontaneous seizures in epileptic dogs, spontaneous seizures in amygdala-kindled rats, and poststatus epileptic seizures after prolonged electrical stimulation of the basolateral amygdala have all been shown to be pharmacoresistant in subgroups of the experimental animals. To rely on these models for drug developments is difficult, since it requires tedious testing of the animals with different agents. Further, pharmacodynamic analysis in animal models (dogs, cats, rodents) may be of questionable relevance given that the metabolism of antiepileptic drugs has often been found to differ from that in humans.

Given such difficulties in intact animal models, attempts have been made to use in vitro seizure models (i.e., brain slices obtained from normal adult animals) to identify pharmacoresistance mechanisms and to develop more effective drugs. In such preparations, seizurelike events comparable to those recorded in vivo can be induced, e.g., by lowering magnesium and calcium, or by elevating potassium concentration. The application of different drugs, such as veratridine and 4-aminopyridine (4-AP) also induces seizurelike events. However, when tested for sensitivity to current antiepileptic drugs, these models, in general, exhibit drug-sensitivity. An exception to these observations has been seen with prolonged treatment with low Mg (and sometimes also with 4-AP); these treatments resulted in a change in activity pattern, from seizurelike events to late recurrent discharges – which were pharmacoresistant. Activity mimicking late recurrent discharges could also be readily induced by the combined application of 4-AP and bicuculline, or by the combined application of low Mg with bicuculline. These results suggest that transition to late recurrent discharges is due to a strong reduction in efficacy of GABAergic synaptic transmission, potentially due to consumption of GABA in the GABA shunt of the tricarboxic acid cycle. This observed reduction in GABAergic synaptic transmission is not likely to be dependent on malfunction of GABA receptors, since the application of muscimol or GABA can reverse late recurrent discharges into drug sensitive seizurelike events and eventually block this activity.

A somewhat different in vitro system has also provided a window onto pharmacoresistance. In organotypic slice cultures (from rats and mice), most (if not all) cell types present in the intact animal are preserved. These preparations maintain physiological connectivity, but can also develop aberrant connectivity similar to that in human hippocampus with hippocampal sclerosis. The induction of epileptiform activity by 4-AP or lowering of extracellular Mg concentration induces electrical activity patterns that are similar to seizures in intact animals. In this in vitro model, we have noted that seizurelike events were typically resistant to AEDs which interfere with cellular excitability (such as carbamazepine, phenytoin, and valproate) or which augment the efficacy of synaptic inhibition (such as benzodiazepines and barbiturates). The activity was also resistant to gabapentin.

Finally, seizurelike events have been induced by elevating potassium concentration in human tissue (hippocampus, neocortex, and subiculum) obtained after surgical interventions. Interestingly, this activity was suppressed by antiepileptic drugs if slices came from patients with no epilepsy or where patients had seizures which were controlled by AEDs. In contrast, in most cases where slices came from patients with drug refractory epilepsy, seizurelike events were found to be insensitive to carbamazepine and valproic acid. These studies suggest that such tissue is suitable for analysis of mechanisms of drug refractoriness.

The Development of Tolerance

Even without the occurrence of chronic seizures or status epilepticus, the development of resistance in the course of treatment is observed for a number of substances, and has been termed 'tolerance.' For instance, tolerance is observed following treatment with GABA-mimetic drugs. In the case of barbiturates, this effect may be related to the induction of liver enzymes. However, the fact that withdrawal seizures can occur when other GABA-mimetic agents are used indicates that the central mechanisms of tolerance development must also be involved. These mechanisms may be part of homeostatic plasticity. Thus, in dissociated neuronal cultures, blocking of $GABA_A$ receptors leads to upregulation of $GABA_A$ receptors, while down regulation of GABA receptors was observed following an increased activation by GABA agonists. Such adaptation of central GABA

receptors may lead to a reduction in the efficacy of inhibitory mechanisms after a long-term use of GABAmimetic drugs and could explain withdrawal seizures after abrupt cessation of delivery of GABA mimetic drugs. Such withdrawal seizures have also been noted in subjects that do not have epilepsy (such as in drug addicts consuming alcohol, barbiturates and/or benzodiazepines). Thus, the development of tolerance may be independent of the presence of chronic epilepsy.

The mechanisms underlying the development of tolerance to benzodiazepines are not well understood, but may involve changes in the GABA receptor subunit composition, changes in GABA synthesis, and also in other neuromodulatory systems. The loss of efficacy has been proposed as the basis for tolerance to vigabatrin. These data collectively suggest that development of resistance/tolerance can occur purely as a consequence of long-term treatment with certain drugs, without effects of the epileptogenic process. Such a scenario is less likely for drugs such as valproic acid (VPA), phenytoin (PHT), and carbamazepine (CBZ), for which tolerance has not been observed in nonepileptic subjects. These agents have been used for a number of indications other than epilepsy, including neuralgias and depressions.

Drug Targets in Chronic Epilepsy

Clearly, the development of tolerance does not seem to be a common feature of long-term application of all anticonvulsant drugs. Thus, it appears likely that epilepsy (seizure)-related preexisting or acquired changes contribute to drug resistance. As indicated earlier, two main concepts have evolved to explain disease-related drug resistance in chronic epilepsy. The target hypothesis suggests that the molecular targets for anticonvulsant drugs may be functionally or genetically altered, and that these modifications subsequently render them insensitive to AEDs. A large number of ion channels and receptors have been identified that are targets for anticonvulsant drugs. Voltage-gated ion channels are involved in the anticonvulsant effects of drugs such as PHT, CBZ, VPA, and lamotrigene (LTG). Transient and persistent sodium channels are sensitive to PHT, CBZ, LTG, VPA; calcium channels are sensitive to ethosuximide (ESM), VPA, PHT, and gabapentin. Recent data now suggest that potassium channels can be potential target for anticonvulsants, such as retigabine and perhaps also for gabapentin.

Another large group of drugs acts to enhance the efficacy of synaptic inhibition. This effect can be achieved by increasing affinity for GABA at the receptors (e.g., by benzodiazepines (BDZ)), by increasing Cl channel mean open time (barbiturates, neurosteroids), by increasing presynaptic GABA release (vigabatrin), and by reducing GABA uptake (tiagabine). There are some drugs which act by not-yet-fully-resolved mechanisms, such as leviracetam and topiramate, which may affect mitochondrial metabolism but also appear to have other molecular targets. It should be noted that the list of targets for established anticonvulsant is also likely incomplete, and may expand as their actions on novel targets are examined. This diversity of drug mechanisms of action presents a conceptual problem: explaining concurrent resistance to multiple anticonvulsant drugs, which is frequently encountered in epilepsy patients, would require the modification of a large number of very different drug targets or drug-targeting mechanisms.

Methods

The study of mechanisms underlying drug resistance requires suitable models. Most, if not all, in vivo models of drug resistance are post hoc models (i.e., drug resistance of the model can be determined only after one studies effects of a drug), and therefore rather tedious to apply to investigations of the underlying mechanisms. In contrast, there are now in vitro models in which drug resistance can be predicted with high reliability. In neonatal whole hippocampal preparations, seizurelike events induced by low magnesium have been shown to be drug-refractory. Organotypic slice cultures, prepared between postnatal days P2 and P12 and tested a week after preparation, also present with drug resistance of seizurelike events induced by 4-AP or by low magnesium This drug resistance persists for months after preparation. In status epilepticus in vitro models, prolonged exposure of entorhinal cortical tissue to low Mg leads to pharmacoresistance; the same holds true when bicuculline and 4-AP are applied in combination. Finally slices from human patients can be prepared and seizurelike events, induced (e.g., by elevation of extracellular potassium concentration); these seizurelike events are drug-sensitive when the tissue comes from patients which had been responding to antiepileptic drugs, but not (in most cases) when the patients have been drug-refractory. As is the case with low Mg-induced activity and combined application of 4-AP with bicuculline, this human drug refractory tissue often shows signs of disinhibition.

Results

Drug Resistance in Chronic Epilepsy: The Transporter Hypothesis

The transporter hypothesis contends that the drug levels necessary to prevent ictogenesis are simply not reached within the CNS parenchyma. There are several mechanisms that could give rise to such a phenomenon. For instance, cleaving enzymes that degrade anticonvulsant drugs might be up-modulated. Alternatively, genetic or functional upregulation of drug transporter molecules

could account for decreased permeation of anticonvulsant drugs into the CNS. The drug transporter hypothesis, however, faces the difficulty that virtually many insults and noxious stimuli (e.g., traumatic brain injury, tumors, malformations, hypertension, stress, or seizures) can be associated with an opening of the blood–brain barrier, potentially allowing access by anticonvulsant drugs.

Several genes have been discovered which encode proteins that function as drug efflux pumps, including MDR1, MDR2, and the MRP family (MRP 1–7). Both MDR1 and some of the MRPs have been shown to be expressed by endothelial cells composing the blood–brain barrier, and a large body of evidence now exists which indicates that drug transporter molecules are upregulated in human epileptic patients as well as in experimental models of epilepsy – and that this upregulation correlates with less efficient seizure control. Additionally, expression of drug transporters in epileptic foci was observed in cell types which do not usually express them, such as astrocytes or dysplastic neurons in foci with neuronal dysplasia. If upregulation of drug transporters in endothelial cells underlies pharmacoresistance, then seizurelike events in brain slices from human epileptic brain should be sensitive to AEDs (since the BBB is by-passed in this preparation). This responsiveness is, however, rarely the case in our series of studies on human slice preparations suggesting that upregulation of drug transporters at the blood–brain barrier is only rarely a case of drug resistance in humans. It is important to note, however, that in human and experimental tissue, ectopic expression of drug transporters (on neuronal membranes) has been demonstrated; such ectopic localization could contribute to drug refractoriness, but only if AEDs act from inside the cells.

Drug Resistance in Chronic Epilepsy: The Target Hypothesis

As mentioned earlier, in brain slices from epilepsy patients, elevation of the extracellular K^+ concentration resulted in the expression of seizurelike events. In tissue from patients who were pharmacoresistant to CBZ, such artificially induced convulsions did not respond to CBZ, even at concentrations as high as 100 μM. In marked contrast, seizure activity in patients who were clinically sensitive to CBZ proved sensitive to CBZ. These results appeared to be independent of the type of mesial temporal lobe epilepsy. Because CBZ, along with other anticonvulsants such as PHT and lamotrigine, targets voltage-gated Na^+ channels, it appeared likely that changes in Na^+ channel properties in these tissues might render them unresponsive to these drugs. All three of the drugs mentioned earlier have several concomitant effects on Na^+ channels: (1) They reduce the maximal amplitude of Na^+ currents; (2) They shift the voltage-dependence of inactivation in a hyperpolarizing direction, thus reducing the number of available Na^+ channels; and (3) These anticonvulsants slow the recovery from inactivation of these channels. This third mechanism may be particularly relevant to the treatment of epilepsy because it causes a preferential reduction of Na^+ channel availability during high frequency discharges. Indeed, subtle reductions in the anticonvulsant drug effects on the voltage-dependent inactivation, and more pronounced changes in the recovery from inactivation, have been described, both in animal models of epilepsy and in human epilepsy tissue. Both in the pilocarpine model of epilepsy and in therapy-resistant patients, CBZ effects on the recovery from inactivation were completely lost. These data suggest CBZ loses a major mechanism of action in chronic epilepsy. These results also suggest that there is a fundamental change in the Na^+ channel molecule, which may be related either to altered subunit composition or to different forms of posttranslational modification.

The main pore-forming component of Na channels is the alpha subunit. Nine different alpha subunits have been identified, termed Na_v 1.1–1.9. Of the nine alpha subunits, Na_v1.1–1.3, 1.5, and 1.6 are expressed in the CNS. Na^+ channels also contain auxiliary β subunits (β_{1-4}) that modulate the biophysical properties of the channel and are important for the subcellular localization and transport of Na^+ channel pore-forming subunits. On the mRNA level, a number of changes in Na^+ channel expression have been described following kainite-induced seizures or in human epileptic material – changes that potentially lead to altered drug responsiveness.

Changes in pharmacological properties of Na^+ channels could also be due to various posttranslational modifications of the channel protein. Evidence that metabolic changes can affect sodium channels is sparse. However, effects of unsaturated fatty acids on Na^+ channels have been described, which might be related to scavenging of radical oxygen species. That ROS production is increased in chronic epileptic tissue, and that mitochondrial functions are chronically altered in human and animal forms of TLE, are well documented. Other posttranslational modifications, like glycosylation or phosphorylation, may also alter the properties of Na^+ channels, but whether they modulate the pharmacoresponsiveness of these channels is unknown.

Apart from alterations in Na^+ channels, the most significant advances in determining the potential relevance of changes in drug targets for the development of drug resistance have been made for $GABA_A$ receptors. Several hypotheses have been offered to explain the development of drug resistance to GABA-mimetic drugs, including changes in GABA receptors, alterations in the efficacy of GABAergic synaptic transmission, and reorganization in GABAergic neuronal networks. On the molecular level, GABA receptors in chronic epileptic tissue undergo highly coordinated subunit changes. In some regions of the hippocampus, such as the dentate gyrus, changes in subunit

composition profoundly affect the sensitivity of the GABA receptor complex to intrinsic modulators as well as possible anticonvulsant drugs. For example, the sensitivity of GABA receptors to zinc in epileptic dentate granule cells is considerably increased, while their sensitivity to the benzodiazepine site agonist, zolpidem, is dramatically decreased. Both of these findings have some important consequences. The block of GABA receptors by zinc, which may be released onto granule neurons by recurrent mossy fibers, may render GABA-mimetic drugs less effective. Additionally, the fact that a benzodiazepine site agonist is less effective in these cells implicates a reduced efficacy also of other substances targeting the benzodiazepine binding site. It remains to be seen whether such changes can also be observed in other mesial temporal subregions. Other alterations have been observed in area CA1 which would also suggest a reduced sensitivity of GABA receptors to some anticonvulsants. Most evidence suggests that neither CA1 nor the DG regions are seizure initiation sites, but rather participate in seizure spread. Hence, it would be important to know whether similar alterations in GABA receptors occur in seizure initiation sites such as the medial entorhinal cortex or area CA3.

Studies of seizure-induced alterations in GABAergic synaptic transmission have focused recently on potential alterations of chloride transport. During ontogenesis, GABA-mediated events induce a strong depolarization which may elicit action potentials in immature neurons. Three mechanisms can be involved in this effect: First, the inward chloride transporter NCC1, which is highly expressed in developing brain, establishes a very depolarizing reversal potential for chloride. Second, upregulation of KCC2 – the transporter that moves chloride ions out of the cell – occurs after birth; in rats, KCC2 gains a major influence on the chloride equilibrium potential only after postnatal day 10. Third, as GABA-activated channels are also permeable to bicarbonate ions, the capability of cells to generate $HCO3^{-}$ introduces a further confounding variable. During development, the neuronal carboanhydrase appears only at postnatal day 14. This enzyme shifts the reversal potential of IPSPs again in a depolarizing direction, and may be responsible for GABA-mediated depolarizations during seizures and in response to repetitive stimulation. The known anticonvulsant effect of some carboanhydrase inhibitors, including topiramate, may therefore depend on preventing a seizure-induced depolarizing shift of reversal potentials for GABA. Interestingly, recent studies in human hippocampal tissue have demonstrated that depolarizing GABA-mediated events are involved in the generation of spontaneous epileptiform bursts in the subiculum. If these events occur also elsewhere in human epileptic tissue, any GABA-mimetic drug would augment the depolarizing effect of GABA. The fact that some epileptic patients profit from treatment with carboanhydrase inhibitors suggests that reduced generation of $HCO3^{-}$ – and thereby maintenance of hyperpolarizing IPSP reversal potentials – may indeed be an important therapeutic strategy. However, to what extent GABA-mediated responses are depolarizing in chronic epileptic tissue remains a matter of dispute even in the human subiculum.

Finally, in chronic epileptic tissue, reorganization also affects interaction between interneurones and principal cells. Three classes of interneurones have been described: those that interact with the soma and axon initial segment, and thus control output of the neuron (soma targeting interneurones); those that interact with apical dendrites and control input to principal cells (dendrite targeting interneurones); and those that interact only with other inhibitory interneurones (IN interneurones). When principal cells die and are therefore reduced in number, as in temporal lobe epilepsies with hippocampal sclerosis, surviving interneurones interact more strongly with other interneurones. Therefore, the normal throughput of information through the hippocampus, via glutamatergic mechanisms, may be replaced by interactions between interneurons; information may now be transferred by disinhibition of interneurons. GABA-mimetic drugs, which augment inhibition, might then augment disinhibition and facilitate seizure generation.

Directions for Future Research

The alterations in chronic temporal lobe epilepsy may be such that typical targets for anticonvulsant drugs are no longer appropriate for a pharmacological intervention. This possibility implies that combinations of current drugs – even with different pharmacological targets – may be unlikely to offer an effective therapeutic alternative. If that is the case, then a search for alternative drug targets would be required. Such targets might include adenosine receptors, cholinergic receptors, receptors for certain peptides with proven anticonvulsant potential, glycine receptors, etc. The fact that human tissue slices present with pharmacoresistant forms of epileptiform seizurelike discharges offers the potential to test newly developed drugs in human tissue from pharmacoresistant patients. This opportunity would increase the potential for discovery of agents which are effective in the treatment of drug resistant seizures.

See also: **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; Preclinical Drug Development in Epilepsy; **Models**: Seizure Models in Acute and Organotypic Slices; **Non-Synaptic Mechanisms**: Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; **Pharmacoresistance**: Animal Models of Drug-Resistant Epilepsy; Brain Imaging of P-glycoprotein Function in Drug-Resistant Epilepsy; Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue.

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Synaptic Reorganization in the Hippocampal Dentate Gyrus

Axon Sprouting in Epilepsy

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Introduction

Temporal lobe epilepsy is the most common form of epilepsy in the adult population, afflicting at least 800 000 Americans. Its pathogenesis is, however, poorly understood. Pharmacotherapy usually fails to achieve adequate long-term control of seizure discharge, in part because the available drugs do not target abnormal mechanisms unique to this disorder. Although temporal lobe epilepsy can arise from numerous causes, most cases are believed to develop after a lesion of the brain. Most of, and sometimes all, the detectable pathology is found in the hippocampus. Within the hippocampus, the most consistent region of neuronal loss is the hilus of the

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dentate gyrus. Therefore many studies – including our own – have focused on mechanisms by which hilar damage leads to periodic spontaneous seizures.

The consequences of brain injury depend not only on the degeneration of neurons and their axonal projections but also on reactive growth triggered by the injury. Synaptic sites denervated as a result of injury do not remain unoccupied, but become reinnervated by axons that survived the injury. For the most part, axon sprouting and neosynaptogenesis appear to restore normal function, because the normal lamination of afferent fibers is preserved. The lost synapses are replaced mainly by fibers of the same pathway and/or by heterologous fibers that normally project to the same lamina. In some instances, however, axon sprouting creates an aberrant pattern of connections. In animal models of temporal lobe epilepsy, the number of monosynaptic recurrent excitatory synapses increases dramatically in several brain regions, including the dentate gyrus, area CA1 of the hippocampus, and the neocortex. Strengthening of recurrent excitatory circuitry is one mechanism that could synchronize neuronal firing, thus amplifying epileptiform discharge and enhancing seizure propagation.

Our research has focused on the dentate gyrus. Aberrant synaptic reorganization in epileptic brain was first described in the dentate gyrus, and this remains the only form of anatomic plasticity documented to occur in human epilepsy. Reorganization of the dentate gyrus is of particular interest because this region normally imposes a high resistance to the propagation of seizures from the highly-excitable entorhinal cortex to the equally excitable CA3 area of the hippocampus. The principal neurons of the dentate gyrus, the granule cells, are difficult to activate, both individually and as a synchronized population, due to their robust synaptic and tonic GABA inhibition, high resting membrane potential, high K^+ conductance, limited Ca^{2+} conductance, low intrinsic burst capability and virtual absence of monosynaptic recurrent excitation. Thus establishment of novel recurrent excitatory circuitry in this brain region is expected to facilitate seizure propagation by providing a mechanism for synchronizing granule-cell firing. Development of monosynaptic recurrent excitation is not the only mechanism that can synchronize granule-cell firing. However, such circuitry serves as the major substrate for synchronization of pyramidal-cell bursts in area CA3, and it would be expected to play a similar role in other brain regions. The ultimate goal of our studies is to identify properties of the novel circuitry that could serve as targets for therapeutic intervention.

Background

Most of our knowledge about reorganization of the dentate gyrus in epileptic brain comes from studies of animal models, although the essential features have been observed also in human hippocampi that were surgically resected to control medically-intractable seizures. Studies of animal models revealed that dentate granule cells become synaptically interconnected through the growth of recurrent mossy fibers. Mossy-fiber sprouting appears to be initiated largely by the hilar lesion. Neurons of the dentate hilus include excitatory mossy cells and several distinct populations of inhibitory GABA interneurons. Both mossy cells and GABA interneurons degenerate. Recurrent mossy fibers then cross the granule-cell body layer and innervate the proximal portion of the granule-cell apical dendrite (**Figure 1(b)**), the same territory normally occupied by synapses of mossy-cell axons (associational–commissural fibers; **Figure 1(a)**). Indeed the extent of mossy-fiber sprouting detected with the Timm heavy metal stain (**Figure 1(g)** and **1(h)**) is directly, and essentially linearly, related to the loss of mossy cells. This change in granule-cell innervation must have profound consequences for information processing by the hippocampus. Both the recurrent mossy fiber and associational–commissural pathways support forms of recurrent excitation. However, the recurrent mossy-fiber pathway creates a monosynaptic circuit oriented in the transverse plane, whereas the degenerated associational–commissural pathway connected granule cells in the longitudinal plane and on different sides of the brain through a disynaptic circuit.

About 10% of dentate granule cells in rodent epilepsy models have a basal, as well as an apical, dendrite. Granule cells in normal rodent brain are much less likely to have a basal dendrite, and basal dendrites that extend a long distance into the hilus appear unique to epileptic brain. Granule-cell basal dendrites provide an additional target for recurrent mossy-fiber synapses (**Figure 1(c)**). In humans, ~25% of dentate granule cells normally have a basal dendrite. Thus, recurrent excitation of granule cells may be a normal feature of human brain, which is then enhanced by mossy-fiber sprouting during epileptogenesis.

Dentate granule cells are unusual in that they continue to be born and differentiate throughout life. Seizures increase the rate of granule-cell neurogenesis markedly. Some studies suggest that postseizure neurogenesis contributes importantly to the progressive worsening of epilepsy. Most of the newly-born granule cells migrate into the granule-cell body layer and differentiate normally (although some retain a basal dendrite, thus accounting for the increased number of bipolar granule cells). However, a sizable subpopulation of newly-born granule cells migrates aberrantly, and many of these cells are later found throughout the dentate hilus (**Figure 1(d—f)**). Few granule cells are located in the hilus normally. Hilar ectopic granule cells (HEGCs) receive mossy-fiber innervation from other granule cells and also innervate other granule cells (**Figure 1(c)**) in addition to the normal mossy-fiber targets (CA3 pyramidal cells and inhibitory interneurons).

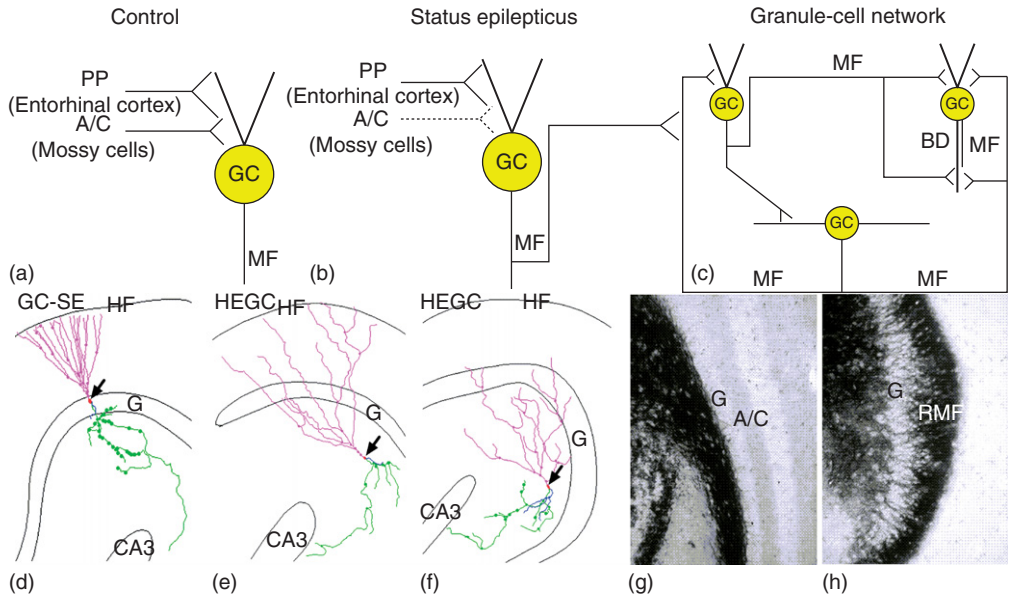


Figure 1 (a) Dentate granule cells (GC) normally receive excitatory innervation from perforant path (PP) and associational–commissural (AC) pathways on the distal and proximal portions of the apical dendrite, respectively. Mossy fibers (MF; granule-cell axons) project into the dentate hilus and area CA3. (b) Status epilepticus kills hilar mossy cells, causing mossy fibers to elaborate recurrent branches that make synapses on the portion of apical dendrite formerly innervated by associational–commissural fibers. (c) The granule-cell network consists of normally-located granule cells, with or without a basal dendrite (BD), and ectopic granule cells. These components are interconnected by mossy fibers. (d–f) Reconstructions of granule cells filled intracellularly with biocytin after whole-cell patch-clamp recording in a hippocampal slice. G, granule-cell body layer; HF, hippocampal fissure. Purple, apical dendrite; red, cell body (indicated by arrow); blue, basal dendrite; green, mossy fiber. (d) After status epilepticus, granule cells (GC-SE) in the cell body layer retain normal cellular morphology, except that a minority have a basal, as well as an apical, dendrite. (e–f) Two HEGCs demonstrating the widespread, but relatively sparsely-branched, dendrites characteristic of these cells. (g–h) Timm-stained dentate gyrus. G, granule-cell body layer. (g) The light staining of the inner molecular layer in controls is characteristic of the associational–commissural (A/C) pathway. (h) Dark staining associated with the recurrent mossy-fiber (RMF) pathway fills the inner molecular layer after status epilepticus. (a–c) Reproduced from Nadler JV (2003) The recurrent mossy fiber pathway of the epileptic brain. *Neurochemical Research* 28: 1649–1658, with permission of Springer Science and Business Media.

Thus normal granule cells, granule cells with a basal dendrite, and HEGCs are synaptically interconnected by recurrent mossy fibers, forming a reverberating network unique to epileptic brain. These anatomical changes are not essential for the development of epilepsy either in humans or in animal models. However, they have been associated with a progressive increase in the frequency and duration of spontaneous seizures.

Methods

Our studies utilize rats that had developed status epilepticus after the administration of the muscarinic receptor agonist pilocarpine. We normally allow status epilepticus to self-terminate without the administration of an anticonvulsant. Animals that experience prolonged (6–8 h) status epilepticus exhibit very extensive and uniform hilar lesions followed by robust recurrent mossy-fiber growth, as observed in many persons with temporal lobe epilepsy. The pilocarpine model also replicates the accumulation of HEGCs observed in those persons. Rats that experience pilocarpine-induced status epilepticus invariably develop unremitting spontaneous seizures after a delay of 1–3 weeks. In these respects, the pilocarpine model produces more consistent outcomes than the alternatives, such as the kainic acid or electrical stimulation models. Finally, pilocarpine-induced status epilepticus kills fewer CA3 pyramidal cells than does kainic acid or electrical stimulation, better replicating the lesion most commonly observed in surgically-resected hippocampi and facilitating the placement of a stimulating electrode in the mossy-fiber bundle of hippocampal slices.

Most of our studies are performed during the chronic epileptic period 10 weeks or longer after status epilepticus. By this time, the granule-cell network appears to be fully mature. Some analyses are also performed 4–6 days after status epilepticus, before the new circuitry can be detected. Hippocampal slices are prepared for electrophysiological studies. Extracellular recording, whole-cell and cell-attached patch-clamp recording, and perforated patch recording are used, as appropriate, to define granule-cell properties, the physiology and pharmacology of mossy-fiber→granule-cell synapses, and the role of the novel circuitry in granule-cell epileptiform activity.

In parallel studies, histological, ultrastructural, immunohistochemical, and immunocytochemical approaches are used to describe and quantitate the cytopathology of the lesion and the anatomical response to the lesion.

Results

Formation of recurrent excitatory circuitry in the dentate gyrus is associated with a reduced threshold for granule-cell synchronization in both humans and animal models. The clearest evidence was obtained in our studies of mossy fiber-evoked granule-cell population bursts. This response can be evoked only in slices with substantial recurrent mossy-fiber growth (**Figure 2(a)**). We have found no experimental conditions under which mossy-fiber stimulation evokes a granule-cell population burst in slices from control animals or in slices prepared from rats that experienced status epilepticus, but had not yet developed a detectable recurrent mossy-fiber pathway.

Although computational models suggest that formation of mossy fiber→granule-cell synapses should reliably enhance synchronized granule-cell bursting, low-frequency stimulation of the mossy fibers evokes population bursts in only 14–25% of hippocampal slices that evidence robust recurrent mossy-fiber growth. Even when the superfusion medium is modified to enhance granule-cell excitability, a population burst may be evoked in only 60–70% of slices. Furthermore, perforant path stimulation may or may not reveal evidence of granule-cell hyperexcitability during the chronic epileptic period, depending on experimental conditions. These observations prompted us to study the factors that regulate excitability in the reorganized dentate gyrus.

Our results revealed that a modest increase in extracellular K^+ concentration, short-term frequency-dependent facilitation (frequencies of 1–2 Hz), corelease of zinc, and feedback activation of presynaptic kainate receptors augment activity in the recurrent mossy-fiber pathway. Activation of presynaptic $GABA_A$ receptors by spillover from nearby GABA synapses and corelease of brain-derived neurotrophic factor (BDNF) from the mossy fibers themselves have been shown to enhance transmission at mossy-fiber synapses on CA3 pyramidal cells and would be expected to enhance recurrent mossy-fiber transmission similarly. Factors that diminish activity in the recurrent mossy-fiber pathway include short-term frequency-dependent depression (frequencies <1 Hz), feedback activation of type II metabotropic glutamate receptors, and neuropeptide Y. Activation of $GABA_B$ receptors by spillover from nearby GABA synapses and activation of presynaptic adenosine A1 receptors and κ opiate receptors by adenosine and dynorphin released from mossy fibers depress synaptic transmission onto CA3 pyramidal cells and would be expected to have similar effects at mossy-fiber synapses on granule cells.

Enhanced GABA inhibition probably accounts for the unexpectedly low excitability of the granule-cell network. Dentate granule cells receive synaptic inhibition from six

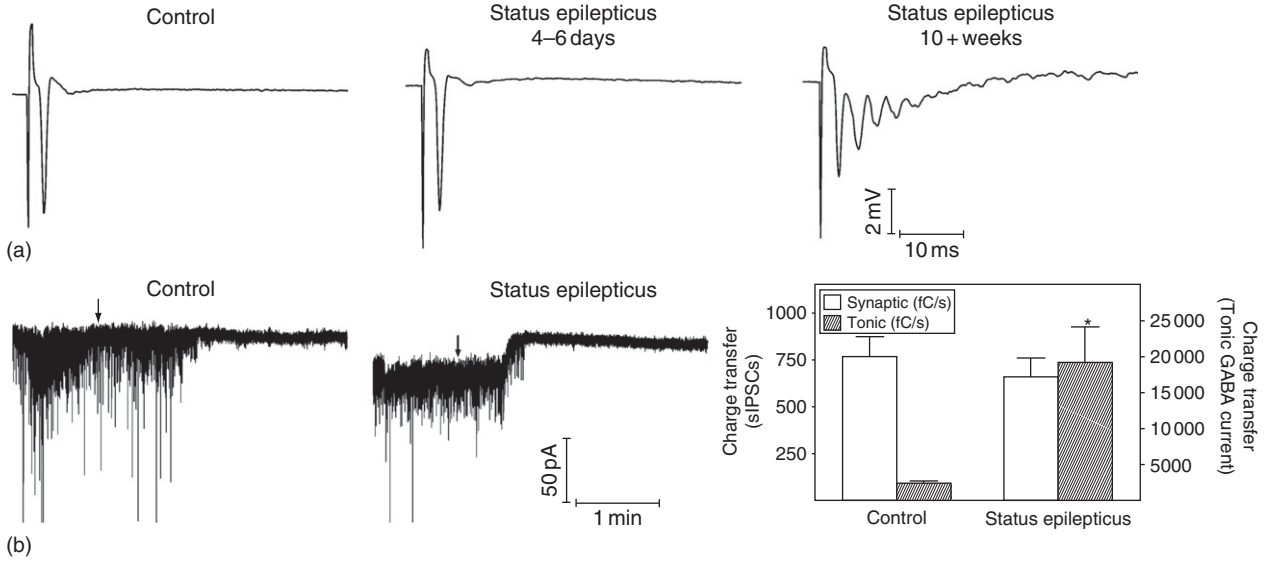


Figure 2 (a) Extracellular recording in the granule-cell body layer reveals evidence of mossy fiber-evoked reverberating excitation only after formation of a substantial recurrent mossy-fiber pathway. Reproduced from Okazaki MM and Nadler JV (2001) Glutamate receptor involvement in dentate granule cell epilepti form activity evoked by Mossy fiber stimulation. *Brain research* 915(1): 58–69, with permission from Elsevier. (b) Whole-cell patch-clamp recording (CsCl-based internal solution, $V_{hold} = -70$ mV) in the presence of glutamate antagonists reveals changes in synaptic and tonic GABA inhibition. The amplitude and charge transfer per second of tonic GABA current increases in dentate granule cells by 10 weeks after status epilepticus and the charge transferred by spontaneous IPSCs per second is only slightly depressed, despite the 70–80% loss of hilar GABA interneurons. Note the differential change in membrane current produced by the addition of the $GABA_A$ receptor antagonist SR95531 (100 μM) to the superfusion medium (arrows). $^*p < 0.05$.

distinct interneuron populations. In addition, dentate granule cells have a tonic GABA current produced by ambient extracellular GABA and released GABA that escapes the synaptic cleft. Prolonged pilocarpine-induced status epilepticus destroys 70–80% of the inhibitory interneurons located in the dentate hilus. As a result, feedback (mossy fiber-evoked) inhibition of granule cells declines substantially and granule-cell excitability increases. Both of these effects reverse with time. Results from our laboratory and others suggest several mechanisms that, in combination, probably explain the recovery of inhibition. Reverberating excitation among granule cells may allow the surviving dentate gyrus interneurons to be driven repeatedly by a mossy-fiber volley rather than just once. The charge transfer per second of spontaneous IPSCs recovers substantially and the magnitude of tonic GABA current increases (**Figure 2(b)**). These effects appear to arise in part from the increased spontaneous firing of surviving interneurons and/or the facilitation of GABA release. Finally, recurrent mossy fibers make some contacts with inhibitory interneurons. Although they account for only 5% of all synapses made by recurrent mossy fibers, their formation might represent a significant percentage increase in the mossy-fiber innervation of those interneurons.

Properties of HEGCs suggest that they could serve as triggers for recruitment of the entire granule-cell population into reverberating excitation – and thus epileptiform activity. We find that most HEGCs possess intrinsic burst capability dependent on retention of the large T-type Ca^{2+} current characteristic of immature granule cells. In addition, both ultrastructural and electrophysiological evidence suggest a much sparser inhibitory innervation than normal granule cells, although their excitatory innervation is of normal size. Hyperexcitable HEGCs may play a role in the initiation of granule-cell population bursting similar to that of the relatively excitable CA2/CA3a pyramidal cells in the initiation of CA3 bursting. In contrast, granule cells that are born after seizures and migrate normally into the granule-cell body layer appear to exhibit reduced excitability compared with granule cells born earlier. Therefore, to the extent that postseizure neurogenesis enhances the frequency and duration of subsequent spontaneous seizures, the newly-born neurons responsible for this are probably the ectopic granule cells.

Future Goals

A central issue is the extent to which the strengthening of recurrent excitation accounts for seizure initiation and/or propagation in the lesional epilepsies. The limited studies of this issue to date have focused mainly on the dentate gyrus and the results have been mixed. Hyperexcitability of dentate granule cells caused by dysfunctional BK-type Ca^{2+}-dependent K^+ channels predisposes to seizures that emanate from the temporal lobe, and at least some spontaneous limbic seizures in animal models involve the dentate gyrus at an early stage. These results support the view that any change that increases granule-cell excitability should have a proepileptogenic effect. However, other seizures appear to propagate directly from the entorhinal cortex to area CA1, bypassing the dentate gyrus. In these instances, the enhancement of recurrent excitation in area CA1 would seem to be of greater importance. In addition, seizure-induced plasticity produces both proepileptogenic and antiepileptogenic effects. Given that homeostatic mechanisms at least partially restore granule-cell excitability toward normal, we still need to define the circumstances under which recurrent excitatory activity overcomes these restraints and synchronizes population bursts.

The mechanism(s) by which dentate gyrus inhibition recovers after the loss of hilar interneurons is also of interest. Studies of CA1 pyramidal cells indicated that both the spontaneous firing of the surviving interneurons and GABA release per impulse increases in pilocarpine-treated rats. Do these changes occur also in dentate gyrus interneurons? If so, what is the underlying mechanism? Which specific interneuron populations respond to the lesion in this manner?

HEGCs have properties that favor a key role in population bursting of granule cells in epileptic brain. However, direct evidence of such a role is needed. Do HEGCs serve as triggers for bursting of the larger granule-cell population or are they primarily followers of such activity? How much do they contribute to the activation of CA3 pyramidal cells? Studies of these issues require, at a minimum, correlating the occurrence and magnitude of population bursts with the number of HEGCs (which can vary among animals by more than an order of magnitude) and the use of agents that act preferentially on HEGCs. It will also be necessary to discriminate the effects of ectopic granule cells from those of newborn granule cells that migrate normally into the cell body layer.

Finally, neuronal loss and axon sprouting create markedly abnormal circuitry in several brain regions, even if the overall level of excitability recovers to normal. An extensive and somewhat controversial literature documents cognitive/behavioral dysfunction in persons with lesional epilepsy. To what extent might reorganization of brain circuitry account for these dysfunctions?

See also: **Granule Cells**: Properties of Dentate Granule Cells and their Relevance to Seizures; **Neurogenesis**: Adult Neurogenesis and Mesial Temporal Lobe Epilepsy; **Plasticity**: Circuitry Reorganization, Regeneration, and Sprouting; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; Synaptic

Reorganization in the Hippocampal Dentate Gyrus; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Circuitry Reorganization, Regeneration, and Sprouting

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Introduction

Pharmacological therapy for intractable partial-onset epilepsy is aimed to relieve, symptomatically, seizure frequency and severity. Almost 30 000 compounds have been examined using two animal models of acute evoked convulsions, and these tests have provided excellent anticonvulsants; however, to date, these compounds appear to be less effective against other seizure types, and ineffective in altering the progression of epileptogenesis. Advancement in the design and discovery of therapies that alter the development of epileptogenesis has been hindered by our lack of understanding of the fundamental mechanisms of epileptogenic processes – i.e., the pathophysiological process underlying the development of spontaneous seizures. Understanding the mechanisms of epileptogenesis might lead to the discovery of compounds that effectively modify the disease, by altering the natural history of symptomatic partial-onset epilepsy. This article critically summarizes synaptic reorganization in the hippocampal circuitry as a potential mechanism for epileptogenesis. We have focused on the most common, pharmacologically intractable partial-onset epilepsy, mesial temporal lobe epilepsy. Within this context, synaptic reorganization of the hippocampal circuitry progressively enhances limbic hyperexcitability and may also contribute toward pharmacological intractability in partial-onset epilepsy.

Background

Mesial temporal lobe epilepsy (MTLE) is the most common epilepsy syndrome with pharmacologically intractable partial-onset seizures. This epileptic syndrome is

Reorganization in the Hippocampal Dentate Gyrus; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Introduction

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Background

Mesial temporal lobe epilepsy (MTLE) is the most common epilepsy syndrome with pharmacologically intractable partial-onset seizures. This epileptic syndrome is

associated with acute neurological insults, such as status epilepticus (SE), traumatic brain injury, brain tumors, and stroke; it is highly associated with febrile seizures, particularly complex or prolonged febrile seizures. However, many patients with MTLE have no obvious brain insults, other than, the cumulative effect of repeated brief partial-onset seizures. The natural history of epileptogenesis is clearer in those patients with an acute neurological insult, which provides an initial substrate for a progression to MTLE. After an acute insult, there is typically a latent period (before the onset of spontaneous seizures) that can span several weeks, but often lasting months to years. Severe insults, such as prolonged febrile convulsions and generalized convulsive SE, are associated with a shorter latency to the onset of spontaneous seizures (compared with other less severe insults such as simple febrile seizures). The latent period between the precipitating event and the onset of epilepsy is one of the hallmarks of the pathophysiological process underlying the progression of epileptogenesis in MTLE.

Most patients with intractable MTLE have a seizure onset within the hippocampal formation. After resective surgery to remove the hippocampus, electrophysiological studies of the resected tissue demonstrate evidence of cellular hyperexcitability in the subiculum and the dentate gyrus. Subicular pyramidal neurons and dentate granule cells are hyperexcitable, but only under conditions that impair inhibitory mechanisms. Such studies have also shown a correlation between the degree of granule cell hyperexcitability and the extent of mossy fiber (dentate granule cell axons) synaptic reorganization. This type of morphological plasticity resulting in rewiring of the hippocampal circuitry is a feature of intractable MTLE (and has been shown in many experimental models), suggesting that the spontaneous epileptic seizures might be associated with these pathological alterations. However, causative relationships between the structural and functional phenomena observed in human MTLE cannot be determined, because the available resected tissue reflects only the late stage of human epileptogenesis, many years after the onset of spontaneous seizures.

Numerous neuropathological abnormalities have been described in the epileptic hippocampus from patients with intractable MTLE. Widespread neuronal injury produces a pattern of hippocampal neuronal loss known as mesial hippocampal sclerosis. The neuronal circuitry in the dentate gyrus undergoes synaptic reorganization, primarily involving the mossy fibers. These neuropathological abnormalities might be consequences of repeated seizures or the result of the initial precipitating brain insult that led to repeated seizures. Further, each of these abnormalities might also reflect a potential mechanism that contributes toward the process of epileptogenesis, which ultimately results in pharmacologically intractable MTLE. Understanding the relative contribution of these pathological abnormalities in epileptogenesis has been considerably improved by utilizing experimental models of MTLE, in which the relationship between the morphological plasticity, the pathological abnormalities, and other potential mechanisms can be systematically tested.

Methods and Experimental Approach

Several strategies are used to study the process of epileptogenesis in experimental models of MTLE. One approach utilizes chemoconvulsants to induce a prolonged period of convulsive SE and an acute excitotoxic insult in limbic structures including the hippocampus. This acute treatment gives rise to the later development of spontaneous brief seizures. The experimental chemoconvulsant SE models generally offer a latent period (between initial SE and the onset of spontaneous seizures) of several days to weeks before the onset of partial seizures. Two frequently used acute chemoconvulsants that employ this approach are the kainic acid and pilocarpine. Rats that have experienced several hours of convulsive SE induced with these chemoconvulsants exhibit neuropathological and neurophysiological abnormalities similar to those observed in intractable MTLE. Adult rats that have experienced SE demonstrate prominent neuronal damage and gliosis in hippocampal pyramidal neurons and the hilar polymorphic neurons of the dentate gyrus. After hippocampal neuronal loss, these regions demonstrate morphological reorganization of the neuronal circuits, and associated enhanced excitability. A problem with this approach is the considerable variability and mortality among experimental animals, and the severity of these pathological abnormalities (which mimics only the late stages of intractable human MTLE).

Another strategy utilizes a chronic model of repeated exposure to seizures, using electrical stimulation of limbic pathways (kindling) or chemoconvulsants to produce frequent, short insults that lead only to brief repeated seizures. In general, these chronic models of MTLE exhibit considerably less injury, and a reduced severity of pathological or physiological abnormalities (compared with the models that require an initial episode of SE). Although the chronic 'repeated exposure' models might more appropriately mimic the frequency of seizure in patients with intractable MTLE, they offer less frequent late spontaneous seizures and a less discrete latency period as compared to the kainic acid or pilocarpine model of MTLE.

Sprouting and synaptic reorganization have been investigated primarily in hippocampal pathways; however, neuronal loss has been demonstrated in multiple limbic areas. An advantage of the acute chemoconvulsant models of MTLE is that the time course of progression of the neuropathological and neurophysiological abnormalities can be studied in relation to the development of spontaneous partial-onset seizures. Following an acute excitotoxic insult,

one can study the time course and sequence of development of the different forms of morphological plasticity, as well as other neuropathological abnormalities that occur during the latent period – i.e., before the emergence of cellular hyperexcitability and the onset of spontaneous seizures. During the latent period, there are transitory functional impairments of inhibitory control that recover during the chronic state. Other neuropathological abnormalities and plasticity that appear in the latent period are permanent. It has been hypothesized that these permanent alterations underlie the mechanisms of epileptogenesis of intractable MTLE (**Figure 1**). Synaptic reorganization of the mossy fibers – i.e., sprouting into the inner molecular layer of the dentate gyrus – is the most studied form of circuitry reorganization induced during the latent period. The time course of development of mossy fiber synaptic reorganization correlates temporally with the emergence of cellular hyperexcitability in the dentate granule cells, and precedes the occurrence of spontaneous seizures. Mossy fiber synaptic reorganization has been described as a permanent alteration of the hippocampal circuitry virtually in all models of MTLE and human intractable MTLE.

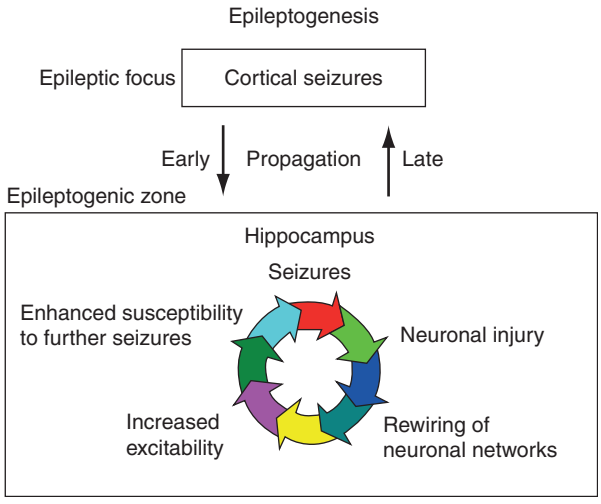


Figure 1 Mechanisms of epileptogenesis. The schematic diagram describes the relationships between pathophysiological phenomena and brain location during epileptogenesis in mesial temporal lobe epilepsy. Partial-onset seizures might initially originate in neocortical areas, but after the establishment of intractability, there is a vicious circle of interrelated pathophysiological phenomena within the hippocampal circuitry that self-sustains intractable seizures with propagation to neocortical structures. Modified from Cavazos JE and Cross DJ (2006) The role of synaptic reorganization in mesial temporal lobe epilepsy. *Epilepsy & Behavior* 8: 483–493.

Recent Results

Mossy Fiber Sprouting

The most commonly documented circuitry reorganization in MTLE is in the mossy fiber pathway of the dentate gyrus. Most studies in experimental models of MTLE have shown sprouting in the mossy fiber pathway into the inner molecular layer of the dentate gyrus (DG) and sprouting of the distal mossy fiber projection to the CA3 region. Synaptic reorganization induced by seizures has been studied extensively in the mossy fiber pathway due to the ease of detecting changes in the laminar pattern of this pathway using Timm histochemistry or Dynorphin-A immunocytochemistry. These changes in laminar pattern have been confirmed in experimental models of MTLE, using techniques that trace the axonal projections of the mossy fibers from individual dentate granule cells.

Timm histochemistry depicts the projection pattern of the mossy fibers in the hippocampus due to the high zinc content of their synaptic terminals. In normal rats and humans, there are few Timm granules in the inner molecular layer of the dentate gyrus. However, after many repeated seizures or SE, a dense band of Timm granules appears in the inner molecular layer of the dentate gyrus (**Figure 2**). Investigations have demonstrated that this

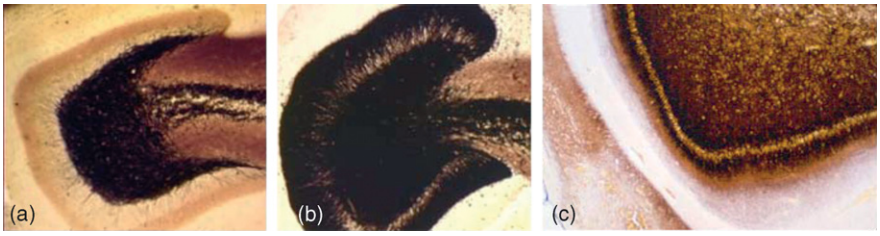


Figure 2 Mossy fiber sprouting. These are three photomicrographs from histological sections of the dentate gyrus (DG) stained with Timm histochemistry. Dark punctate granules depict the projection pattern of mossy fiber terminals that originate from granule cell axons. (a) The DG from a normal rat shows dense staining in the hilus (area within the U-shape of the DG) and in the CA3 region, with an absence of dark punctate granules in the molecular layer of the DG (arrow). (b) The DG from a rat that experienced SE-induced with kainic acid demonstrates prominent staining in the molecular layer. (c) Human DG obtained surgically during a standard anterior temporal lobectomy for the treatment of pharmacologically intractable mesial temporal lobe epilepsy. Note that the molecular layer of the DG has prominent staining, demonstrating mossy fiber sprouting into that region. Modified from Cavazos JE and Cross DJ (2006) The role of synaptic reorganization in mesial temporal lobe epilepsy. *Epilepsy & Behavior* 8: 483–493.

new Timm-positive band is due to the appearance of mossy fiber collaterals, which sprout into the inner molecular layer to form new synaptic contacts with primarily onto spines and dendrites of granule cells but also onto the dendrites of interneurons. Light and ultrastructural examination of Timm-processed tissue has been used to assess the time course of development and permanence of these reorganized axonal projection. In one kindling experiment, adult Sprague–Dawley male rats (250 g) were implanted with electrodes into the perforant path, and the entorhinal projection to the molecular layer of the dentate gyrus was stimulated twice daily to evoke five after-discharges over 3 days. Tissue from these animals was examined histologically (with Timm histochemistry) on days 1, 5, 21, and 90 after the first stimulation. A small degree of mossy fiber sprouting into the inner molecular layer was found as early as day 5, and had a maximum development by day 21 (with no further changes on day 90). In other kindling experiments, mossy fiber sprouting was observed to last as long as 18 months (when the age of the rats was over 21 months), suggesting that this morphological plasticity is permanent circuitry reorganization.

In acute chemoconvulsant models with exposure to SE early in development, there is a relative resistance to neuronal injury and lack of mossy fiber sprouting into the inner molecular layer. There seems to be a developmental age threshold in rats – around postnatal day 21 – after which exposure to SE leads to the pathological abnormalities and mossy fiber plasticity, as observed in adults. However, it should be noted that the lack of mossy fiber sprouting into the inner molecular layer does not imply an absence of circuitry reorganization. For example, SE induced with kainic acid on postnatal day 15 results in mossy fiber sprouting at the distal projection to the CA3 region, as well as, at the distal projection from the CA1 region to the subiculum.

Among the neuropathological alterations strongly associated with MTLE is hilar polymorphic neuronal loss, a possible trigger for mossy fiber synaptic reorganization. In the kainic acid model of MTLE, the acute excitotoxic insult results in neuronal degeneration in the hilus that can be recognized as early as 6 h after the onset of SE. Terminal degeneration in the inner molecular layer can be detected 24 h prior to the development of mossy fiber sprouting.

Experimental manipulations that prevent terminal degeneration from hilar polymorphic neurons also prevent the development of mossy fiber sprouting. Degeneration of axon terminals in other central (and peripheral) neural systems results in sprouting in the absence of seizures, suggesting that denervation per se is a sufficient trigger for sprouting. However, there is evidence that seizures also induce a small degree of neurogenesis of newly formed granule cells, perhaps migrating to ectopic locations and developing aberrant axonal projections.

Mossy fiber sprouting alters the organization of the dentate gyrus circuitry, making available new recurrent excitatory connections within a structure that is considered to be the 'gate' to hippocampus, and which is normally under a high degree of inhibitory control. Normally, there are few recurrent excitatory connections between granule cells. However, in epileptic animals, the sprouted mossy fibers provide direct excitatory feedback to neighboring granule cells (and to some interneurons). In the kainic acid and pilocarpine models, most of the newly formed synaptic terminals in the inner molecular layer are on granule cell spines, primarily creatinga recurrent excitatory collateral circuit. The axons from a sprouted granule cell form ~500 new synaptic contacts with granule cells, and fewer than 25 contacts onto interneurons. The chronic model of repeated kindling of limbic structures has been used to study the early stages of mossy fiber sprouting into the inner molecular layer; these studies suggest that the initial postsynaptic targets of sprouting mossy fibers are dendrites of interneurons. In Timm histochemistry, the light microscopy shows collections of Timm granules aligned in few 'strings' along the granule cell layer and the inner molecular region, presumably in association with interneuron dendrites. As many more kindling seizures are evoked, the pattern spreads along the inner molecular layer until forming a dense band of granules. Thus, it is likely that in its initial stage, mossy fiber sprouting is a homeostatic compensatory mechanism aimed to restore inhibitory control by providing feedback synaptic connections onto inhibitory basket cell interneurons. However, as the repeated seizures continue, the terminal degeneration in the inner molecular layer creates many postsynaptic targets on dendrites, spines, and somata of granule cells, providing a substrate for the formation of recurrent excitatory collaterals onto principal excitatory neurons. Computer models of the epileptic dentate gyrus demonstrate that an increase from 1% to 5% in the number of recurrent excitatory collaterals (i.e., sprouting mossy fibers) is sufficient to explain the degree of cellular hyperexcitability observed in the epileptic dentate gyrus.

Seizure-induced reorganization of the mossy fiber pathway is not limited to the formation of aberrant recurrent collaterals into the inner molecular layer of the dentate gyrus. The mossy fiber pathway also increases its branching within the hilus and expands the granule cell connectivity between the two blades of the dentate gyrus and along the septotemporal axis of the hippocampus. The net effect of this latter connectivity is the involvement of other hippocampal lamella (see below). Recurrent collaterals are also formed (along the septotemporal axis of the hippocampus) in the terminal projection of the mossy fibers to the CA3 region, particularly into the basal dendrites and spines of the CA3 pyramidal neurons in the stratum oriens.

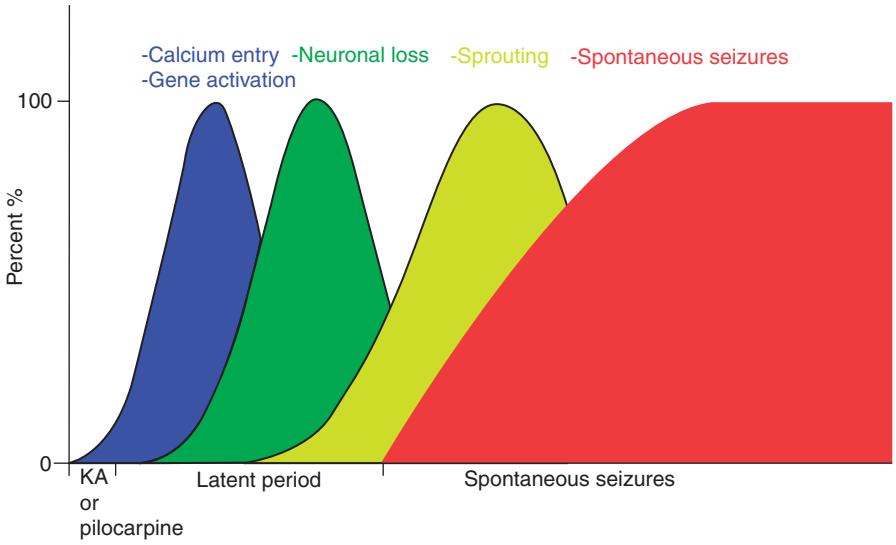


Figure 3 Time course of morphological alterations during the latent period.

To summarize, the latency of the development of morphological plasticity matches the time course of development of spontaneous seizures in acute and chronic experimental models of MTLE (see **Figure 3**). Hilar neuronal loss and terminal degeneration of their projections precede the development of mossy fiber sprouting into the inner molecular layer in acute models of MTLE; there is a direct correlation between the amount of hilar neuronal cell loss, the extent of mossy fiber sprouting into the inner molecular layer, and the degree of granule cell hyperexcitability. However, the physiological consequences of hilar polymorphic neuronal loss and mossy fiber sprouting remain a controversial subject.

Synaptic Reorganization in CA1 and Subiculum

In human MTLE and experimental models, pyramidal neurons of CA1 and subiculum also show persistent cellular hyperexcitability, and extensive neuronal loss is seen in the CA1 pyramidal neurons. Examination of the time course to formation of sprouted CA1 recurrent collaterals in acute models of MTLE suggests that seizure-induced CA1 hyperexcitability might be due to the formation of recurrent excitatory collaterals. Although hyperexcitability in CA1 neurons has been proposed to result from circuitry reorganization, these results were demonstrated in hippocampal slices, in which axonal projections perpendicular to the plane of the slice are severed. Importantly, hippocampal slices preserve the trisynaptic circuitry but do not recapitulate the complexity of the anatomical topography of the CA1 projection to the subiculum and the stratum lacunosum-moleculare of CA1.

A recent study examined the possibility that the distal axonal projections of epileptic CA1 pyramidal neurons reorganize outside of its normal laminar boundaries within the subiculum and CA1 hippocampal region (**Figure 4**). Synaptic reorganization of the CA1 projection to its primary output, the subiculum, might provide a mechanism for the enhanced and persistent cellular

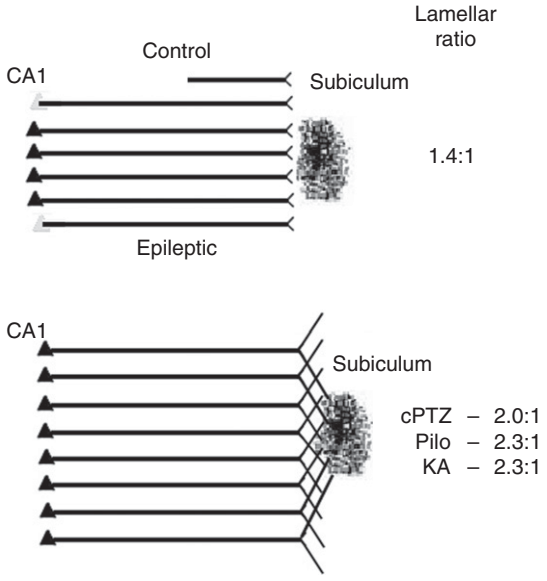


Figure 4 Synaptic reorganization in the CA1 projection to subiculum. There is prominent reorganization of the lamellar projection of the CA1 axonal pathway to the subiculum in several animal models of mesial temporal lobe epilepsy using retrograde tracers. In control rats, the extent of CA1 retrograde labeling from an injection site is limited to a couple of lamellae above and below the injection site in subiculum. In contrast, in epileptic rats, the retrograde labeling extends beyond several CA1 lamellae above and below the normal projection. This is direct evidence that axonal terminals from neurons in those layers extend their axons into the area of injection. Modified from Cavazos JE, Jones SM, and Cross DJ (2004) Sprouting and synaptic reorganization in the subiculum and CA1 region of the hippocampus in acute and chronic models of partial-onset epilepsy. *Neuroscience* 126: 677–688.

hyperexcitability in this region – an observation made in several studies using different techniques. In five animal models of MTLE, neural tracing experiments (using a retrograde labeling technique) showed that the distal branches of axons of CA1 pyramidal neurons extend 42–67% beyond their normal lamellar projection to include lamellae above and below the injection site. This study demonstrated substantial circuit reorganization of the CA1 projection to the subiculum along the septotemporal axis of the hippocampus, a finding that would support increased transverse connectivity among hippocampal lamellae above and below the normal circuitry. This type of circuit reorganization might allow epileptic activity arising in a given hippocampal lamella to recruit and synchronize activity in additional hippocampal lamellae, amplifying the epileptic response and playing a role in the persistent hyperexcitability observed in intractable partial-onset epilepsy (Figure 5).

The mechanisms triggering circuitry reorganization of the CA1 axons projecting to the subiculum are not known. In the acute chemoconvulsant models of MTLE, there is some degree of neuronal degeneration in the subiculum, but it is considerably less than in the CA1 pyramidal layer or in the hilus of the dentate gyrus. As indicated above, neuronal loss is not observed in these acute chemoconvulsant models of MTLE, if the exposure to SE occurs prior to postnatal day 21 in rats. Interestingly, in an experiment inducing SE with kainic acid on postnatal day 15 rats, investigators showed a larger extent of CA1 reorganization than in adult animals – despite the absence of overt neuronal loss in the subiculum or the CA1 pyramidal region. Furthermore, in the immature brain's response to excitotoxic insult, circuit reorganization did not occur in hippocampal pathways proximal to the site of axon origin (i.e., mossy fiber sprouting into the inner molecular layer; sprouting of CA1 axons into the stratum lacunosum-moleculare of CA1) in response to excitotoxicity in the immature brain. In contrast, circuit reorganization of hippocampal pathways distal to the site of axon origin (i.e., mossy fiber projection to CA3; CA1 projection to subiculum) displayed

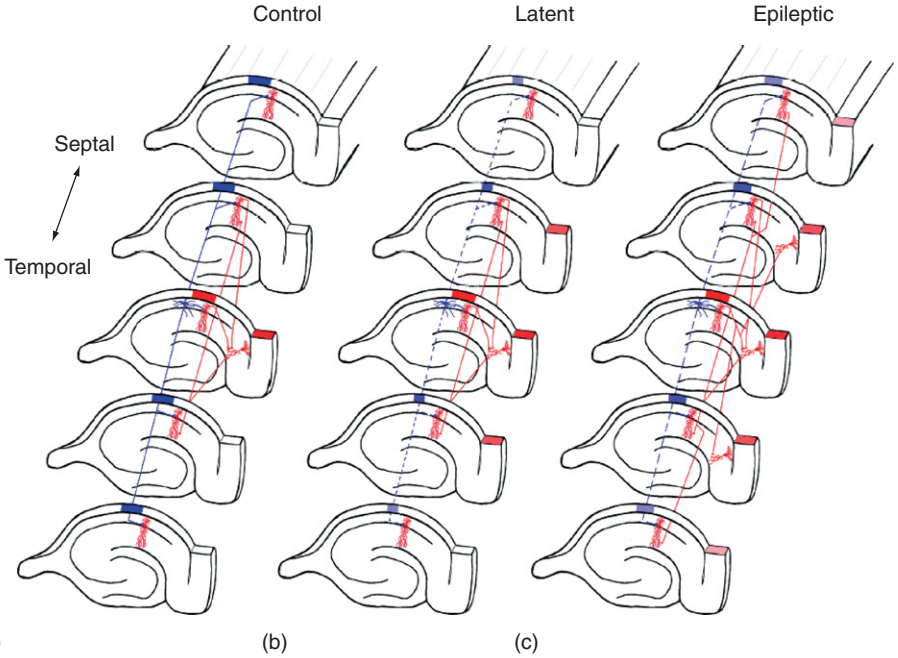


Figure 5 Synaptic reorganization in CA1 projection to the subiculum results in translamellar hyperexcitability in the hippocampal formation. The schematic drawings illustrate normal hippocampal circuitry and abnormal circuitry during the latent state and after development of spontaneous seizures in the kainic acid model of mesial temporal lobe epilepsy. The red neurons and axons are excitatory neurons; the blue neurons are inhibitory interneurons. (a) In the normal hippocampus, activation of the CA1 pyramidal neurons in a lamella results in limited activation of subicular neurons shown also in red, and the CA1 interneurons inhibit CA1 pyramidal neurons from lamellae above and below the activated lamella (shown as a blue block over those lamellae). (b) During the latent state, inhibitory mechanisms are functionally impaired, with slow improvement in the inhibitory tone (perhaps, in part, due to synaptic reorganization of inhibitory pathways). There is a mild degree of disinhibition in lamellae above and below the activated lamella (shown as smaller blue block in those lamellae). Furthermore, mild disinhibition results in a greater degree of activation in subiculum (shown as a greater number of activated subiculum sections). (c) Once spontaneous seizures develop in epilepsy models, there is prominent synaptic reorganization of the CA1 pyramidal axons, making synaptic contacts with additional CA1 pyramidal neurons and subicular neurons in sections (lamellae) above and below their normal projection pattern. The resulting increased recurrent excitatory connectivity between principal neurons in the hippocampus and within hippocampal lamellae results in translamellar sprouting. Modified from Cavazos JE and Cross DJ (2006) The role of synaptic reorganization in mesial temporal lobe epilepsy. *Epilepsy & Behavior* 8: 483–493.

a similar type of reorganization in the immature brain as it is seen in the adult brain in response to excitotoxicity. Therefore, there are significant differences between the immature and the adult brain (when exposed to excitotoxicity and SE) in distribution and extent of hippocampal circuit reorganization. The differences suggest different mechanisms for the development of seizure-induced circuit reorganization in the immature and mature brain.

Future Directions

Neuronal loss induced by excitotoxicity or repeated seizures is widespread in limbic structures. However, synaptic reorganization has been investigated primarily in the mossy fiber pathway (and to a lesser extent in the CA1 projection to the subiculum). It is likely that circuit reorganization occurs in other limbic regions, which need to be systematically studied. The physiological connectivity among these regions also requires further investigation. Such studies should focus on whether the increased connectivity within the epileptic networks (e.g., in the hippocampus) actually provides the basis for increased neuronal synchrony and facilitates the propagation of epileptic discharges beyond the epileptic focus. The mechanisms underlying the formation, development, and permanence of circuit reorganization need further investigation, as they might become targets for disease modification and prevention of intractability for MTLE. Lastly, further analysis of the differences between early life insults and insults during adulthood might provide a window for understanding critical age-dependent mechanisms of plasticity. Circuit reorganization of limbic pathways appears to be a key element in the process of epileptogenesis of MTLE. Finding strategies to prevent, stop, or reverse this process might lead to a cure of MTLE.

Acknowledgements

This review was supported by a grant from NINDS (NS02078) and a Veterans Administration Merit Review grant to JEC.

See also: **Plasticity**: Axon Sprouting in Epilepsy; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; Synaptic Reorganization in the Hippocampal Dentate Gyrus; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy.

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Dendritic Abnormalities in Epilepsy

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Introduction

Studies of dendritic abnormalities in epilepsy have a long history. Microanatomical studies of human epilepsy were first conducted more than 100 years ago, and described a number of dendritic abnormalities, including a marked decrease in the number or even a complete loss of dendritic spines. In the intervening years, these observations have been reproduced by numerous investigators studying both human tissue and tissue from various animal models of epilepsy. Dendrites and their spines are well known to be major sites of excitatory synaptic transmission. Thus, these results generally suggest that this form of synaptic transmission may be significantly compromised in the epileptic brain. However, such a conclusion raises an important paradox in epilepsy research. How can one explain the presence of epilepsy – a state of chronic neuronal/network hyperexcitability – when many of the synapses that mediate excitatory synaptic transmission are presumably absent? One approach to this paradox is to consider the numerous comorbidities associated with many forms of epilepsy – perhaps most prominently cognitive disabilities. Glutamatergic synapses are thought to be sites of memory consolidation and storage, and well-known synaptic plasticity mechanisms – mediated by NMDA and mGluR receptors at these synapses – are thought to participate in important ways in learning and the formation of memories. A significant decrease in the normal complement of glutamatergic synapses in the hippocampus or neocortex, and an associated loss of these 'memory molecules,' would be expected to compromise the ability of a neuron to store memories. Thus, it is important to understand the cellular and molecular events that produce dendritic abnormalities in the epileptic brain and their functional consequences. Only by combining microanatomical and neurophysiological studies with behavioral analyses in animal models, will we be able to determine if the dendritic changes that are so prominent in the epileptic brain contribute to the learning and memory deficits observed. And perhaps only then may we be able to develop an explanation for 'the epileptic dendrite paradox,' where anatomical biomarkers predict decreased neuronal/network excitability despite the presence of epilepsy.

Background

The majority of the studies of dendrites from human brain have been carried out on tissue removed during surgery to control intractable epilepsy, and thus represents effects of persistent partial and intractable seizures on hippocampal and/or neocortical neurons. In animals, experiments have been done primarily in models of partial seizures, where alumina cream or ferric chloride was injected locally into neocortex to produce an epileptic focus, or pilocarpine or kainate was injected systemically to produce prolonged limbic motor seizures. Thus the results that have been reported so far may not apply to generalized epilepsy and may not be observed, for example, in animal models of absence seizures.

Spine loss is only one of the dendritic abnormalities found in epilepsy. In focal or partial epilepsy, decreases in dendritic arbor complexity have been commonly reported, an abnormality that could result from both a decrease in the number of dendritic branches and decreases in the length of individual dendrite segments. However, in most instances, neuronal reconstructions have not been conducted and thus a quantitative analysis has not been performed. Nonetheless, the reported effects appear to be quite dramatic; thus, in addition to spine loss, decreases in dendritic arbor complexity are thought to occur. This type of change would be expected to result in a further reduction in the number of glutamatergic synapses in affected brain regions.

Another dendritic abnormality seen in epilepsy is varicose or nodular swellings along the length of dendrites. This phenomenon gives dendrites a 'string-of-beads' appearance. Such dendritic beading is easily evoked in culture systems by application of glutamate receptor agonists – such as NMDA or kainate. Thus, dendritic beading is thought to be one hallmark of early excitotoxic injury. However, dendritic beading is readily reversible in neuronal cultures, as long as the concentration of the agonists is kept reasonably low and neuronal cell death does not occur. Recently, dendritic beading has also been demonstrated in response to kainate-induced status epilepticus in the intact animal. Again, beading appeared to be transient; after 30 m of status, dendritic beading was quite evident, but beading disappeared 1–2 h after cessation of the seizures.

The relationship between dendritic beading and spine loss (as well as excitotoxic injury) in epilepsy has been the subject of some discussion. Currently, evidence favors the view that these are independent phenomena, although they can occur simultaneously. This view is based on the observation that the time course of beading and spine loss can differ. Some investigators have observed spine loss preceding beading, but others have reported that it follows the formation of these nodular swellings. In the latter case, spines were observed emanating from swellings, and only later did some of the spines disappear. The mechanisms underlying dendritic beading and spine loss are likely to differ. There is evidence from excitotoxicity studies (as well as from epilepsy investigations) that spine loss can be prevented by pharmacologically inhibiting the activity of the calcium and calmodulin-dependent phosphatase, calcineurin. These and other results implicated increases in intracellular calcium in depolymerization of F-actin, which is a major component of the cytoskeleton of dendritic spines. Other experiments, however, have shown that dendritic beading is not calcium-dependent, instead is most likely mediated by the movement of Na^+ and Cl^- intracellularly – with beading the result of water moving intracellularly to maintain osmolarity. In these experiments, replacing Na^+ or Cl^- with impermeant cations or anions (respectively) prevented bead formation. Overall, experimental results support the notion that dendritic beading is a transient process that occurs when neurons receive intense excitatory synaptic input – such as that occurring during status epilepticus. Once seizure activity ceases, the acute changes (e.g., in Na^+ and Cl^- and water) should rapidly dissipate. However, spine loss could be longer lasting; indeed, in the recent study of kainate-induced status epilepticus, most dendritic beads disappeared within 1–2 h but spine loss persisted for at least 24 h – the maximum length of time for which images were obtained.

In summary, over 100 years have passed since dendritic abnormalities in epilepsy were first described. It is remarkable how little progress has been made in understanding the cellular and molecular events that are responsible for these abnormalities. However, in recent years, important experimental tools have been developed that promise to speed discovery in this area of investigation, and thus help unravel the role dendrites play in epilepsy and its cognitive comorbidities. For example, transgenic mice have been created that express a variety of fluorescent proteins in subpopulations of neurons in the brain. The dendrites of these cells can be easily imaged, not only in fixed tissue but also in live preparations – either in vitro or in vivo. These preparations allow investigators to examine the consequences of seizures (or seizurelike activity) on dendrites in exquisite detail. The following section reviews methods commonly used to study dendrite morphology and function.

Methods – Experimental Approaches to the Study of Dendritic Abnormalities

Most studies of human brain tissue have relied on the Golgi technique. Intracellular injections of biocytin have been performed in tissue removed during epilepsy surgery, and biocytin reconstructions have been generated in a number of animal models. The Golgi-stained sections are relatively easy to prepare but the unpredictability of the staining can be problematic, especially when many neurons are stained. The complexity of dendritic branching, and the intermixing of arbors when many cells are stained, make complete reconstructions challenging – particularly when portions of the dendritic arbor are in adjacent sections. The biocytin technique has advantages over the Golgi method, since one cell is usually filled with the dye, and the neurophysiological properties of that neuron are also available for analysis. However, the requirements of electrophysiological equipment and expertise can be a significant barrier for the use of the biocytin technique in some laboratories.

The discovery of green fluorescence protein (GFP), and the creation of numerous derivatives with their own excitation and emission spectra, have revolutionized the study of the morphology of neurons – and in particular, investigations into how seizures may impact on dendrites. Transfection of cells with GFP can be readily achieved using a variety of techniques, including lipofection, electroporation, viral transduction, and biolistic transfer. The latter procedure uses the so called 'gene gun,' whereby microscopic gold particles coated with a plasmid (in the simplest case, the DNA for GFP itself) are shot into cells. This technique is particularly advantageous for slice culture experiments where the morphology of individual neurons can be studied in detail with confocal or two (or multiphoton) microscopy. Dendrite reconstructions can be carried out once slices are fixed or can be done in living neurons. Time-lapse imaging can be employed in living tissue to study the time-dependent effects of seizurelike activity, induced in a variety of ways (e.g., zero magnesium, high potassium, disinhibition, etc.). More recently the Diolistic techniques have been introduced, in which tungsten microparticles are coated with a variety of lipophilic flurochromes like DiI and DiO. When these particles are shot into the tissue, neurons that come in contact with the particles are filled with the dye and reveal the detailed morphological features of cells. Diolistic methods can also be used in lightly fixed brains, where sections can be shot and the morphology of individual cells, studied.

The creation of an ever growing number of transgenic mice that express GFP (or a derivative) in specified neurons of the CNS has made possible more detailed, cell-specific studies of seizure effects (and pathologies associated with many other brain disorders) on neuronal

morphology. Mice that express fluorescent proteins in subpopulations of GABAergic interneurons, hippocampal and neocortical pyramidal cells, and Purkinje cells (among many others) are now readily available. When these mouse strains are crossed with genetic models of CNS disorders, insight into microanatomical abnormalities can become readily accessible. In addition to genetic models of epilepsy, the acquired epilepsies can also be studied in these transgenic mice. For instance, kainate or pilocarpine status epilepticus can be induced and the effects on neocortical pyramidal cells dendrites, studied. Alterations can be evaluated using advanced imaging techniques and quantitatively assessed by dendrite reconstructions in fixed brain tissue. And as mentioned earlier, acute morphological alterations of dendrites produced by seizures can be described in living mice using multiphoton microscopy.

To evaluate microanatomical changes in dendrites, quantitative analysis is most often required. Historically, camera lucida techniques were used for this purpose – where neurons were drawn on paper with the aid of a drawing tube that was attached to a microscope. Data were quantified by manually counting or measuring features of interest from the paper. This procedure has now been replaced by computer systems where morphological features are entered – most often still by tracing – into computer memory. Computer programs then rapidly quantify morphological features of neurons. More recently, morphological studies have generated stacks of confocal images, which are imported into computer programs; neuronal processes can be traced on the computer monitor, as one follows their course while stepping through a series of images acquired in the z axis. Large data sets are generated relatively rapidly by these methods and numerous graphical and statistical algorithms have been developed to further analyze results.

As in all areas of the neurosciences, anatomical data can only make suggestions about the functional consequences of the observed changes. Neurophysiological recordings from effected neurons – and when possible, from dendrites themselves – will ultimately lead to a clearer understanding of the consequences seizures have on neuronal function and physiological properties of dendrites. Behavioral studies will provide insights into the role dendritic abnormalities play in cognitive deficits. Molecular studies will be required to ultimately understand how seizures produce their effects.

Results – The Impact of Seizures on Developing Dendrites

As mentioned earlier, cognitive deficits – including mental retardation – are comorbidities commonly associated with severe childhood epilepsy. Such comorbidities are particularly problematic in the catastrophic epilepsies of childhood. The frequent seizures that characterize these conditions have often been implicated as contributors to cognitive disabilities. However, since many of these children have a preexisting neuropathology, and are also treated with multidrug anticonvulsant therapies, it has been difficult to establish a clear causal relationship between recurrent seizures and deficits in learning and memory. Studies in a variety of animal models have attempted to address the relationship between early-life seizures and cognition. Results have uniformly shown that brief but recurring seizures in early-life can produce spatial learning deficits. One of the models used for this purpose was the tetanus toxin model, which is characterized by frequent brief seizures during the second to third week of postnatal life in rats. As adults, these animals have marked spatial learning deficits but neuronal cell death is not apparent in the hippocampus. However, consistent with results reviewed earlier, the dendrites of hippocampal pyramidal cells from these animals show a marked loss of dendritic spines and decreases in the number of branch points in basilar dendrites. Biochemical studies of the flurothyl model, another model of early-life seizures, have reported marked decreases in the expression of proteins primarily expressed postsynaptically in dendrites. Similar results have been obtained from slice cultures where $GABA_A$ receptor antagonists have been used to induce epileptiform activity. Additional experiments have shown that these effects in slices were completely suppressed by NMDA receptor antagonists.

The mechanisms underlying these effects on dendrites in developing hippocampus have been the subject of discussion and speculation. It is conceivable that the changes observed in dendrite spine density and branching complexity could be the result of excitotoxicity in dendrites. As described earlier, application of glutamate receptor agonists to neurons in culture produces beading of dendrites and spine loss (although these two effects are likely mediated by different molecular mechanisms). Similar effects have been reported in vivo in adult rodents during status epilepticus. Thus, one possibility is that the dendritic abnormalities observed in vivo and in vitro developmental epilepsy models are the product of dendrotoxicity – a sublethal impact of massive amounts of glutamate released during seizures on dendrites. However, dendritic beading – a surrogate marker for dendrotoxicity – has not been demonstrated in developing neurons subjected to brief/recurring seizures activity. Alternatively, differences in spine density and dendritic arbor complexity, observed between control and neurons that have undergone early-life seizures, might be explained by seizure-induced suppression of dendrite growth. Recent studies from our laboratory support such a hypothesis. Here, recurring epileptiform activity was induced in hippocampal slice cultures prepared from

1 week old YFP-H mice. These mice express yellow fluorescent protein (YFP) in a subset of CA1 pyramidal cells. Figure 1 summarizes results from a set of experiments. Reconstructions of basilar dendrites over a 4 day period (3–7 days in vitro-DIV) showed that during this time, dendrites undergo rapid growth; new branches are added and the length of individual segments increases. However, when slices were treated with the $GABA_A$ receptor antagonist bicuculline (or high potassium) to induce epileptiform discharging, the growth of dendrites observed in sister cultures was markedly suppressed. This result was further shown to be an NMDA receptor-dependent process.

Challenges for the Future

Based on these results, experiments in vivo are needed to verify that recurrent seizures in infant rodents produce similar results. Such a demonstration would open up

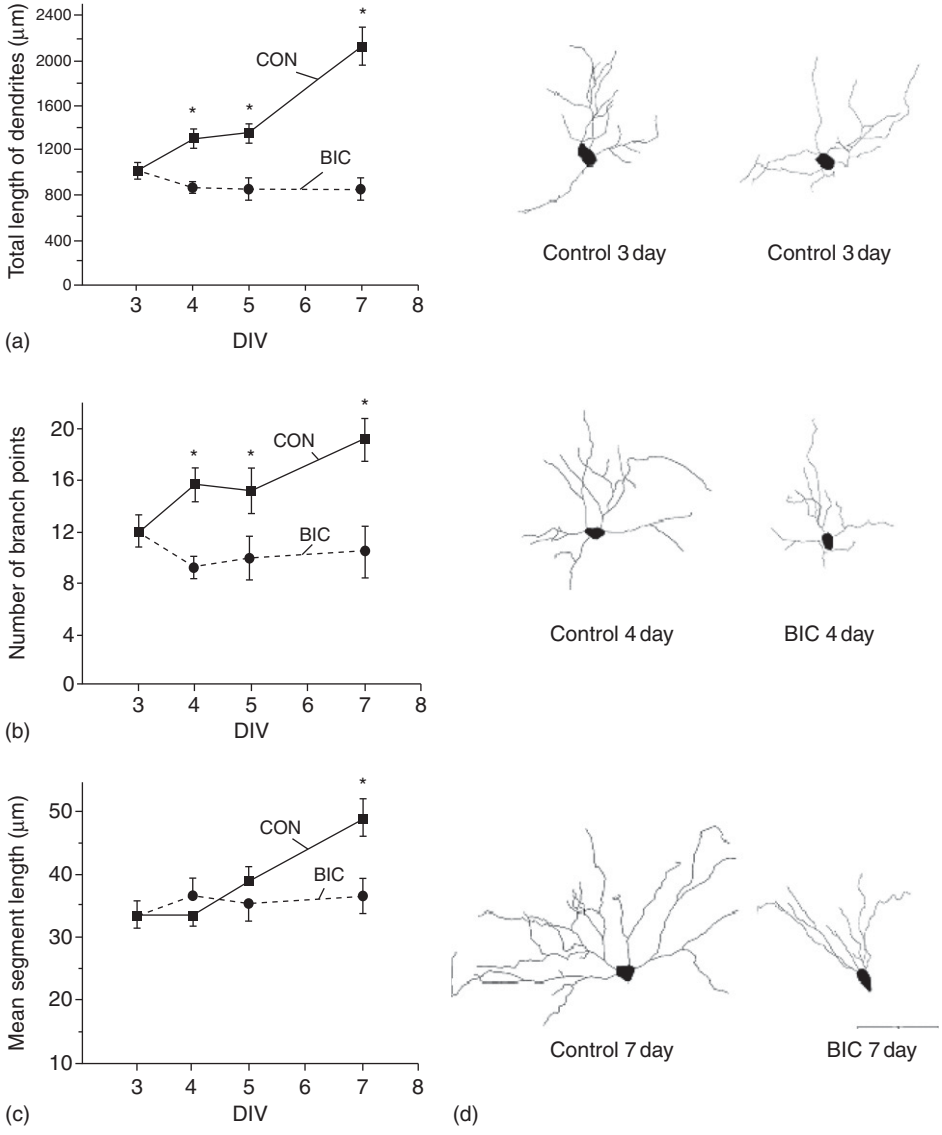


Figure 1 Chronic disinhibition suppresses the growth of CA1 hippocampal pyramidal cell dendrites. (a–c) Between 3 and 7 days in vitro (DIV), the basilar dendrites of CA1 pyramidal cells in control slices gradually increased in: total length, number of branch points and mean segment length. However in bicuculline treated slices, dendrites did not increase in length or branching complexity. (d) Representative Neurolucida reconstructions of dendrites of YFP positive CA1 pyramidal neurons at selected times in vitro. The soma and basilar dendrites of these cells are shown. Reproduced from Nishimura M, Owens J, and Swann JW (2008) Effects of chronic network hyperexcitability on the growth of hippocampal dendrites. *Neurobiology of Diseases* 29: 267–277, with permission from Elsevier.

many new avenues of investigations into the molecular mechanisms that are responsible for growth suppression. Indeed, in recent years a number of important molecular signaling pathways have been identified as important players in normal dendrite growth. Some of these pathways are directly regulated by neuronal activity. Alterations in one or more of these pathways would be a candidate mechanism for seizure-induced dendritic growth suppression. Normally, neuronal activity is thought to promote dendrite growth in early life; based on this hypothesis, one would think that the hyperexcitability of recurring seizures would result in an accelerated growth or even overgrowth of dendrites. However, such accelerated growth is not observed, either clinically or in laboratory studies. Instead, one might speculate that growth suppression (if it really occurs in children with epilepsy) is a compensatory and neuroprotective response of growing/developing neurons to abnormally high levels of neuronal activity.

During development dendrites eventually stop or greatly decrease their rate of growth. 'Stop growing signals' are likely produced to shut off molecular developmental programs mediating dendrite growth. Levels of neuronal activity are likely to be one of a number of environmental and genetic cues integrated by neurons to signal the end of the rapid phase of dendrite growth. If so, it is easy to envision how excessive neuronal activity that characterize seizures could produce a premature induction of a stop growing signal. Such a process could also be viewed as neuroprotective, since decreasing dendrite arbor length and complexity should result in a decrease in the number of excitatory synapses onto pyramidal neurons, which would in turn result in diminished neuronal and network excitability – something that might be advantageous in intractable childhood epilepsy. However, another effect of such neuroprotection would be a decrease in the complement of glutamatergic synapses (and their molecular machines) that are thought to be critical contributors to learning and the storage of memories.

We are at the early stages of understanding the impact seizures have on dendrites in both the mature and immature nervous systems, as well as the contributions these changes make to epileptogenesis and coexisting cognitive and psychiatric difficulties. However, many new experimental tools have become available in just the past five years, and new methods and techniques in molecular biology, genetics, and imaging are being added at impressive rates. Many of these new approaches should be directly applicable to epilepsy research and promise to provide interesting answers to many long-standing questions.

Acknowledgements

The work of our laboratory has been supported by grants from NIH (NINDS) the Partnership for Pediatric Epilepsy Research and an Epilepsy Foundation Fellowship to MN.

See also: **Cell Damage/Excitotoxicity**: Excitotoxicity and Neurodegenerative Disease; **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; **Models**: The Tetanus Toxin Model of Temporal Lobe Epilepsy; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; **Plasticity**: Seizure-Related Changes in Dendritic Spine Structure; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Inositol Lipid Signaling in Synaptic Activity, Neuronal Plasticity and Epileptogenesis

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Introduction

Excessive glutamatergic neurotransmission is a major factor underlying the pathophysiology of seizures and of epilepsy. The use of ionotropic glutamate receptor antagonists as potential antiepileptic drugs has shown therapeutic potential in animal models of epilepsy, but this strategy has failed in early clinical trials. Recent studies have suggested that the modulation of metabotropic glutamate receptors (in addition to drug effects at glutamatergic ionotropic receptors) could ameliorate seizures. Specifically, metabotropic glutamate (mGlu) receptor (possibly $mGlu_5$) agonists (as accessed in basolateral amygdala neurons from brain slices of kindled rats) activate the Na^+–Ca^{2+} exchanger, suggesting that the mechanism for removal of the excess intracellular Ca^{2+} occurs during periods of high glutamate release such as seizures. mGluR1 and mGluR5, both linked to phosphoinositide hydrolysis, show perturbations in mRNA expression in hippocampus of amygdala-kindled animals, studied at different time points after the last generalized seizure (stage 5 from Racine's score). Inositol hydrolysis leads to sustained increase in protein kinase C activity following amygdala kindling, suggesting that these agonists trigger inositol lipid hydrolysis, and enhance protein kinase C activity during early stage of epileptogenesis. Given these results, there is an interesting potential for ameliorating seizures with the combination of mGlu receptor ligands with other glutamatergic (and non-glutamatergic) agents.

The exploration of second-messenger signaling, mediated through activation of mGlu receptors, will expand our understanding of the modulation of seizures and epileptogenesis. mGlu receptor activation is correlated with an increase of intracellular diacylglycerol (DAG) because of hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (**Figure 1**, wild type). The molecular species 1-stearoyl-2-arachidonoyl-*sn*-glycerol comprises 80% of brain PIP_2. Arachidonoyldiacylglycerol (AA-DAG) is a second messenger derived from phosphatidylinositol 4,5-bisphosphate and generated by stimulation of glutamate metabotropic receptors linked to G protein and activation of phospholipase C. AA-DAG signaling is terminated by its phosphorylation to phosphatidic acid, catalyzed by diacylglycerol kinase (DGK). The significance of DGK family in synaptic function has been investigated in mice with targeted disruption of the DGKε gene. $DGK\varepsilon^{-/-}$ mice showed a higher resistance to electroconvulsive shock, with shorter tonic seizures and faster recovery than do $DGK\varepsilon^{+/+}$ mice. The phosphatidylinositol 4,5-bisphosphate-signaling pathway in cerebral cortex was greatly affected by the DGKε gene deletion, leading to lower accumulation of AA-DAG and free AA. Also,

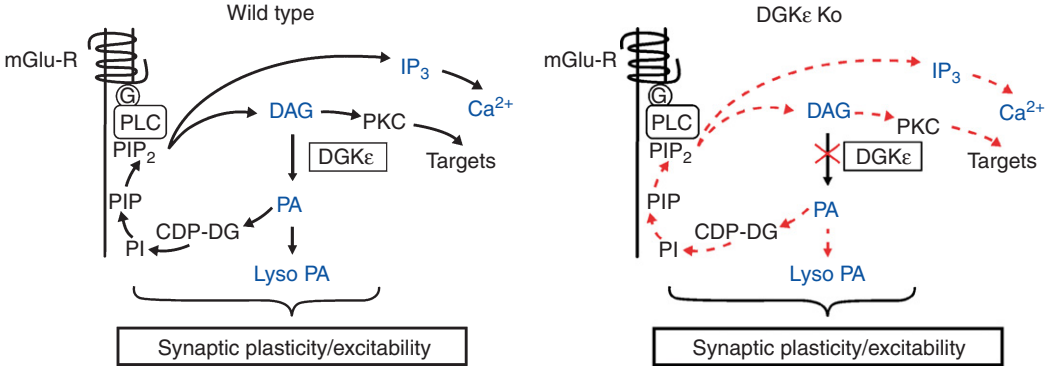


Figure 1 Diagram depicting seizure-induced inositol lipid signaling in wild-type and DGKε deficient mice (DGKε KO). In wild-type mice, metabotropic glutamate receptor interacts with G proteins, which in turn activate PLC, resulting in the hydrolysis of PIP_2 that generates DAG and IP_3. DGKε is a lipid kinase that modulates multiple signaling functions related to epileptogenesis, such as synaptic plasticity and excitability. DGKε catalyze the phosphorylation of DAG to PA. Inositol cycle is interrupted leading to decreased availability of bioactive molecules: DAG, PKC, IP3, CA2, PA and lyso-PA. PI phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5 phosphate; PLC, phospholipase C; G, G protein; IP_3, inositol 1,4,5 phosphate; CDP-DG, cytidine diphosphate-diacylglycerol; PA, phosphatidic acid; lyso-PA, lyso-phosphatidic acid.

long-term potentiation (LTP) and kindling epileptogenesis were attenuated in these DGKε$^{-/-}$ mice. On the basis of these studies, we propose that DGKε contributes to modulate neuronal signaling pathways linked to synaptic activity, neuronal plasticity, and epileptogenesis.

Background

Inositol lipid-derived diacylglycerols (DAGs) are generated by the excitatory neurotransmitter glutamate through the activation of phospholipase C (PLC). Activation of metabotropic receptors (mGluRs), coupled to G proteins and PLC, increases DAG concentration because of the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). A subsequent increase in DAG pool size follows as a result of phospholipase D (PLD)-catalyzed hydrolysis of phosphatidylcholine to phosphatidic acid (PA) and its conversion to DAG. The early and late DAG peaks are temporally dissociated, and their fatty acid composition is different. Inositol phospholipids are enriched in arachidonate at the sn2 position of the glycerol backbone, whereas in phosphatidylcholine this position is occupied mainly by monounsaturated fatty acid chains (i.e., oleic acid, 18:1n-9).

AA-DAG activates protein kinase C (PKC) in vitro and plays second messenger functions in vivo. Moreover, there is evidence that targets of DAG can distinguish between different DAG species. Thus, the distinct fatty acid composition of inositol phospholipids in neuronal cell signaling may be important for the maintenance of cellular functional integrity. Enzymes responsible for inositol lipid synthesis selectively use AA-containing lipids as substrates. DAG kinase ε (DGKε), which selectively phosphorylates AA-DAG to generate PA, is one candidate for this selective function. Eight other mammalian DGKs, with no significant selectivity, have been identified.

Activation of inositide lipid signaling through metabotropic glutamate receptor (mGluR) and PKC is involved in synaptic plasticity, learning, memory, LTP, and long-term depression (LTD). Moreover, alterations of this signaling have been implicated in epilepsy, Alzheimer's disease, and depression. To explore the mechanisms underlying metabotropic glutamate-mediated lipid signaling in epilepsy, the targeted deletion of the DGKε gene was explored as an effective approach to determine whether DGKε contributes to arachidonate enrichment – and to determine the functional consequences of DGKε gene deletion with respect to seizure responses and LTP. The AA-DAG signal generated from mGluR-activated PIP_2-PLC pathway is switched off by DGKε that generates PA. The PA, in turn, is used for the synthesis of phosphatidylinositol and the replenishment of the precursor by phosphorylations to phosphatidylinositol 4-phosphate (PIP) and PIP_2.

Methods

Electroconvulsive Shock (ECS)

Two- to three-month-old wild-type, heterozygous (+/−), and homozygous (−/−) DGKε mice (20–25 g, male/female) were acutely implanted with two platinum electrodes under the scalp (parallel, 1 cm apart) and then stimulated. A single stimulation train of square pulses was delivered at 50 V DC and 0.5-ms pulse duration at 100 Hz; train duration was 200 ms, and train rate was 0.750 per second.

Lipid Analysis

Mice were killed by high-frequency, head-focused, microwave irradiation, their heads were cooled quickly in ice-cold water, and the brains were dissected. Lipids from the right cerebral cortex were extracted with hexane/isopropanol, 3:2 (vol/vol), for the analysis of free fatty acids (FFA) and DAG. Polyphosphoinositides (PPI) were extracted from the left cerebral cortex with acidified chloroform/methanol. Lipid classes by TLC were isolated and their acyl groups were analyzed by gas liquid chromatography.

Electrophysiological Recordings

Hippocampal slices were prepared from DGK-deficient mice (either sex) and age-matched controls. Whole-cell patch–clamp recordings were made with an Axoclamp-2B patch–clamp amplifier in bridge mode. Excitatory postsynaptic potentials (EPSPs) in response to stimulation of the perforant path (at a frequency of 0.05 Hz) were recorded, and the amplitude range of the evoked EPSPs was adjusted to 2–6 mV (<30% threshold for generating an action potential). LTP in the perforant path was induced by high-frequency stimulation (HFS). HFS consists of eight trains, each of eight pulses at 200 Hz, with an intertrain interval of 2 s. LTP was operationally defined as >20% increase above baseline for the amplitude of ESPSs from 26 to 30 min after HFS.

Rapid Kindling Epileptogenesis

Bipolar electrodes were implanted in the right dorsal hippocampus of male DGKε$^{+/+}$ and DGKε$^{-/-}$ mice. Ten days post surgery, kindling was achieved by stimulating six times daily for 4 days with a subconvulsive electrical stimulation protocol (10-s trains, of 50-Hz biphasic pulses, 75–200-μA amplitude) and a 30-min intertrain interval. After 1 week, mice were rekindled. Hippocampal evoked responses immediately after subconvulsive stimulations were recorded and analyzed to characterize epileptogenic events, such as spike, sharp waves, or abnormal amplitude and rhythms.

Histology

The right hippocampus was analyzed by histological and immunocytochemical techniques (Timm staining, NPY, and GFAP immunoreactivity), and using TUNEL staining for DNA fragmentation, following provider protocols.

RNA Isolation and Quality Control

Left and right hippocampal total RNA was isolated using Trizol reagent (Invitrogen Life Sciences, Carlsbad, CA) and RNA quality was screened and quality controlled using RNA LabChips (Caliper Technologies, Mountain View, CA). Each total RNA sample was scanned from 220 to 320 nm using a GeneSpec III spectrophotometer (Hitachi Genetics, Alameda, CA) and the $A_{260/280}$ of total RNA (based on peak area) was typically >1.8. RNA samples were next analyzed for spectral purity and concentration (ng μl^{-1}) using RNA Nano LabChip analysis Chips and a 2100 Bioanalyzer (Caliper Technologies, Mountainview, CA; Agilent Technologies, Palo Alto, CA).

RNA Labeling and DNA-Array Analysis

Total RNA was labeled using the Enzo Bioarray High Yield RNA Transcript Labeling system (Affymetrix, Santa Clara, CA), hybridized to the Test3 and analytical GeneChips, washed, and scanned.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analysis was performed with the unpaired Student's *t* test. One-way ANOVA with Fisher's PLSD post hoc was used for statistical comparison when appropriate. Statistical analysis of the frequency of the behavioral responses to ECS was carried out with the χ^2 test. Differences were considered significant when $P < 0.05$.

Recent Results

DGKε Expression in Mice

Murine DGKε displayed similar selectivity for AA-DAG as human DGKε; thus, the enzymatic selectivity of DGKε seems to be conserved in mammals. This AA-DAG-specific DGK may be significant in terminating signals transduced through AA-DAG generated in the PIP_2 cycle. However, the tissue distribution patterns of murine and human DGKε are different, as demonstrated by Northern blotting; murine DGKε is primarily expressed in brain, but human DGKε is predominantly expressed in testis. This difference may reflect the involvement of murine and human DGKε in different signaling pathways. The cellular distribution of murine DGKε in brain, as seen with in situ hybridization, was similar to that of the mGluR1 in cerebellar Purkinje cells, mitral cells of the olfactory bulb, hippocampal interneurons, and neurons of the thalamus and substantia nigra. G protein-coupled mGluR1 leads to PIP_2 hydrolysis catalyzed by PLC and the subsequent activation of PKCβ.

Downregulation of AA–Inositol Lipid Signaling and Resistance to Seizures

Since ECS and ischemia activate the PLC-mediated release of AA-DAG, and since DGKε terminates AA-DAG signals, we investigated whether deficiency of DGKε (in the mouse knockout) would affect the seizure response to ECS. In fact, male and female DGKε-deficient mice were more resistant to ECS, displaying shorter tonic seizures and faster recovery than do $DGK\varepsilon^{+/+}$ mice. This behavioral response was paralleled by decreased degradation of brain PIP_2 and decreased accumulation of DAG and FFA after ECS (in DGKε-deficient mice compared with wild-type controls). Moreover, DAG returned to basal levels within 1 min after ECS in $DGK\varepsilon^{-/-}$ mice, but a more sustained accumulation was observed in $DGK\varepsilon^{+/+}$ mice (**Figures 2** and **3**).

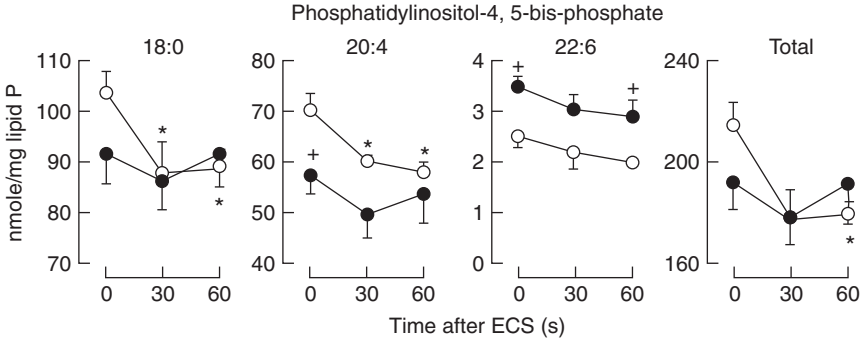


Figure 2 ECS-induced degradation of PIP_2 is decreased in the $DGK\varepsilon^{-/-}$ mice. Mean values ± SEM are shown ($n = 4$–6). Statistically significant differences (Student's *t* test, $P < 0.05$) are indicated: *, with respect to control (0 time); +, $DGK\varepsilon^{-/-}$ mice (●) vs. $DGK\varepsilon^{+/+}$ mice (○). Modified from Rodriguez de Turco EB, Tan W, Tophan MK, *et al*. (2001) Diacylglycerol kinase epsilon regulates seizures susceptibility and long term potentiation through inositol lipid signaling. *Proceedings of the National Academy of Sciences USA* 98: 4740–4745, with permission from National Academy of Sciences.

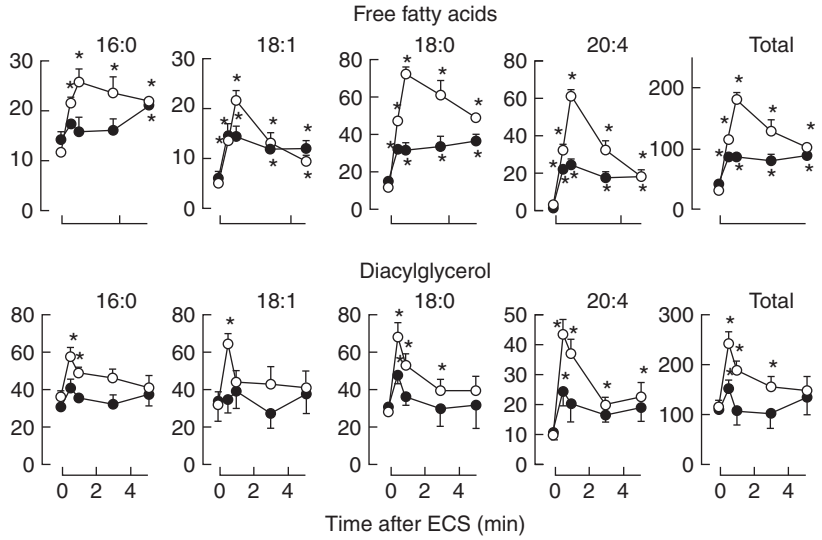


Figure 3 ECS-induced accumulation of FFA and DAG in wild-type (○) and DGKε$^{-/-}$ mice (●). Mean value ± SEM are shown. Basal values (time 0) are the average of 16 samples. Other time points are from 5–8 individual samples. * denotes values significantly different from basal ($P < 0.05$, Student's *t* test). Modified from Rodriguez de Turco EB, Tan W, Tophan MK, *et al*. (2001) Diacylglycerol kinase epsilon regulates seizures susceptibility and long term potentiation through inositol lipid signaling. *Proceedings of the National Academy of Sciences USA* 98: 4740–4745, with permission from National Academy of Sciences.

Although resting levels of PIP and PIP_2 were similar in cerebral cortex from DGKε$^{-/-}$ and DGKε$^{+/+}$ mice, some differences were detected. Neuronal PPI is maintained by de novo synthesis via PA, whereas the DAG–DGKε pathway contributes to their resynthesis after synaptic activity-induced PIP_2 degradation. Activation of the mGluRs regulates the operation of this cycle through PLC. In DGKε$^{-/-}$ mice, only AA-PIP_2 displayed decreased resting levels. Therefore, despite the deficiency in DGKε selective for AA-DAG phosphorylation, other DAG kinase(s) and/or the de novo synthesis pathway may partly compensate to generate AA-PA (that is then channeled to inositol lipids) in DGKε$^{-/-}$ mice. Moreover, DGKε$^{-/-}$ mice did not show enrichment in AA-DAG, and only 31% of the animals displayed higher resting levels of total DAG as compared with DGKε$^{+/+}$ mice. It is likely that the resting AA-DAG pool of the PPI pathway is very small compared with the pool of AA-DAG linked to the turnover of other phospholipids, and therefore changes in this pool may be masked within the total DAG pool.

The ECS-induced 18:0- and AA-DAG accumulation in the cerebral cortex, at 30 s post-ECS, was lower in DGKε$^{-/-}$ mice than in DGKε$^{+/+}$ mice. However, removal of DAG showed similar kinetics in both DGKε$^{-/-}$ and DGKε$^{+/+}$ mice, suggesting that even after stimulation, other DGK(s) may compensate for the DGKε deficiency. The DAG pathway is terminated by DGK through its phosphorylation to PA, and/or by DAG lipases with the generation of FFA. However, the rapid removal of DAG in DGKε$^{-/-}$ mice was not paralleled by a further increase in FFA, supporting the notion that DGKs other than DGKε, rather than DAG lipases, are involved in the efficient removal of DAG after ECS in DGKε$^{-/-}$ mice (**Figure 3**).

Interestingly, DGKε$^{-/-}$ mice displayed very low PIP_2 degradation after ECS, suggesting impairments in mGluR function linked to G proteins and PLC activation. It is possible that to cope with the deficiency of the DGKε, adaptive and/or compensatory changes are developed, including a persistent PKC binding to the membrane in close association with the mGluR–G protein–PLC complex, where AA-DAG accumulates. Because PKC controls the PPI cycle by feedback inhibition of PLC, this sustained translocation would inhibit the PPI–PLC signaling, as reflected in the low ECS-induced AA-DAG release.

In DGKε$^{-/-}$ mice, a significant decrease of 18:0- and AA-PIP (not observed in DGKε$^{+/+}$ mice) took place after ECS. Because stimulation activates degradation and resynthesis of inositol lipids, the levels of PIP and PIP_2 reflect the balance of these two pathways. In DGKε$^{+/+}$ mice, degradation of PIP_2 occurs at a faster rate than its replenishment from PIP by PIP 4-phosphate 5-kinase, while PIP is being replenished through the DAG–PA–phosphatidylinositol pathway. This results in a decrease of PIP_2 and no detectable changes in PIP. In DGKε$^{-/-}$ mice, the decrease observed in PIP may indicate its active phosphorylation to PIP_2 and its slower replenishment from the DAG–DGKε$^{-/-}$ pathway. Because type I PIP 4-phosphate 5-kinase is highly expressed in the brain and greatly stimulated by PA, deficiency of DGKε will likely not force PIP_2 resynthesis. Therefore, other metabolic dysfunctions should be considered in DGKε deficiency, including PIP degradation by PLC and/or its dephosphorylation to phosphatidylinositol.

In DGKε$^{+/+}$ mice, there was also accumulation of 16:0- and 18:1-DAG after ECS, while levels of 16:0- and 18:1-PIP_2 remained unchanged (data not shown). These changes are consistent with degradation of other phospholipids through a PLD pathway that contributes to the sustained accumulation of DAG. In DGKε$^{-/-}$ mice, however, no change in 16:0- and 18:1-DAG was observed. Thus, the disruption of the inositol lipid cycle, and the consequent lack of DAG-stimulated PKC-mediated activation of PLD, may lead to the loss of PLD response to stimulation. These results suggest impairments in multiple signaling pathway response to ECS in DGKε$^{-/-}$ mice that may contribute to the observed faster recovery of DGKε$^{-/-}$ mice from tonic–clonic seizures. The magnitude of free AA and other FFA accumulation after ECS in DGKε$^{-/-}$ mice (compared with DGKε$^{+/+}$ mice) reveal potential alterations in the calcium-dependent $cPLA_2$ pathway. Degradation of 18:0- and AA-DAG generated from PIP_2 after ECS may contribute to the FFA pool. However, the release of AA after ECS, when PIP_2 levels start to recover, implicates the activation of $cPLA_2$ targeting with high selectivity of AA-phospholipids.

NMDA Receptor Activation Leads to Calcium Influx and $cPLA_2$ Activation

The intracellular mobilization of calcium by IP_3 also contributes to sustained activation of $cPLA_2$. The $cPLA_2$ signaling has a profound impact in responses to stimulation because AA by itself, and eicosanoids generated by COX-1–COX-2, are involved in the modulation of synaptic activity. Moreover, $cPLA_2$ generates lyso-PAF, the precursor of PAF, a neuromodulator of LTP that stimulates glutamate release from presynaptic terminals and activates transcription of genes. In cultures of hippocampal neurons from DGKε$^{-/-}$ mice, glutamate stimulation resulted in lower accumulation of intracellular Ca^{2+} compared with cells from DGKε$^{+/+}$ mice (unpublished results). In this context, it is relevant that sustained increase in intracellular Ca^{2+} appears to be required for $cPLA_2$ translocation to the membrane and full enzymatic activity. Decreased activity of $cPLA_2$, and consequently lower release of AA and PAF production in DGKε$^{-/-}$ mice, is also supported by the observation that LTP is attenuated in perforant path–dentate granular cell synapses.

In summary, changes in lipid messengers generated in the brain after one ECS are complex and suggestive of alterations in different signaling pathways (i.e., PLA_2, PLC), related to glutamate receptors activation. Knockout mice with a deficiency in DGKε have helped to explore the roles of metabotropic glutamate receptor–inositol lipid signaling. The lack of the DGKε blocks the availability of several lipid mediators (DAG, PA, lyso-PA) and other signaling molecules (IP3, Ca, PKC) during seizures, which likely are involved in epileptogenesis (**Figure 1**).

HFS-Induced LTP is Reduced in DGKε-Deficient Mice

Synaptic transmission and plasticity were examined in dentate granular cells of DGKε-deficient mice. There were no abnormalities in basic membrane properties, including resting membrane potential, input resistance, and action potential generation in cells in hippocampal slices from enzyme-deficient mice. However, the potentiation of EPSP amplitude by high-frequency stimulation (HFS) was significantly reduced in cells from DGKε$^{-/-}$ mice (potentiation, mean ± SEM, 135 ± 11% of baseline from 26 to 30 min after HFS) when compared with that in the DGKε$^{+/+}$ mice (224 ± 23%). These DGKε$^{-/-}$ mice displayed lower release of AA-DAG induced by ECS, suggestive of lower $cPLA_2$ activity necessary for PAF synthesis. Since PAF enhance neuronal excitability, the attenuation of LTP seen in DGKε$^{-/-}$ mice may be the consequence of diminished PAF synthesis. However, low production of other AA–inositol lipid-derived signaling molecules also may contribute to alterations in synaptic plasticity. Thus, alterations in the AA–inositol lipid cycle take place in limbic structures that display high DGKε expression and may contribute to synaptic dysfunction and pathology.

Diacylglycerol Kinase Epsilon Modulates Kindling Epileptogenesis

In a rapid kindling paradigm, DGKε$^{-/-}$ mice had significantly fewer motor seizure and epileptic events compared with DGKε$^{+/+}$ mice from the second day of stimulation (**Figure 4**). These differences were maintained during rekindling. DGKε$^{-/-}$ mice also exhibited lower-amplitude spike-wave complexes, shorter spreading depression events, and predominant lower-frequency (1–4 Hz) EEG bands throughout stimulation (compared with DGKε$^{+/+}$ mice), as determined by power spectral analysis. Further, DGKε$^{-/-}$ mice displayed no sprouting in the supragranular area or NPY immunoreactivity in the hilus, and had weak astrocyte reactivation in all hippocampal areas. No TUNEL-positive cells were detected in any group of mice.

DGKε modulates kindling epileptogenesis through inositol lipid signaling. Arachidonate-containing diacylglycerol phosphorylation to phosphatidic acid is selectively blocked in DGKε$^{-/-}$ mice. This finding suggests that the shortage of arachidonoyl-moiety inositol lipids, and/or the messengers derived from there, is a key signaling event in plasticity during epileptogenesis.

Diacylglycerol Kinase Epsilon Deficiency Upregulates COX-2

Gene-expression patterns can be effectively displayed by plotting the data as 'volcano plot' (V-plot) representations.

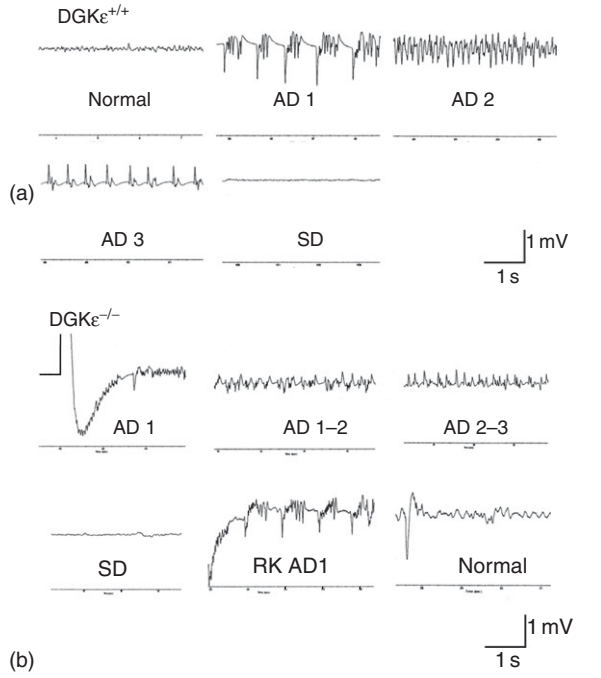


Figure 4 (a) Distinctive EEG patterns of afterdischrage (AD) obtained from DGKε$^{+/+}$ mice during stages 4–5. Repetitive, synchronized bursts of high-amplitude spikes followed by polyspikes-waves were observed immediately after the stimulation (AD1); continuing this pattern, repetitive spikes (AD2) and spike-wave complexes (AD3), with opposite polarization compared with AD1, were observed. A spreading depression was observed at the end of each AD. (b) In contrast, DGKε$^{-/-}$ mice had few spikes and merged AD1–AD2 profiles or merged AD2–AD3 profiles (AD 1–2; AD 2–3), which were observed only sporadically in the rekindling session. The spreading depression was shorter or became a normal EEG after the last spike. Modified from Musto A and Bazan NG (2006) Diacylglycerol kinase epsilon modulates rapid kindling epileptogenesis. *Epilepsia* 47: 267–276, with permission from Blackwell Publishing.

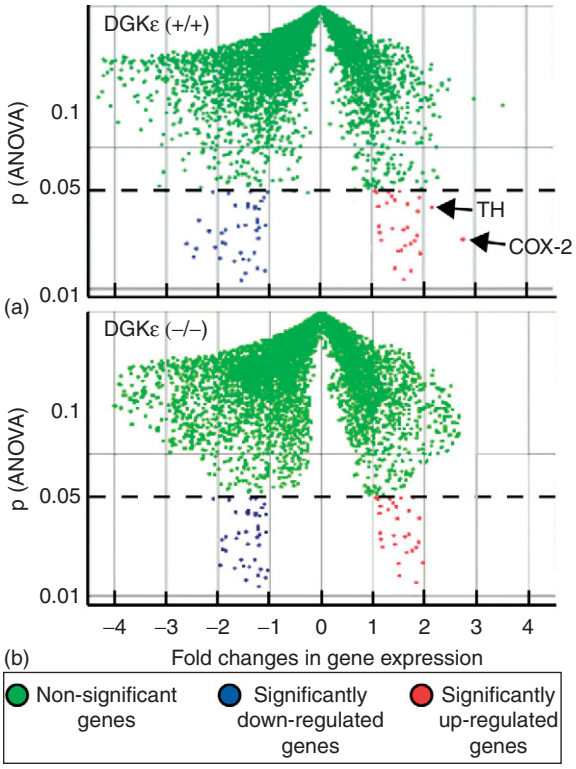


Figure 5 Volcano plots of (a) kindled DGKε$^{+/+}$ vs. control DGKε$^{+/+}$ mice and (b) kindled DGKε$^{-/-}$ vs. control DGKε$^{-/-}$ mice in whole hippocampus. Arrows point to upregulated oxygenase-encoding genes TH and COX-2; for clarity the data of nonsignificant genes in the region $P \leq 0.05$ (dashed horizontal line) and fold-changes ≤ 1.0 are not shown. Modified from Lukiw WJ, Cui JG, Musto AE, Musto BC, and Bazan NG (2005) Epileptogenesis in diacylglycerol kinase epsilon deficiency up-regulates COX-2 and tyrosine hydroxylase in hippocampus. *Biochemical and Biophysical Research Communications* 338: 77–81, with permission from Elsevier.

Using the experimental cut-off of $P \leq 0.05$ and changes greater than or equal to twofold (either up- or down-regulated), we found that changes in only four genes achieved significance in comparing kindling vs. control conditions in DGKε$^{+/+}$ animals; two of these genes encoded the oxygenases COX-2 and tyrosine hydroxylase (TH). Interestingly, animals kindled with electrodes in the right dorsal hippocampus showed higher levels of both COX-2 and TH in the ipsilateral (left) hippocampus, when compared against βTUBIII RNA levels in the same sample; no changes in the levels of the 'housekeeping' genes βTUBIII or COX-1 RNA were noted in either the left or right hippocampus after kindling (**Figure 5**).

Future Goals

Arachidonic acid and AA-DG mediators should be further studied as modulators of synaptic plasticity and seizure susceptibility. Exploration of these signaling pathways, specifically their downstream targets (e.g., protein kinases and phosphatases, transcriptional factors, ion channels, receptors, adhesion proteins) and the organization of pathways at the subcellular level in both neurons and astrocytes, is likely to contribute critical mechanisms relevant to neuronal hyperexcitability.

Damage and death of neurons and astrocytes may occur as consequences of an imbalance of neuronal signaling pathways activated by excessive excitatory and/or deficient inhibitory stimulation. However, if the seizures do not result in cell death, but instead cause functional damage as a consequence of aberrant signaling – e.g., over-stimulation of metabotropic glutamate receptors and subsequent inositol lipid signaling – the resulting processes (even those associated with epileptogenesis) could trigger homeostatic or neuroprotective signaling. The identification of the pathways and targets of inositol lipid signaling could, therefore, contribute to the identification of novel potential therapeutic interventions.

Acknowledgements

Supported by National Institutes of Health, National Center for Research Resources grant P20 RR016816 and the NIH, National Institute of Neurological Disorders and Stroke grant R01NS23002.

See also: **Glutamate**: Metabotropic Kainate Receptor Regulation of Pyramidal Cell Excitability; Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; **Metabolic Changes**: Cell Signaling by Polyunsaturated Fatty Acids in Epileptogenesis; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures.

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LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy

H L Grabenstatter, J Ockuly, and T P Sutula, University of Wisconsin, Madison, WI, USA

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Introduction and Background

Kindling is a robust form of seizure-induced plasticity that refers to the induction of progressive, permanent increased susceptibility to seizures in response to repeated seizures. The phenomenon of kindling was recognized by Graham Goddard during experiments investigating the effects of electrical stimulation of neural pathways on learning paradigms in rats. These experiments unexpectedly revealed that repeated periodic trains of electrical stimulation – evoking seizures – induced progressive permanent increases in susceptibility to additional seizures. He referred to this phenomenon as 'kindling.' Several years later, Bliss and Lomo reported that high frequency trains of tetanic stimulation, applied to the perforant path (PP) in anesthetized rabbits, induced long-lasting increases in synaptic transmission in granule cells of the dentate gyrus – a phenomenon referred to as 'long-term potentiation' or LTP.

Following these pioneering observations, it was subsequently appreciated that *either* tetanic stimulation across of range of frequencies *or* network synchronization during seizures acutely induces long-lasting increases in synaptic transmission. The observation that induction of LTP or kindling increases synaptic efficacy suggests that cellular mechanisms of activity-dependent synaptic modification in response to patterns of coincident synaptic activation (LTP) – widely regarded as a physiological basis for

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information storage in synapses, a mechanism underlying memory – are potentially linked to mechanisms of synaptic modification associated with network synchronization and seizures – a feature of epilepsy. The increases in synaptic transmission induced by LTP and kindling are both long-lasting in the case of kindling, these changes are permanent. While the permanent alterations in neural function induced by kindling can be regarded as a form of information storage in neural pathways, the repeated seizures evoked by kindling (unlike LTP) lead to the pathological outcome of permanently increased seizure susceptibility – and eventually progression to spontaneous recurring seizures (i.e., epilepsy). Although the outcomes of kindling and LTP are thus clearly distinct, the fact that both LTP and kindling increase synaptic transmission suggests that activity-dependent modification of synaptic transmission underlying synaptic information storage and memory may contribute to network dysfunction associated with epilepsy. Given this apparent overlap, analysis of these two forms of activity-dependent plasticity is of considerable interest to determine the differential bases for normal plasticity vs. pathological plasticity.

Experimental and Methodological Details: LTP vs. Kindling

Induction Methods (LTP vs. Kindling)

While high frequency trains of stimulation can be used to induce LTP or kindling, there are distinct differences in the specifics of the stimulation paradigms that induce these phenomena of activity-dependent plasticity. LTP can be induced either in vivo or in vitro by application of high-frequency 1 s stimulus trains, typically consisting of pulses of 0.05–0.1 ms in duration delivered at 50–400 Hz. Timing of activation in the theta range (5–12 Hz) is also reported to be effective, and may more closely mimic ongoing neuronal firing and cerebral rhythms. The induction of LTP after application of tetanic stimulation is detected by physiological measurements of synaptic transmission. Using extracellular recording methods as in the original description of Bliss and Lomo, increases in synaptic transmission induced by LTP are indicated by an increase in the slope of the population EPSP or amplitude of the population spike evoked by test pulses of constant intensity (compared to responses evoked by test pulses prior to the tetanic stimulus train). Increases in synaptic transmission may be detected using intracellular recording techniques by assessing the amplitude of the evoked EPSP (or EPSC) recorded in response to stimulation of an afferent with a test pulse of constant intensity.

The increases in synaptic efficacy which are observed after tetanic stimulation trains are time-dependent. Immediately after the tetanic stimulation there is an initial increase (in the population EPSP slope, the amplitude of the population spike, or the intracellular EPSP) which is referred to as post-tetanic or short term potentiation of synaptic transmission. This initial increase in synaptic transmission is followed by lower amplitude but persisting increases (LTP). The latter long-term increases in synaptic efficacy (LTP) require protein synthesis and typically persist for the duration of recording in brain slices, or for hours to days when recorded in vivo, in awake animals. LTP can also be induced in vitro by pairing postsynaptic depolarization with low frequency stimulation of presynaptic afferents, implicating coincident neural activity in the resulting strengthening of synaptic connections, as originally postulated by Hebb in 1949.

In contrast to LTP, kindling is (by definition) an exclusively in vivo phenomenon induced in awake, freely behaving animals by stimulation that typically consists of 1–2 s trains of 1.0 ms biphasic pulses delivered at 10–150 Hz, once or twice daily. The duration of the stimulus pulses used for kindling (1 ms biphasic) are typically longer than the stimulus pulses used to induce LTP (e.g., 0.05–0.1 ms). Stimulation trains of 60 Hz are particularly effective in evoking afterdischarges (ADs), which are required for the progression of kindling. ADs are repeated high amplitude discharges and spikes (recorded by EEG) which outlast the stimulus train and typically propagate beyond the immediate synaptic target of the stimulated pathway. The evoked ADs are accompanied by behavioral seizures which are initially focal, but gradually evolve and progress to secondary generalized tonic–clonic (Class V) seizures. The increased susceptibility to ADs and to evoked Class V seizures, induced by repeated evoked seizures, is permanent.

The key difference in the stimuli that induce LTP vs. kindling is that the tetanic trains that induce LTP do not evoke the sustained ADs that are required for kindling (**Figure 1**). While occasional short ADs may be observed after stimulation trains that induce LTP, these episodes are not sustained and are quite brief. Indeed, when afterdischarge-like phenomena are evoked in the context of LTP experiments, there is often a 'depotentiation' or loss of synaptic enhancement. The observation that LTP can be induced in specific synapses by pairing stimulation of presynaptic afferents with postsynaptic depolarization also emphasizes that LTP can be induced in a limited population of synapses in the absence of propagating ADs.

The stimulation trains that induce LTP or evoke kindled seizures *both* increase synaptic efficacy in the immediate synaptic target of the activated afferent pathway, but the increases in synaptic efficacy induced by stimulation trains evoking LTP are typically confined to those synapses which are directly activated by the stimulus trains. The effects of stimulation that induces kindling, in contrast, propagate significantly to secondary and downstream synapses and circuits – during the initially

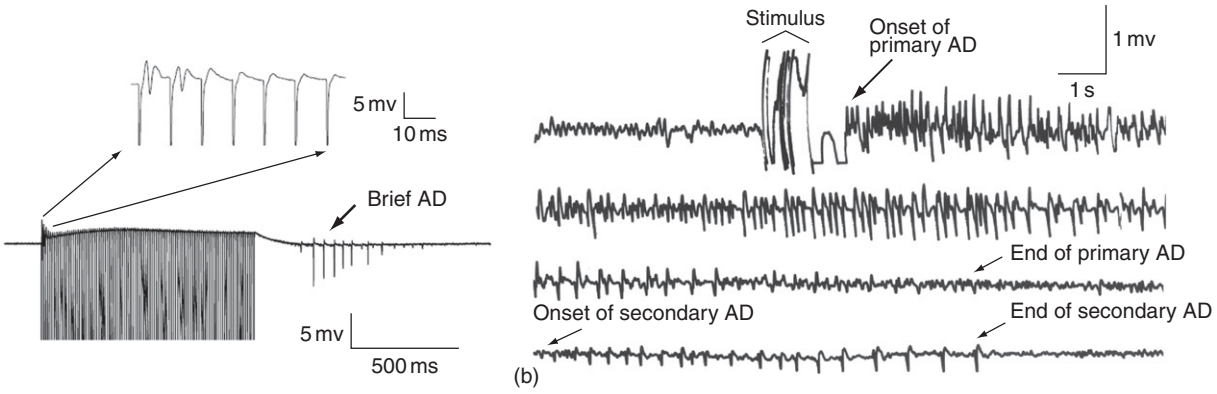


Figure 1 Examples of evoked activity and afterdischarges (ADs) in response to stimulus trains typically inducing LTP (a) or kindling (b). In (a), extracellular recording of the evoked field potential in the dentate gyrus of hippocampal slice from a rat in response to a 1 s 100 Hz train of 0.05 ms pulses delivered to the PP is followed by a brief AD that rapidly decrements within 500 ms. Inset at faster sweep speed demonstrates that the first two pulses evoke population spikes superimposed on the population EPSP. In (b), depth EEG recording in the PP of an awake freely moving rat with an electrode implanted in the region of the angular bundle demonstrates normal baseline activity and a stimulus artifact induced by a 1-s train of 60 Hz biphasic 1.0 ms pulses. The stimulus is followed by continuing artifact and then by a prolonged AD consisting of repetitive rhythmic spikes which continue for ~29 s and by a secondary AD of lower amplitude.

evoked ADs as well as in advanced stages of kindling. The progression of kindled seizures into advanced motor manifestations, such as bilateral tonic–clonic movements with loss of postural tone (Class V seizures) is presumed to represent propagation of long-lasting (permanent) *transynaptic* changes into motor and brainstem circuitry. The rate of progression to Class V seizures is reliably related to the site of stimulation that evokes the seizures, further implicating seizure-induced alterations in specific pathways and activated circuitry as the substrate for the permanent alteration in neural function induced by kindling.

Distinctive Functional Consequences of Increases in Synaptic Plasticity Induced by LTP vs. Kindling

In the original description of kindling, Goddard emphasized the potential importance of kindling as a form of permanent seizure-induced plasticity with relevance to epilepsy. Nevertheless, the gradual induction of incremental but permanent modification of neural function induced by kindling was also initially regarded as an example of information storage in neural pathways, a process that might be potentially relevant to mechanisms of learning and memory formation. In this respect, initial speculative perspectives about kindling included suggestions that long-lasting cellular alterations induced by kindling might also be relevant to memory, as was also postulated for LTP. It is now apparent that kindling induces permanent modification of neuronal and circuit functions spanning molecular to systems levels, and produces a predictable pattern of evolving molecular and cellular alterations that include alterations in transcription and translation, changes in composition and biophysical properties of membrane receptors, alterations in signal transduction pathways, neuronal cell loss in the hippocampus and other limbic structures, and sprouting and reorganization of neuronal circuitry, gliosis, and neurogenesis.

Furthermore, the functional consequences of kindling include not only progressive permanent induction of increased susceptibility to seizures, but also permanent memory disturbances and cognitive dysfunction in behavioral tasks (such as hippocampal-dependent spatial memory, which increases in severity with progression of kindling and in association with seizure-induced neuronal loss in the hippocampus). It is thus clear that kindling is not a model of memory, but rather *causes* memory dysfunction.

Relationships between Synaptic Plasticity (LTP) and Seizure-Induced Plasticity (kindling)

While there are systematic differences in the stimulation paradigms that induce LTP and kindling, and both distinctive immediate effects of the inducing stimulus trains (i.e., induction of sustained ADs) and different long-term effects (induction of a predictable sequence of neuronal and circuit alterations leading to spontaneous seizures), the stimulus trains that induce LTP or ADs that evoke kindled seizures *both* induce long-lasting increases in synaptic transmission. The relationship between synaptic plasticity induced by LTP and kindling has been studied in particular detail in the synapses of the PP projection to granule cells in the dentate gyrus, where the phenomenon of LTP was originally described. In rats undergoing kindling, synaptic potentiation resembling LTP develops in the monosynaptic PP projection to the dentate gyrus in

association with an initial evoked AD. The synaptic potentiation induced by repeated ADs is, in many respects, quite similar to LTP. However, repetitive induction of LTP, with high frequency stimulation trains (64 pulses of 0.1 ms duration at 400 Hz) delivered once daily for 20 days, is not sufficient to produce kindling – but did facilitate subsequent kindling (by ~90%) when stimulation trains that evoke ADs (60 pulses of 1 ms duration at 60 Hz) were applied. These observations indicate that repeated trains of stimulation that induce LTP are not sufficient to induce kindling, thus further highlighting the distinctive differences between these forms of activity-dependent plasticity. Nevertheless, synaptic plasticity (LTP) does have effects on seizure-induced plasticity (kindling), as the rate of kindling is increased after prior induction of LTP in the PP.

Conversely, in the studies cited above, low frequency stimulation with an equivalent number of pulses (64 pulses of 0.1 ms duration at 0.33 Hz) delivered once daily for 20 days reduced the rate of kindling. Low frequency stimulation paradigms are now recognized to induce long-term-depression (LTD), another form of activity-dependent modification of synaptic efficacy. LTD serves, in part, to restrain or prevent activity-driven increases in synaptic efficacy (LTP) from reaching saturating levels, which would preclude a dynamic range of functional synaptic modification. LTD is typically induced by application of low-frequency stimulus pulses at 1 Hz, which reduce the amplitude or slope of EPSPs evoked by test pulses of constant duration and intensity. A typical stimulus paradigm for induction of LTD is 600–900 pulses of 0.5–0.1 ms duration at 1 Hz. LTD may also be induced in vitro by pairing postsynaptic hyperpolarization with low-frequency stimulation of presynaptic afferents. The strength of synaptic connections between neurons which are firing together is increased as a consequence of LTP conversely the strength of synaptic connections between neurons which fire independently decreases as a consequence of LTD.

Recent Results

More recent studies have demonstrated that activity-dependent reduction of synaptic efficacy, by trains of stimulation that induce LTD, reliably reduce rates of kindling. This result further supports the view that activity-dependent synaptic plasticity has potentially important influences on circuit function, including susceptibility to network synchronization, seizure generation, and epilepsy. The rate of kindling of the amygdala is reduced by prior low frequency stimulation of the piriform cortex, and low frequency stimulation of amygdala also reduces behavioral seizure scores when the low frequency stimuli are applied prior to stimulation trains evoking kindled seizures. It is thus clear that activity-dependent synaptic plasticity can modify susceptibility to network synchronization and seizures. These effects have been most clearly demonstrated in terms of chronic seizure susceptibility as represented by kindling, but may also be contributing to the emergence of network synchrony underlying acute susceptibility to seizures evoked by other methods.

Directions for Future Research

The evidence that activity-dependent synaptic plasticity, including LTP and LTD, influences acute seizure generation and chronic epileptogenesis is consistent with the possibility that deep brain stimulation (through implanted electrodes) could be of potential therapeutic benefit for epilepsy. More generally, the demonstration that LTP and LTD can modify network function suggests that the modification of this process (i.e., of activity-dependent plasticity in synapses and circuits) may have potential as a therapeutic target for a novel treatment of epilepsy. Recent studies have provided evidence that activity-dependent plasticity is controlled through mechanisms of synaptic scaling and homeostatic plasticity. The relevance of these emerging phenomena for epilepsy has not been explored, and could possibly provide new therapeutic approaches.

See also: **Interneurons**: Plasticity and Reorganization of GABA Neurons in Epilepsy; **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; **Plasticity**: Axon Sprouting in Epilepsy; Circuitry Reorganization, Regeneration, and Sprouting; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; Synaptic Potentiation and Inhibition after Partial Seizures; Synaptic Reorganization in the Hippocampal Dentate Gyrus.

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Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus

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Introduction

Status epilepticus (SE) can be a devastating medical emergency. It is known in humans that neuronal injury from severe SE is often followed by the development of epilepsy. Research with animal models has shown that regardless of how SE is induced, if it is allowed to proceed for prolonged periods (e.g., >1 h), neuronal death in the hippocampus and elsewhere is followed by the development of spontaneous recurrent seizures (i.e., acquired epilepsy). The goal of this article is to examine some of the key hypotheses, concepts, and controversies relating to how epileptogenesis occurs. In particular, we consider how animal models of SE have been used to examine the hypothetical mechanisms of epileptogenesis, with a focus on synaptic reorganization (the progressive formation of new recurrent excitatory circuits) following SE-induced selective loss of specific, vulnerable, GABAergic interneurons. We propose that these two processes – selective interneuron loss and circuit reorganization – alone may account for some of the critical changes associated with injury-induced epileptogenesis.

Background

Models of Temporal Lobe Epilepsy

One of the primary reasons why a considerable amount of experimental epilepsy research has focused on models of SE is that these models are viewed as a possible approach to reproduce some aspects of temporal lobe epilepsy (TLE), which is arguably the type of epilepsy most refractory to antiepileptic drug (AED) treatment. As is briefly discussed later, another model of TLE involves 'kindling,' but most recent research in this field has centered on either electrically evoked or chemoconvulsant-induced SE. The models involving chemoconvulsants generally rely on either kainic acid (i.e., kainate) or pilocarpine injections. One reason for the interest in these models is that SE leads to injury in the hilus of the dentate gyrus and in the CA3 and CA1 areas of the hippocampus, similar to the pattern of cell loss found in many cases of human TLE. Further, these animal models lead, after a latent period of several days to a week or two, to the development of spontaneous recurrent seizures (i.e., acquired epilepsy). Considerable debate continues on whether such animal models of SE accurately recapitulate the condition in humans.

Definition of Epileptogenesis

The process of epileptogenesis is widely believed to consist of a collection of molecular, cellular, and systems-level changes that ultimately lead to spontaneous recurrent seizures. The process is generally considered to be time-dependent in that chronic epilepsy does not generally occur immediately after the injury, but rather takes time to develop and mature; indeed, the occurrence of spontaneous recurrent seizures in humans can be weeks, months, or even years after the precipitating injury. For our description, in this article, the term 'epileptogenesis' does not include the acute seizures that occur when brain tissue is injured, does not include those alterations in animals or isolated tissue where seizures occur after pharmacological treatments, and also does not include those cases where genetic abnormalities lead to altered seizure susceptibility.

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Definition of Epileptogenesis

The process of epileptogenesis is widely believed to consist of a collection of molecular, cellular, and systems-level changes that ultimately lead to spontaneous recurrent seizures. The process is generally considered to be time-dependent in that chronic epilepsy does not generally occur immediately after the injury, but rather takes time to develop and mature; indeed, the occurrence of spontaneous recurrent seizures in humans can be weeks, months, or even years after the precipitating injury. For our description, in this article, the term 'epileptogenesis' does not include the acute seizures that occur when brain tissue is injured, does not include those alterations in animals or isolated tissue where seizures occur after pharmacological treatments, and also does not include those cases where genetic abnormalities lead to altered seizure susceptibility.

This article considers only those instances in which a defined (i.e., time-limited) brain insult leads to demonstrable spontaneous recurrent seizures. Within this context, some investigators have argued that the brain insult need not necessarily cause neuronal death and cell loss. Indeed, many studies of pediatric epilepsy, particularly involving models of hypoxia, febrile seizures, and even SE in the immature brain, argue that epilepsy can develop without neuronal death. Clearly, that view is not consistent with the hypotheses offered in this article; in the view of this author, most of those studies have not adequately documented the lack of neuronal death or the occurrence of spontaneous recurrent seizures, and have therefore not shown that epilepsy develops without neuronal death.

Synaptic Reorganization

Mechanisms of acquired epilepsy, including but not limited to those dependent on experimental induction of SE, may invoke a wide range of mechanisms, including alterations in neurotransmitter receptors (e.g., $GABA_A$ and NMDA receptors) and/or alterations in voltage-dependent currents (e.g., sodium channels and H-current). While those examples of cellular plasticity may well be important, this article focuses exclusively on local circuit alterations, which can be viewed under the broad heading of synaptic reorganization. Many different hypotheses involving local circuits have been proposed. The two key mechanisms that are discussed here are: (1) the loss of GABAergic interneurons, leading to a decrease in GABA-mediated inhibition (Figure 1); and (2) axon sprouting followed by increased recurrent excitation (Figure 2). The first of these mechanisms would be expected to cause an immediate loss of interneurons as a result of SE – but it is important to note that interneurons could also be continuously lost during the process of epileptogenesis. The second mechanism has garnered considerable attention because it is viewed as process that might occur during a latent period, perhaps involving other progressive changes – that is, that axon sprouting and consequent synapse formation is a time-dependent process.

Loss of GABAergic Interneurons

Nearly half a century ago, numerous investigators showed that pharmacological treatments that lead to a blockade of $GABA_A$-mediated inhibition could evoke abnormal epileptiform activity and even frank seizures. Early studies in both in vivo and in vitro preparations showed that penicillin induces epileptiform activity, and later it was determined that this effect results primarily from a block of $GABA_A$ receptors. Several decades ago, investigators showed that some, but not all, GABAergic interneurons are lost in animal models of temporal lobe epilepsy and in human tissue (hippocampus) resected for the treatment of intractable epilepsy. Some researchers emphasized that most of the GABAergic interneurons were, however, still present, and thus challenged the concept that GABAergic inhibition was severely depressed in this tissue. A variety of more recent studies (reviewed later in this article) have provided strong evidence from both histopathological and electrophysiological experiments that selective interneuron populations are lost. Because the change in the total

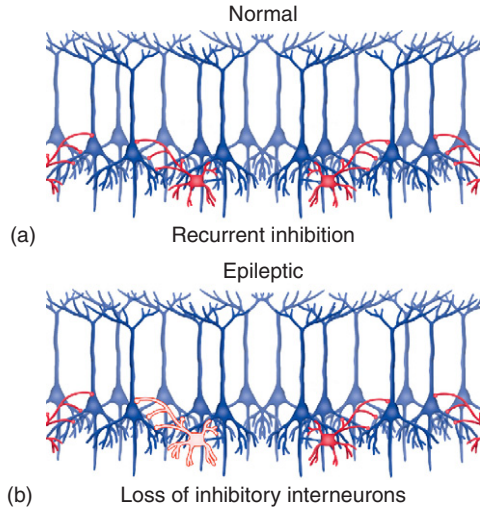


Figure 1 Schematic diagram showing hypothetical loss of interneurons in the hippocampus from a normal (a) and an epileptic brain (b). The two diagrams, which include pyramidal cells and interneuron's, illustrate the hypothesis that the hippocampus from epileptic brain has lost some but not all of the interneurons.

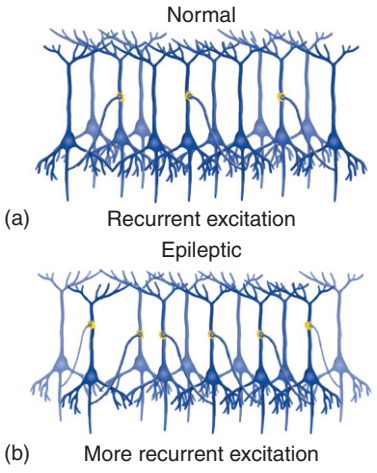


Figure 2 Schematic diagram illustrating the hypothesis of axon sprouting and increased recurrent excitation during epileptogenesis in the hippocampus (e.g., CA1 area). The two diagrams of pyramidal cells compare normal and epileptic brain, and show that although recurrent excitation is present among some pyramidal cells in the normal brain (a), epileptogenesis is associated with increased recurrent excitation (b).

number of interneurons is comparatively small, it can be difficult to demonstrate this loss of interneurons; further, this selective loss leaves intact many inhibitory circuits within relevant cortical networks (Figure 1).

Axon Sprouting and Increased Recurrent Excitation

Roughly 25 years ago, a series of histopathological studies showed that axons of the dentate granule cells (i.e., the mossy fibers) can be labeled with the Timm stain, and that the inner molecular layer of the dentate gyrus shows a robust increase in Timm stain in both tissue resected from humans with intractable TLE and in various animal models (most notably the kainate-treated rat, and later the kindled rat). This histopathological observation led to the hypothesis that dentate granule cells form new recurrent excitatory circuits, and that this reorganization would lead to positive feedback among dentate granule cells. A wide range of studies have confirmed that the mossy fibers synapse on other granule cells in this tissue. Some researchers, however, have suggested that interneurons receive mossy fiber synapses (an unlikely possibility given available quantitative analyses) – and have suggested that mossy fiber sprouting is associated with increased inhibition (a view based on data obtained using indirect techniques, such as paired-pulse stimulation, which is simply not a reliable approach to this question). In contrast, a variety of electrophysiological studies from several laboratories (using antidromic stimulation of the mossy fiber axons, analyses of spontaneous excitatory postsynaptic potentials and currents, focal glutamate microstimulation, and dual intracellular recording) have provided strong evidence that granule cells form new recurrent excitatory synapses with other granule cells.

The discussion regarding axon sprouting and epileptogenesis has been plagued by the view that mossy fiber sprouting in the dentate gyrus is the sole location in the brain where synaptic reorganization would occur following SE. However, numerous studies have now suggested that the CA1 pyramidal cells show similar changes (Figure 2), and other research implicates a similar mechanism after a variety of injuries to the neocortex and elsewhere. Histopathological evidence for the role of axon sprouting and increased recurrent excitation in epileptogenesis has now been supported by electrophysiological studies performed with whole-cell recording under conditions that exclude other possible mechanisms.

Methodological Approaches

Animal Models of SE

Several animal models of SE are available, and each of them has advantages and disadvantages – although the overall results from the models are probably more similar than different. In all cases, it is generally believed that a 'self-sustaining' component of the SE must occur in order to generate neuronal death and subsequent epilepsy. Experimental status epilepticus has been induced with the chemoconvulsants kainate or pilocarpine (with or without pretreatment with lithium). Both of these compounds are generally given as a single intraperitoneal injection, although some studies have used repeated low-dose protocols in an effort to enhance survival and to increase the proportion of animals that experience a sufficiently prolonged period of SE to cause neuronal death and subsequent epileptogenesis. Kainate first received substantial attention because it was shown that kainate injections cause a relatively selective death of neurons in the hilus and in the CA3 area. Similarly, pilocarpine induces SE and is commonly associated with neuronal death in the hilus and in the CA1 area. Both of these chemoconvulsants, however, induce cell death not only in hippocampal fields (hilus, CA3, and CA1) but also in many other areas of the brain. Similarly, electrically evoked SE can be used, and this technique has the potential advantage that one can more easily adjust the input (i.e., the electrical stimulation parameters), and thus one is, in theory, less likely to cause significant mortality. The disadvantage of the electrical stimulation protocol is that one must implant the stimulating electrodes – unless, of course, one wants to undertake chronic electrophysiological recording. If one aims to perform experiments without electrodes implanted in the brain, then the chemoconvulsant approaches may be advantageous, or at least easier. Some researchers argue that the chemoconvulsant itself is a problem for the experiment, although one could argue that electrical stimulation is also abnormal.

All of these techniques lead to SE; some researchers attempt to terminate the SE with injections of a benzodiazepine, such as diazepam.

These studies are meant to provide a general model of TLE, although some cases of TLE are quite different, histopathologically, from the animal models employing SE. In these cases of TLE, there is relatively little neuronal loss or evidence of mossy fiber sprouting, and this observation has fueled a debate about the homogeneity of TLE.

Some studies have used the kindling model, where electrical stimulations that evoke seizures on a daily basis lead to an increased seizure susceptibility or lowered threshold, but without the occurrence of spontaneous seizures. This approach, it has been argued, is advantageous in providing a more controlled experimental situation. However, because the kindling model does not generally involve spontaneous recurrent seizures (the hallmark of chronic epilepsy), it fails to recapitulate a key feature of epilepsy. Thus, there are several important advantages to the use of SE as a model of acquired epilepsy, if not TLE, but there are also disadvantages that need to be accounted for when designing experiments.

Histopathological Techniques

Most research concerning hypothetical changes in synaptic circuitry has involved histopathological studies, and oftentimes these anatomic techniques have been used before electrophysiological experiments. Thus, early studies showed that mesial temporal sclerosis involves profound neuronal death in structures such as the CA1 and CA3 areas of the hippocampus; this cell loss was readily seen with traditional Nissl stains. In addition, this profound neuronal death has been associated with extensive gliosis, which can also be studied (somewhat indirectly) with Nissl stain. Studies of altered GABAergic inhibition, and particularly, the loss of GABAergic interneurons began with immunohistochemical techniques, initially using GAD as a marker of GABAergic interneurons, but more recently involving other markers for specific subclasses of GABAergic interneurons. The studies of axon sprouting and other forms of synaptic reorganization began with use of the Timm stain, which is a marker for heavy metals that are mostly located in the mossy fibers of the dentate granule cells. Other studies in the dentate gyrus, and particularly the CA1 area of the hippocampus, have used intracellular staining with markers such as Neurobiotin; such studies have revealed an increase in axon collaterals months after SE. Thus, several different types of histopathological techniques have all been used to develop and test hypotheses about synaptic reorganization.

Electrophysiological Experiments

Electrophysiological experiments are plagued with the problem that intact animals are essentially required to study spontaneous recurrent seizures, and these only occur when animals are freely behaving (i.e., not anesthetized). Yet, mechanistic studies in an intact animal generally require anesthesia, and are most feasible with in vitro experiments involving single-cell recordings in brain slices. Relatively few studies have been done on the detailed properties of the SE that occur after an induction treatment (outlined earlier) to determine the degree to which the characteristics of SE are responsible for neuronal death and subsequent epileptogenesis. Many experiments have been done in vivo on anesthetized animals after experimental SE. These studies have the important advantage of preserving three-dimensional circuitry through extensive networks – circuitry that is always damaged in a brain slice preparation. Unfortunately, the presence of anesthesia compromises these experiments. These studies also generally involve extracellular recordings of hippocampal field potentials, which can be extremely difficult to interpret in terms of the underlying mechanism. Brain slice experiments have traditionally used tissue in which electrodes are positioned based on gross landmarks, but more recent experiments have used thinner slices and 'visualized patch' techniques (where cell-level landmarks and individual neurons can be seen with infrared illumination and differential interference contrast optics). In order to study cellular mechanisms of the epileptogenic processes, brain slice techniques are essentially required. In vivo techniques are necessary to study the development of epilepsy. What has been problematic is the use of in vivo techniques to study cellular mechanisms.

Recent Results

Loss of Interneurons

A large body of data strongly and directly support the hypothesis that GABAergic inhibition is depressed because of a modest loss of a specific set of GABAergic interneurons (**Figure 1**). These data are derived from several anatomic and physiologic techniques, from a variety of laboratories, and from experiments on both human tissue and in animal models (based primarily, but not exclusively, on SE). The techniques of immunohistochemistry and in situ hybridization have been used to show, in the dentate gyrus and in the hippocampus proper, that specific types of GABAergic interneurons are lost in animals that have been shown to generate spontaneous recurrent seizures after SE. Several studies have used immunohistochemical antibodies against neuropeptides that are coexpressed with GABA as evidence for a selective loss of interneurons. The loss of somatostatin-containing interneurons in the hippocampus is probably the best documented, but parvalbumin-positive interneurons are also often reduced. Even with Nissl staining, one can see that some of the neurons outside of the cell body layers (i.e., cells that are most likely to be GABAergic interneurons) are lost; this observation provides indirect evidence that the reduced number of neurons stained by immunohistochemical techniques is not a result of staining artifact. Because the same, or at least very similar populations of interneurons, appear to be lost in several models, investigators have concluded that some interneurons are much more vulnerable than others. The observation that 'hyperexcitability' is present shortly after SE is explained, in part, from this loss of interneurons – although other mechanisms, (such as redistribution of chloride ions so as to reverse the polarity of GABA-mediated inhibitory potentials) could also contribute to this effect.

Axon Sprouting and Increased Recurrent Excitation

The major experimental observation that led to the generation of the axon sprouting-recurrent excitation hypothesis (i.e., that injury-induced axon sprouting leads

to increased recurrent excitation, which in turn causes seizures) was that Timm staining appeared in the inner molecular layer of the dentate gyrus of epileptic animals after SE (and also in humans with TLE). This pattern of Timm staining is not seen in controls. As this technique is relatively easy, it has been used extensively, and has essentially been equated with axon sprouting and increased recurrent excitation. One important result that has been made not only in animal models of SE but also in the kindling model, is that the amount of Timm stain in the inner molecular layer of the dentate gyrus increases with time after the precipitating insult. This observation is important within the context of these models, inasmuch as one could envision that gradual growth and connectivity would create a latent period after a brain insult, and that this mechanism would generate progressive or time-dependent changes in excitability and epileptogenesis. Early studies examined the functional consequences of this structural change in experiments with GABAergic inhibition left intact – despite the fact that numerous studies (e.g., on the CA3 area, which normally has recurrent excitation) showed that $GABA_A$-receptor-mediated inhibition *masks* the effects of recurrent excitation. Since GABAergic inhibition itself is hypothesized to be changed, in this tissue, one must block GABAergic inhibition (at least partially) in both the experimental and control group to test the hypothesis that there is enhanced excitability due to axon sprouting (**Figure 2**).

Several studies, using antidromic stimulation of the mossy fibers to bypass the major synaptic input from the perforant path, showed that animals that have been subjected to kainate-induced SE (and have been observed for several months to document the development of epilepsy) show burst responses with long and variable latencies. The burst generation mechanism (i.e., with a long and variable latency) to low-intensity stimulation is a hallmark of recurrent excitation, and differentiates it from the results of traditional dendritic excitatory synaptic input. Furthermore, recent experiments using caged glutamate and focal photo-stimulation to active specific granule cells in the dentate gyrus, showed that local excitatory circuits increase with time after kainate-induced SE. When GABAergic inhibition is suppressed, the stimulation of the granule cells anywhere in the cell body layer can evoke a synchronous epileptiform burst throughout the dentate gyrus in slices from kainate-treated rats with chronic epilepsy, but not in controls. More recent studies have also shown, with paired intracellular recordings, that dentate granule cells are synaptically connected when animals have developed epilepsy after pilocarpine treatment. These experiments, and several others, strongly argue that axon sprouting leads to the formation of new recurrent excitatory circuits, which in turn promotes seizures.

It has been reported that pretreatment of rats with cycloheximide (i.e., a protein-synthesis inhibitor) can block mossy fiber sprouting; and furthermore, spontaneous recurrent seizures can still be observed several months after status epilepticus. These results, however, have not been reproduced in two subsequently published studies; in fact, cycloheximide pretreatment had no effect on mossy fiber sprouting. Nonetheless, although mossy fiber sprouting is often seen in a variety of animal models of epilepsy, this mechanism in this structure is almost certainly *not necessary* for epilepsy to occur after injury. Not only synaptic reorganization, including increased recurrent excitation, occurs in other brain areas, but also the spontaneous recurrent seizures that have been observed within a couple weeks after status epilepticus are present before much sprouting has occurred, and may well be the result of a reduction in interneurons.

Other Mechanisms

At least two other possible mechanisms of synaptic reorganization have been invoked to account for epileptogenesis. One of them is the so-called dormant basket cell hypothesis, which states that basket cell interneurons are still present, but that they have lost glutamatergic input (i.e., they are inadequately activated, and their output is therefore diminished). Virtually all the evidence to support this hypothesis comes from paired-pulse recording, which has numerous interpretational problems. Another hypothesis is that GABAergic interneurons sprout axon collaterals, and that this leads to increased GABAergic inhibition. Although this remains possible, studies examining the frequency of mIPSCs shortly after kainate-induced status epileptics versus several months later (when the animals are epileptic) showed that the reduced interneuron input (i.e., mIPSC frequency) to granule cells persisted for several months, when the animals were having spontaneous recurrent seizures, a finding that argues against this hypothesis. Several other possible mechanisms involving reorganization of synaptic circuits could contribute to epileptogenesis – or could reflect compensatory changes that reduce epileptogenesis.

Challenges for the Future

How to Better Analyze Local Synaptic Circuits During Epileptogenesis?

The fundamental problem of testing hypotheses about synaptic reorganization is precisely how to study it. Most experiments have used extracellular electrical stimulation as a means of examining the level of excitation/inhibition in the circuit. This approach is fraught with problems, including the fact that extracellular electrical stimulation will almost certainly activate fibers of passage that may project from areas completely outside of the area of study. Also, electrical stimulation generally activates

action potentials in both directions (i.e., orthodromic and antidromic). Indeed, even the acute isolated slice preparation contains living axons projecting from sites outside of the slice. Extracellular stimulation certainly does not specifically activate those neurons that remain intact in the slice preparation. Many studies also use field-potential recordings, which have relatively little resolution to differentiate cellular mechanisms. In particular, the paired-pulse technique (as a primary tool of extracellular studies) is fraught with a variety of mechanistic problems. Another approach is to study spontaneous or miniature inhibitory and/or excitatory postsynaptic currents (m- and s-IPSCs and EPSCs). The sEPSCs and sIPSCs are difficult to interpret, since these events reflect both action potential-independent transmitter release (i.e., as seen with mPSCs) and action potential-dependent release; changes that cause death of neurons or changes in the activity of neurons necessarily impact the amplitude and frequency of sPSCs. The alternative, to use mIPSCs and mEPSCs as a measure of circuitry integrity (as described earlier), assumes that spontaneous synaptic release is unaltered by SE, and that we can record all mPSCs (while most of them are probably below the resolution of whole-cell recording). The most direct approach is dual recording, but this method is particularly difficult, not so much for the technique itself, but because only a small proportion of individual neurons are directly connected with each other. Given these methodological difficulties with electrophysiological analysis, it is hoped that various imaging techniques will be useful (although to date these methods, too, lack the resolution needed to study the activity of individual neurons).

Experimental Isolation of Local Synaptic Circuits

Another important challenge is differentiating alterations in local synaptic circuits from mechanisms that are intrinsic to individual neurons, which in turn can be divided into two types – voltage-dependent ion channels and transmitter receptors that are on postsynaptic neurons (e.g., $GABA_A$ and NMDA receptors). Nearly any study that aims to identify alterations in local synaptic circuits should account for possible changes in intrinsic properties. Dual recording is the technique most likely to address these possibilities, but even this approach has some interpretational difficulties in separating these two classes of mechanisms.

Can One Prevent Epileptogenesis by Blocking Reorganization of Local Synaptic Circuits

One of the most pressing problems in experimental epilepsy research is how to block the development of epilepsy after a brain injury. If one assumes that epileptogenesis depends on alterations in membrane receptors or ion channels, one can envision that it might be possible to target those receptors and channels selectively, or to prevent their expression after injury. However, if epileptogenesis arises – at least in part – from the death of interneurons (e.g., **Figure 1**), the only approach would be to replace these cells. And indeed, many researchers are starting to use stem cell approaches to reestablish GABAergic interneurons. Although this approach seems worthy of future experimentation, it is unclear how these exogenously introduced interneurons will be engineered to project to the right locations. Similarly, if axon sprouting and the formation of new recurrent excitatory circuits are a critical mechanism (**Figure 2**), then one must devise a way to block this reorganization. Several studies are underway to develop such interventions. Extrapolation of these and other approaches, from the bench to the bedside, is a major goal in our efforts to develop better treatments for humans at risk for acquired epilepsy.

See also: **Interneurons**: GABAergic Interneurons of the Dentate Gyrus and Entorhinal Cortex; Plasticity and Reorganization of GABA Neurons in Epilepsy; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; **Plasticity**: Axon Sprouting in Epilepsy; Circuitry Reorganization, Regeneration, and Sprouting; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Synaptic Reorganization in the Hippocampal Dentate Gyrus.

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Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling

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Introduction

The brain is unique among all organs in the body, being composed of many different cell types each with discrete functions. This complexity represents a major challenge for studying events at the molecular level, which are involved in a multifaceted disease process such as epilepsy. The heterogeneity of brain cell types makes it important to focus on analysis of specific brain regions, cell types, and even subcellular fractions. The kindling model of focal epilepsy perturbs neuronal networks that impinge on the hippocampus, among other structures. Although an animal may be kindled even in the absence of a hippocampus, when the hippocampus is involved in the kindling process, there is a well-documented long-term (perhaps permanent) increase in hippocampal excitatory neurosecretory activity. Our underlying hypothesis has been that the associated molecular transformation involves alteration of the synaptic machinery, particularly the proteins underlying the dynamics of vesicle docking and fusion. These alterations are most easily studied in well-characterized systems, such as the hippocampus; however, such alterations may occur at other nodes in neuronal networks involved with kindling, so findings in hippocampus may be generalized to similar processes of plasticity and beyond the hippocampus.

To date, application of proteomic approaches to the study of neurological disorders has focused primarily on neurodegenerative conditions such as Alzheimer's and Parkinson's diseases, where human tissue is readily available. However, the increasing use of surgical resection to treat some epilepsy patients, studies comparing cerebral cortical samples from patients with temporal lobe epilepsy (TLE) and age-matched nonepileptic subjects (suffering trauma, tumor, etc.) has given rise to demonstrations of distinct differences in protein representation in affected tissue, including reduction in levels of Mn-SOD and alterations in levels of a number of housekeeping and cytoskeletal proteins in mesial sclerosis-associated TLE.

Background and Methods

Procedures for Proteomic Analysis of Protein Contents of Brain Tissues

Gel-based procedures

The development of commercially available immobilized pH gradient (IPG) strips formed with covalently attached prefocused ampholines is the primary advance that has revolutionized 2D gel procedures. The reproducibility in electrofocusing separations of proteins afforded by these IPG strips has been essential for comparative 2D gel mapping studies. IPG strips are available in a number of different pH ranges that permit high-resolution separation of different protein subsets. Initial separations are routinely performed using the widest range available, pH 3–10.

Proper sample preparation is critical to achieving high-resolution and reproducible separation in the first dimension IPG separation. Samples must be concentrated to a small volume as well as desalted prior to IPG separation. In our experience, simple precipitation with ice cold acetone in water (8:1) followed by centrifugation has proved to be superior to all other procedures. This procedure removes bulk salts and lipids and concentrates the sample. Precipitated samples are redissolved in a minimum volume of standard 6 M urea/2 M thiourea buffer, and this solution is used to rehydrate the IPG strips of appropriate pH range for specific applications. Isoelectric focusing (IEF) is generally performed using a series of timed voltage ramps, up to a final high voltage for a fixed total of volt-hours. Tracking dyes are routinely included in samples to ensure that focusing is proceeding uniformly. The pH gradient can also be calibrated by inclusion of known IEF standards available from commercial vendors.

Second dimension separations are performed by SDS-PAGE. The IPG strip is removed from the focusing tray and equilibrated in standard SDS-PAGE buffer containing reducing agent. A second equilibration follows in the same buffer but with 1.5% iodoacetamide substituted for the reducing agent in order to irreversibly block cysteinyl groups preventing formation of disulfide bonded dimers. The strip is then transferred to an SDS-PAGE gel, overlaid with 0.5% Agarose in standard SDS-PAGE buffer and separation is performed in an appropriate 2D slab gel apparatus. For most applications, laboratory-cast 10–15% SDS-PAGE gels give excellent reproducibility at low cost. Molecular weight standards are included in a separate lane on every gel. As proof that low molecular weight proteins have been retained, it is essential to keep the tracking dye on the gel.

For high sensitivity detection and quantification, gels are stained with SyproRuby (Molecular Probes) and detection and quantification performed using a fluorescent gel scanner. Other high sensitivity staining methods also can be used depending on the availability of suitable gel scanning apparatus (e.g., Coomassie R250 with infrared scanning). The

data acquired from all methods can be analyzed by any of several software packages that are now available commercially (e.g., PDQuest from Bio-Rad). These procedures are of sufficient sensitivity to detect and quantify proteins in low nanogram quantities and are linear over a broad concentration range (up to micrograms). Spots of interest can be excised subsequently by either eye or using a robotic spot cutter with fluorescence detection. Excised spots are digested with trypsin after reduction and alkylation and the resulting peptides analyzed by mass spectrometry (MS).

A major concern for these types of analyses is the reproducibility of sample application, which makes it extremely difficult to perform absolute quantification on any given gel. For this reason, comparison of intensities for a given spot from one gel to the next is best done by normalizing spot intensities detected to total gel fluorescence (equivalent to total protein), then comparing these relative spot intensities among different gels. Reproducibility of electrophoretic separations and quantification of spot intensities after SyproRuby staining by repetitive analysis of cytosol, particulate, and vesicle fractions prepared from whole rat brain is $\pm 5\%$ in almost all cases across a wide range of spot intensities. The use of total gel fluorescence for normalization of data circumvents the problem of unequal capture of sample during the rehydration of IPG strips. It is also feasible to use a selected group of spots such as actin, tubulin, or other prominent proteins in a sample for normalization. However, it is possible that these proteins are not equally recovered from one sample to the next or may vary because of a given type of treatment.

New methods for comparative protein profiling studies are based on differential labeling of samples for comparison with Cyber dyes with distinct fluorescence excitation wavelengths. Labeled samples are mixed, then coelectrophoresed and analyzed by differential fluorescence scanning densitometry. This methodology can overcome the problem of loading artifacts but suffers from being extremely expensive and from the fact that the proteins modified with different CyDyes do not comigrate precisely with each other or the unmodified protein making it more difficult to excise spots for MS.

For studies of protein phosphorylation, 2D gels are stained first with the phosphoprotein fluorescent stain Pro-Q Diamond (Molecular Probes). After scanning, the gels are destained then restained with SyproRuby and scanned to determine protein content. Pro-Q Diamond is more sensitive then SyproRuby and specific to proteins containing pSer, pThr, and pTyr. For quantitation, it is most accurate to use the absolute values for spot intensities obtained from scans. As shown later in studies of the 14-3-3 family, the ratio of Pro-Q Diamond staining to SyproRuby staining reflects the molar phosphorylation state for each spot in the gel. However, phosphorylation causes a shift in p*I* giving a set of closely migrating spots at approximately equivalent migration rates (MRs; phosphorylation can also alter migration in SDS-PAGE in some cases). Therefore, absolute quantification of the level of phosphorylation of a protein requires identification of all phospho- and dephospho-forms of a given protein on the 2D gel.

Identification of proteins by mass spectrometry

In most instances, the approaches for MS available to an investigator will be dictated by the corresponding facilities at each institution. In general, identification involves fragmenting the intact protein, usually through trypsin digestion as noted earlier, then analysis of the resulting peptides. Mass peptide mapping approaches involve determining the exact masses of as many peptides from the digest as possible, then comparing these masses with in silico generated peptide mass databases for identification. The more exact approach is to determine the amino acid sequences of a number of peptides in the digest by partial fragmentation, and subsequent mass analysis with comparison of the resulting peptide sequences to established databases. It should be noted that most facilities operate on a fee for service basis. Thus, the MS component of proteomic studies must be planned carefully to avoid becoming cost prohibitive.

Important Results to Date

Proteomic Analyses of Alterations of Synaptosomal Protein Complements in Control and Kindled Rat Hippocampi

We have previously reported initial studies to establish 2D gel maps for rat hippocampal synaptosomes and subfractions (synaptosol, plasma membrane, and vesicle fractions) in both the control and fully kindled state. **Figure 1** shows 2D gel analyses of gradient-purified synaptosomes compared with the synaptosol, synaptic vesicle, and particulate (SPM, mitochondria, etc.) fractions isolated by further Percoll gradient centrifugation following isotonic lysis. Although major cytoskeletal proteins such as actin and tubulin are common constituents of synaptosomes and whole cell homogenates, over 100 of the protein spots in the upper left panel appear to be enriched in the synaptosome, compared with whole hippocampus (gel not shown). Furthermore, the 2D gel profiles for the synaptosomal subcompartments are also largely unique from one another. As would be expected, the pattern obtained for the synaptosol most closely resembles that of the intact synaptosome, while the patterns for the vesicle and particulate fractions are distinct.

We have used this 'divide and conquer' approach to identify proteins that are altered in specific synaptosomal compartments. A number of protein spots in the 2D gel of the synaptic vesicle fraction are either increased or decreased in intensity relative to total gel fluorescence, in kindled samples than in controls. The identities of several of these proteins were determined by LC–ESI–MS/MS

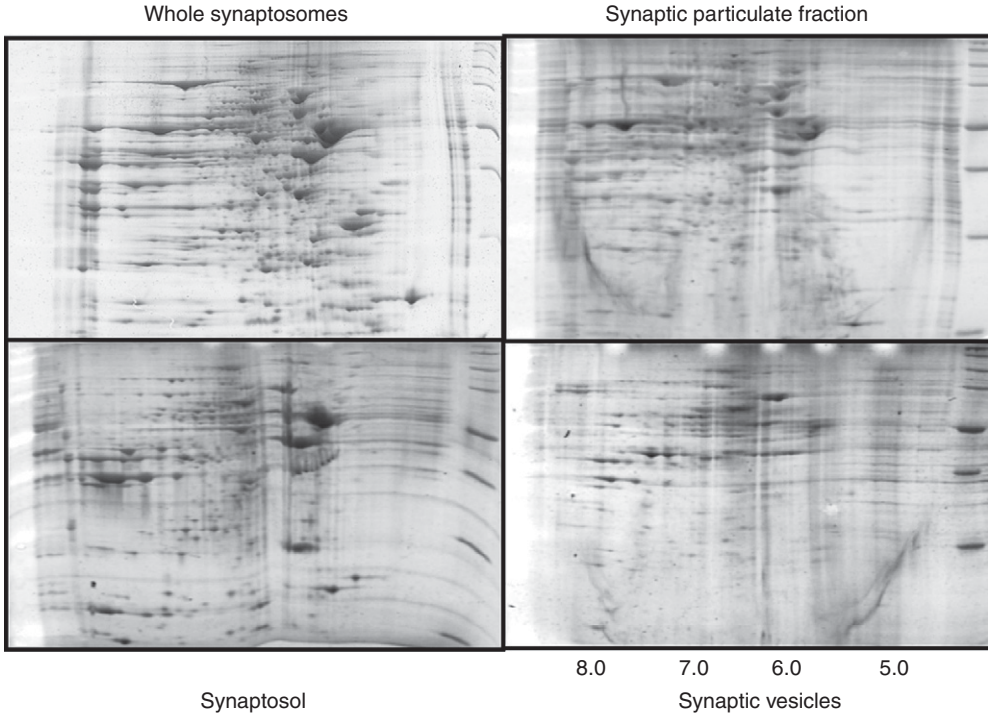


Figure 1 2D gel analysis of synaptosomal proteins. Samples were prepared by density gradient centrifugation and protein precipitates isolated by centrifugation following acetone precipitation. Precipitated proteins were redissolved in 6 M Urea, 2 M thiourea isoelectric focusing buffer and resolved on 11 cm IPG strips followed by 11 cm SDS-PAGE. The resulting gels were stained with SyproRuby and scanned on a Storm phosphorimager.

(liquid chromatography–electrospray ionization mass spectrometry/mass spectrometry), following excision of the spot from the gel and trypsin digestion. The bulk of the proteins appeared to be unchanged 1 month subsequent to stage 5 kindling, including essential proteins such as β-tubulin and the PI3 kinase adaptor protein. However, a major group of proteins around an isoelectric point of 6 increased in concentration in the kindled vesicle sample, including the synaptic vesicle H^+-ATPase required for neurotransmitter uptake.

We previously reported (see Further Reading) that a number of proteins, in the pellet fraction from disrupted kindled synaptosomes, are altered compared with controls – including increases in both α- and β-subunits of the mitochondrial ATP synthase complex, with a corresponding loss of the regulatory d-subunit. A number of proteins in the synaptosol fraction also appear to be altered substantially in kindling but as yet are unidentified. A major limitation of protein profiling studies such as those described earlier is the inherent complexity of proteomes, even of subcellular compartments. An alternative approach described later is to examine specific families of proteins in order to further enhance detection capabilities.

Studies of Synaptosomal 14-3-3 Proteins

Members of the 14-3-3 family of acidic ~30 kDa regulatory proteins exist as homo- and heterodimers that recognize specific phosphorylated Ser/Thr motifs of the type RSXpS/TXP (mode 1) and RXXXpS/TXP (mode 2). They act as both chaperones and regulatory molecules for a host of proteins in the cell, including members of numerous signaling pathways, as recently demonstrated in detail by global proteomic analyses. Among these chaperoned and/or regulated proteins are protein kinase C isoforms and CaM kinase, for which the 14-3-3s serve as inhibitors. Brain is the richest source of 14-3-3 proteins, where all but the σ isoform appear to be expressed. Two 14-3-3 isoforms, β and ζ, can be phosphorylated, at least in brain tissues, apparently inhibiting the formation of dimeric species.

We have examined the 14-3-3 protein complement of rat hippocampal synaptosomes by a combination of 2D gel analysis with both protein and phosphoprotein stains and immunoblotting, coupled with mass peptide mapping studies to identify the isoforms present and to quantify their levels and phosphorylation states, in control versus kindled hippocampus. As shown in **Figure 2**, the most prominent 14-3-3 isoforms present in rat hippocampal synaptosomal preparations are the phospho- and dephospho- forms of the β and ζ isoforms and 14-3-3ε. The amounts of these isoforms and their relative phosphorylation status did not change substantially either 1 month after completion of entorhinal kindling or with simple depolarization (as shown in the included bar graphs). Identification of all isoforms was performed with trypsin digests of the excised spots both by SELDI–TOF

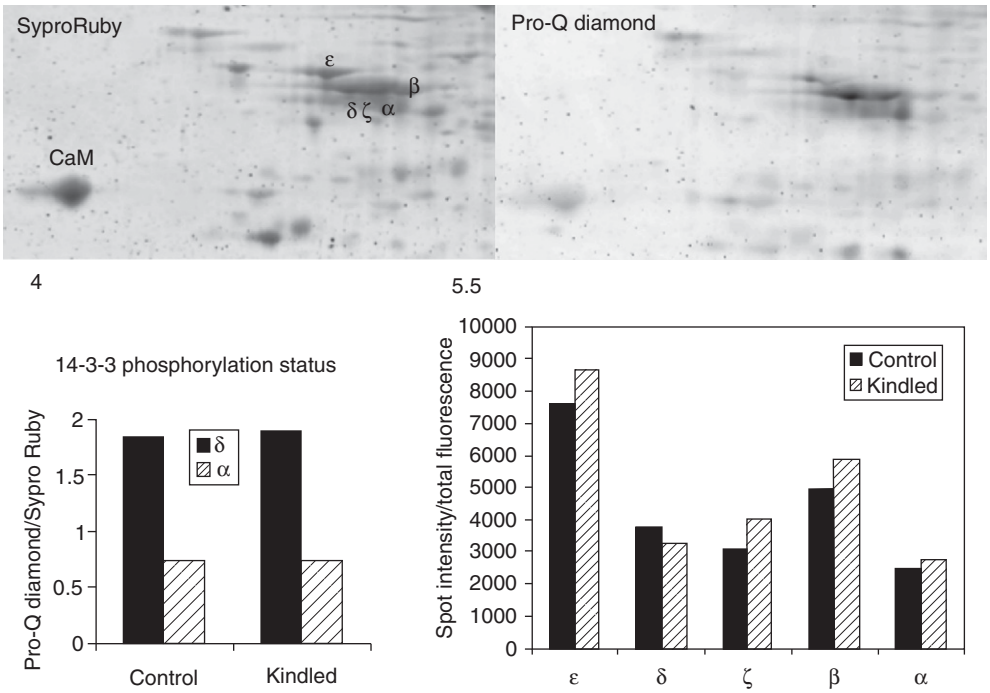


Figure 2 Entorhinal kindling does not alter levels or phosphorylation status of 14-3-3 isoforms in hippocampal synaptosomes. The two upper panels show SyproRuby (left) followed by Pro-Q Diamond staining of a representative of four different 2D gels run with samples of synaptosomes from kindled versus control rat brains. The gel shown was that obtained with an ipsilateral kindled animal. Quantification with a Typhoon phosphorimager showed no significant difference among any sets of samples in the 14-3-3 region of the gel. Average right and left values for Pro-Q Diamond/SyproRuby ratio (left) and for relative spot intensity with SyproRuby (right) are shown in the lower bar graphs.

(surface-enhanced laser desorption ionization –time of flight) mass peptide mapping and LC–MS/MS analysis. Immunoblot analyses of 2D gels (in parallel with the run shown in Figure 2 with a 14-3-3 pan-specific antibody) detected only the spots identified as 14-3-3 isoforms (data not shown). The large SyproRuby staining spot running at a p$I \sim 4.2$ and MR ~ 17 kDa is calmodulin (CaM) which serves as an excellent internal standard for quantification.

Other investigators have reported an apparent decrease of total 14-3-3 levels in cytosol (25%) and microsomal (50%) but not mitochondrial fractions isolated from rat hippocampus 4 h following seizures induced by intraamygdala administration of kainic acid. This change was correlated with a corresponding ipsilateral-specific increase in the proapoptotic factor BAD, suggesting that alterations in 14-3-3 might be triggering neuronal cell death. One may speculate that the differences between these results and our data (reported earlier) reflect the fundamental difference between electrical kindling and kainic acid-induced status epilepticus, and specifically the attendant pathological morphologic changes of the latter. It should also be noted that we have recently reported studies showing a number of kindling-dependent changes in secretory proteins at the synapse that are independent of the site of stimulation but identical for a given kindling stage.

Alterations of the CaM System in Epileptogenesis

The preeminent calcium signaling molecule at the synapse is CaM. One of our major hypotheses has been that the heightened state of excitability leading to spontaneous hypersynchronous neuronal activity (as seen in the epileptic state) must involve alterations in the CaM system. Numerous papers have appeared showing apparent alterations in the primary CaM kinase of the synapse, CaM kinase II, in both chemical and electrical models of epilepsy and in human material from epilepsy surgery. For example, a decrease in hippocampal CaM kinase II at both protein and mRNA levels has been reported with septal kindling and pilocarpine treatment. This change is consistent with the observation that homozygous CaM kinase IIα knockout mice show an enhanced epileptic response and spontaneous seizures. Conversely, increased accumulation of CaM kinase IIβ has been reported in response to PTZ-induced seizures, mediated by a nontranscriptional mechanism. Chronic reduction in CaM kinase II activity is most often correlated with status epilepticus in these models, apparently arising from inhibitory phosphorylation at multiple sites and/or conversion of the enzyme to an insoluble form. Other investigators have reported increased levels of CaM kinase II activity in rat hippocampus after

kainate-induced seizures. Given these disparate results, it is likely that the nature of alterations in CaM kinase II expression and activity depends on multiple variables. Other members of the CaM family have been linked to epileptogenesis, including the death-associated (DAP) kinase and the CaM-activated phosphatase calcineurin. Elevated expression levels of DAP kinase in TLE have been posited to be responsible for the increased neuronal cell death accompanying mesial sclerosis.

Surprisingly, a global analysis of the CaM family during epileptogenesis has not been reported. Indeed, we have only recently begun to apply the tools of proteomics to determining the CaM subproteome at the synapse. This type of analysis is made possible by the ability to isolate CaM binding proteins by calcium-dependent interaction, first described by our laboratory in 1976. **Figure 3** shows the 2D gel profiles for the CaM protein complement isolated from detergent solubilized synaptosomal preparations. This soluble fraction was mixed with an excess amount of biotinylated CaM derivative, prepared with a recombinant CaM containing an inserted Cys residue at position 5 (CaM^{T5C}), and processed as described in the legend of **Figure 3**. Proteins were recovered by acetone precipitation then resolved on standard 2D gels (pH 3–10, IPG/10% SDS-PAGE) subsequently stained with SyproRuby. The numbered spots were excised and subjected to LC–MS/MS to identify the proteins indicated in the figure legend. The complexity of the sample is dramatically reduced compared to the whole synaptosome shown in **Figure 1**. The patterns obtained for the control vs. kindled samples also were quite reproducible. In preliminary analyses, it appears that at least one low molecular weight (41 kDa) spot with an isoelectric point of 5 in the control sample is missing in the profile obtained with the kindled sample. Studies are currently in progress to identify which protein this represents, and to determine the mechanism of its kindling-dependent alteration.

Future Goals

The gel methods outlined earlier remain the primary approach used for initial global quantitative proteomic studies. However, this two-tier procedure is time consuming and cumbersome. A number of procedures have been devised recently which permit direct comparative protein profiling by MS alone. Most of these approaches rely on differential mass tagging of proteolytically digested samples which are then mixed and analyzed by MS. Comparison of the relative peak heights for the differentially tagged peptide peaks is used for quantification. This method has the obvious advantage of the high detection sensitivity of MS, and its ability to identify the protein of interest by subsequent fragmentation sequencing in the same analysis. A recent review of these strategies is given in the recommended readings.

We feel that the isobaric tagging for relative and absolute quantification (iTRAQ) tagging strategy has the most promise for proteomic studies of kindling. The iTRAQ reagents are a multiplexed set of up to eight isobaric reagents for quantitative proteomics analysis. They possess an amine-reactive group that labels the N terminus of all peptides, as well as the side chain of

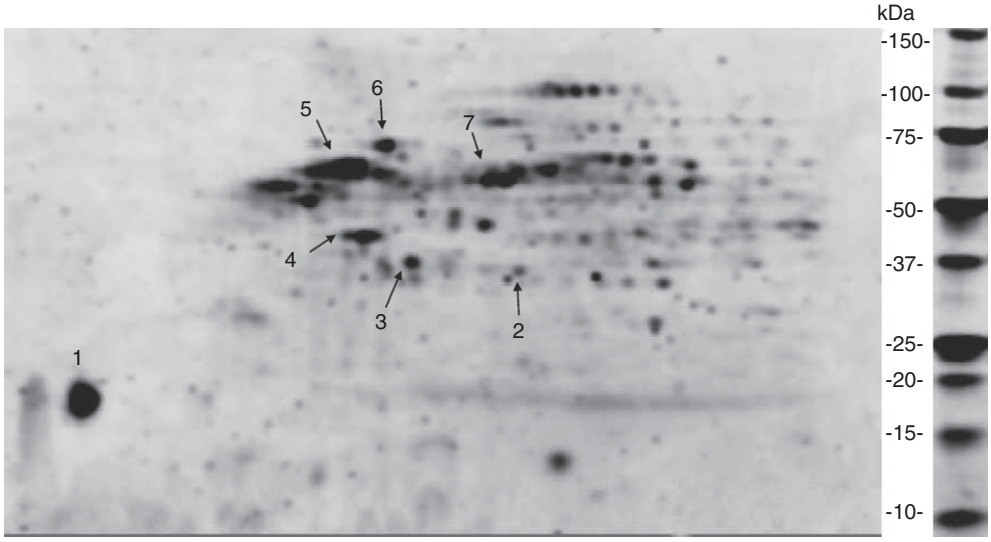


Figure 3 2D gel analysis of CaM binding proteins from hippocampal synaptosomes. Samples of hippocampal synaptosomes were solubilized in 20 mM *N*-ethylmorpholine, 1% TritonX-100, 1 mM $CaCl_2$, pH 7.0, preabsorbed with phenothiazine–Sepharose then mixed with sufficient biotinylated CaM^{T5C} to give 1 mM. After a 10 min incubation, CaM and bound proteins were captured using excess streptavidin magnetobeads, the beads washed with binding buffer then CaM and associated proteins eluted with the same buffer containing 5 mM EGTA in place of Ca^{2+}. Proteins were recovered by acetone precipitation then resolved on standard 2D gels (pH 3–10, IPG/10% SDS-PAGE) subsequently stained with SyproRuby. The numbered spots were excised and subjected to LC–MS/MS as described in Methods to identify the following proteins: (1) calmodulin; (2) annexin 3; (3) G protein β subunit; (4) Actin; (5) CaM kinase II α, β, and γ; (6) Hsp 70/72; (7) dihydropyramidase-related protein 2.

internal lysyl residues. The reagents are isobaric, with a different distribution of isotopes between a reporter and a balance group. Thus, the labeled peptides obtained after modification are identical in MS mode. Upon fragmentation in MS/MS mode, the labeled peptides produce strong, diagnostic, low-mass MS/MS signature ions. Relative areas of these reporter peaks indicate the contribution of each sample to total peptide present, allowing for quantification of up to eight different samples simultaneously. MALDI–TOF/MS (matrix-assisted laser desorbtion ionization–time of flight–mass spectrometry) analysis gives low femtomole to high attomole sensitivity in both MS and MS/MS modes. It should be noted that the iTRAQ approach has been used to demonstrate relatively modest increases in phosphorylation state of CaM kinase II on depolarization of purified rat brain synaptosomes. This demonstration clearly shows the power of the iTRAQ approach in kindling, especially for detecting potential alterations in depolarization dependent protein modifications.

As a final comment, it must be noted that it is essential to confirm any putative kindling-related changes seen with proteomic analyses by traditional quantification methods such as quantitative immunoblotting. We have used this approach quite successfully to study specific members of the secretory protein family. The ultimate goal of these types of studies is to identify changes in the levels of protein at the synapse, reflected by corresponding changes in gene expression, that dictate the state of elevated neuronal activity leading to seizure activity. It is hoped that determining these changes, and the nature of the molecular events and signaling pathways responsible for them, will provide new targets for therapeutic intervention to treat even the most persistent and pernicious seizure conditions in humans.

See also: **Epileptogenesis**: Surrogate Markers for Epileptogenesis; **Neurotransmitter Function**: Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Seizures**: Seizure-Induced Gene Expression.

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The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis

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Introduction

Acquired epilepsy (AE), the development of spontaneous, recurrent seizures following a neurological insult, constitutes approximately half of all cases of epilepsy. The majority of cases of AE are produced by one of the three injuries to the brain: status epilepticus (SE), stroke, and traumatic brain injury (TBI). Although the precipitating events causing these injuries are distinct from one another, it has been shown that each can lead to the development of AE. This process wherein healthy brain tissue is transformed into a hyperexcitable network of

Seizure-Induced Alterations in Motor and Sensory Maps

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Introduction

In vertebrate animals, interconnected networks of large numbers of neurons directly generate behavior. Other causal factors like genes, ion channels, and individual neurons and glia influence behavior because they affect the functioning of neuronal networks. Thus, a comprehensive understanding of behavior requires a complete characterization of neuronal systems. This article presents an overview of several studies conducted to study the neocortex of rats and cats following repeated seizures. These studies have examined network properties, and have shown that neuronal networks have greater interconnection and reduced functional specificity as a consequence of repeated focal seizures.

Background

We have chosen the forelimb area of sensorimotor cortex of the rat and the primary auditory (AI) neocortex of the cat to conduct our studies. The rat sensorimotor neocortex is topographically organized, supports seizure activity, is highly plastic (showing synaptic potentiation and depression phenomena), and is important in the execution of skilled reaching behaviors. Cat AI neocortex exhibits a tonotopic organization that is dynamically regulated and continually altered by ongoing experience, even into adulthood. Cat AI neocortex also presents an ideal structure for examining unit firing characteristics following seizure-induced reorganization because the naïve system has previously been described in detail, and a great deal is known about underlying mechanisms.

The presence of a highly ordered somatotopic map of movement representations was intuited by John Hughlings Jackson, on the basis of his observations of the ordered spread of tonic and clonic activity to adjacent muscle groups during a single ictal event. Penfield later confirmed Jackson's notion and provided the classic descriptions of movement representations within the human precentral gyrus. Much of the initial characterization of these motor maps came from surface stimulation experiments conducted on human patients prior to surgery intended to remove an epileptic focus. More recent work has shown that stimulation-evoked movements in some people with epilepsy could be elicited from regions well outside the classic motor strip located along the Rolandic fissure. This finding suggested that the topography of movement representations within the primary motor cortex could reorganize in response to recurrent seizure activity. I have speculated that Penfield's classic depictions of cortical representations, which Philip Servos found to contain an error (the face representation should be inverted), might be due to the fact that they were obtained from tissue exposed to chronic epileptiform activity.

Neurons in primary AI of the cat respond most vigorously to simple sounds (tone pips) of a particular relatively narrow frequency range. A tuning curve provides a three dimensional plot of the firing rate of auditory cortical neurons over a restricted range of stimulus frequencies and intensities. The frequency to which the neurons are maximally responsive is known as the characteristic frequency (CF). Examination of tuning curves provides a useful way of examining the response properties of single cells within larger neural networks.

Methods and Recent Results

Motor Map Alterations

We decided to directly examine whether experimentally induced repeated seizure activity could reorganize the topography of movement representations. Furthermore, we wanted to determine whether there was a correlation between reorganization and synaptic potentiation, whether the reorganization was persistent, and whether it could be driven by seizures from either cortical or limbic sites. We used the rat sensorimotor neocortex, specifically the forelimb area, to answer these questions. The forelimb area is defined as that area of rat sensorimotor neocortex in which intracortical microstimulation (ICMS) elicits movements of the digits, wrist, elbow, or shoulder of the contralateral forelimb. Under ketamine/xylazine anesthesia, an ICMS procedure involves performing a craniotomy over one hemisphere and removing dura. A grid is placed over a digital image of the brain's surface and is used to identify the points where an electrode is lowered to layer V. Brief trains of high-frequency cathodal current are delivered once per second and the intensity of stimulation is raised until a movement is detected or a limit of 60 μA is reached (see **Figure 1** 'Control').

The primary findings of our studies is that rats that had seizures initiated from the corpus callosum, amygdala, or hippocampus exhibited a profound increase in the area of neocortex from which forelimb movements could be elicited relative to control (i.e., nonstimulated) rats

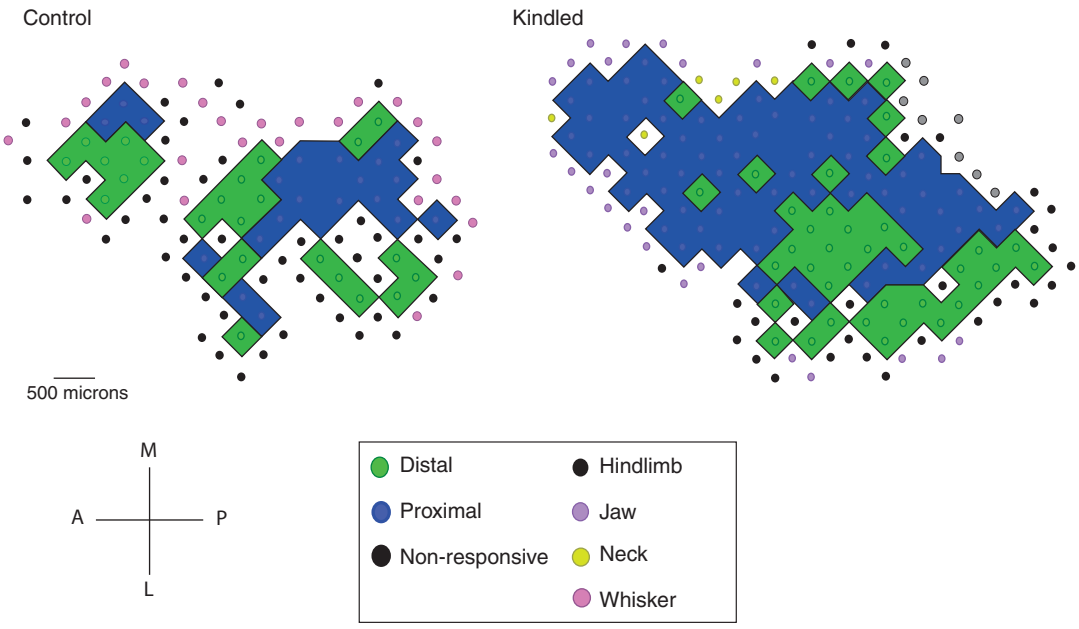


Figure 1 Rat forelimb movement representations. Color-coded representation of the motor map of the rat forelimb areas for a Control and Kindled rat. Each point represents the place where a stimulating microelectrode was lowered 1550 μm into the left hemisphere motor cortex, and stimulation was applied (up to 60 μA) until movement was elicited. Distal is defined as digit or wrist movements while Proximal is defined as elbow or shoulder movements. External map boundaries were always defined as electrode penetrations that failed to elicit movement or nonforelimb area movements (Hindlimb, Jaw, Neck, or Whisker). A–P refers to the direction of the anterior–posterior axis. M–L refers to the direction of the medial–lateral axis.

(see Figure 1 'Kindled'). This change in function facilitates the ability to elicit forelimb movements from regions of neocortex that would not previously produce forelimb movements in response to similar stimulation. At higher ICMS intensities, rats that had seizures also showed a greater number of multiple forelimb muscle responses compared with controls. We have also shown that the size of the maps strongly and positively correlates with both the number of cortical seizures and the degree of cortical synaptic potentiation. Furthermore, seizure-induced motor map expansion is persistent, lasting for at least 5 weeks after the last seizure.

The dramatically expanded forelimb movement representations, in the rats that had seizures, are not a result of alterations in anesthetic levels or ICMS intensity. Because ICMS appears to elicit movement by stimulating a pool of neurons interconnected by horizontal fibers within the neocortex rather than via direct stimulation of corticospinal neurons, enhanced synaptic efficacy may be responsible for the expanded motor representations. We have also shown that high-frequency stimulation-induced synaptic potentiation of horizontal fibers – stimulation that does not result in seizures – results in map expansion. Moreover, low frequency stimulation-induced synaptic depression (of horizontal fibers), that also does not result in seizures, reduces the size of motor maps. Thus, alterations in synaptic efficacy are likely a mechanism underlying functional reorganization within motor cortex.

Although synaptic potentiation phenomena may be necessary for map expansion, a subsequent study detailed below showed that they might not be sufficient. In order to more deeply explore the relationship between epileptogenesis, synaptic efficacy, and the size of motor maps, we used the epileptogenic-prone (FAST) and epileptogenic-resistant (SLOW) rat strains that differ dramatically in their kindling rate. These strains have become a valuable tool for investigating the neurochemical and neurophysiological basis of epilepsy. FAST and SLOW rats received daily repetitive high-frequency stimulation of the corpus callosum in order to induce long-term polysynaptic potentiation. They then underwent high-resolution ICMS in order to assess forelimb motor map size. We observed that high-frequency stimulation induced similar increases in polysynaptic potentiation in both rat strains – and therefore potentiation does not underlie the inherent differences in epileptogenesis between the two strains. Despite the similarities in the magnitude of potentiation, we found that only the FAST potentiated rats showed an increase in the size of their motor maps. SLOW potentiated rats did not manifest an increase in the size of their motor maps compared with SLOW control rats, whereas the FAST potentiated rats had motor maps that were about twice the area of FAST control rats. Thus, our results suggest that although potentiation may be necessary, it is not sufficient to account for map expansion; some other unidentified factor(s) are also involved.

Sensory Map Alterations

We examined the response properties of neurons in AI from cats that had seizures elicited by electrical stimulation through implanted electrodes, cats that were implanted but not stimulated, or nonimplanted controls. Cats were anesthetized with ketamine and the right temporal lobe exposed. In a sound-attenuated room, multiple single units were recorded from layers III/IV across the extent of AI (using one or two 4×2 rectangular electrode arrays) in response to a series of tone pips over a series of frequencies. We observed that the tuning properties of neurons in the primary auditory cortex were altered in the cats that had seizures. In AI of cats, there are normally a high percentage of neurons that express narrow V-shaped tuning curves. In the cats that had seizures, there was a greater tendency for double-tuned single-units (29%) than in the normal controls; the percentage of double tuned neurons in cat AI was only about 8% for the nonimplanted controls and 14% for the sham controls. This change may represent less specification between thalamocortical afferents that provide the normal tuning, and/or the stronger interconnection of cortical horizontal fibers that convey the CF range. Thus, single auditory neurons from cats that had seizures responded vigorously over a broader range of sound frequencies, consistent with the neurons being more strongly interconnected.

Cat auditory cortex normally exhibits a tonotopic organization with a gradual progression from low characteristic frequencies at the posterior ectosylvian sulcus (PES) to high characteristic frequencies towards the anterior ectosylvian sulcus (AES). Substantial changes in the tonotopic maps in AI were observed in all cats that had seizures. The maps from cats that had seizures in AI typically exhibited a lack of a tonotopic gradient, with large portions of AI tuned to the same range of characteristic frequencies (see Figure 2). During spontaneous neural activity, we also observed that neurons recorded from different electrodes within the array show higher intercorrelations in the cats that had seizures. The enhanced spontaneous neural synchrony was not only observed between neurons, but also extended over a greater area of primary auditory cortex. All of these observations can be explained by a greater amount of interconnections mediated by horizontal corticocortical synaptic connections.

We also investigated the effect of seizures on the response properties of neurons in the auditory cortex to periodic stimuli. Temporal coding in the auditory cortex normally manifests itself as the ability to follow the slower amplitude modulations in a complex stimulus, a phenomenon referred to as 'envelope locking.' Envelope locking can be quantified by the ability of neurons to respond to periodic stimuli with varying repetition rates. To examine envelope locking in animals exposed to repeated seizures, we used a relatively simple stimulus: 1 s duration click trains, with click rates ranging from 2 to 64 clicks per second, presented once every 3 s. With this stimulus, we evaluated the capacity of the cortical cells to follow the click periodicity. Neurons from cats that had seizures could not follow the higher-frequency periodic stimuli compared with neurons from control cats.

Mechanisms

Although the neural mechanisms underlying representational plasticity are not fully known, a potential neurochemical mechanism is that preexisting lateral excitatory connections are unmasked by decreased intracortical inhibition. Repeated seizures may induce expansion and reorganization of neocortical representations by altering glutamatergic and GABAergic transmission. A role for

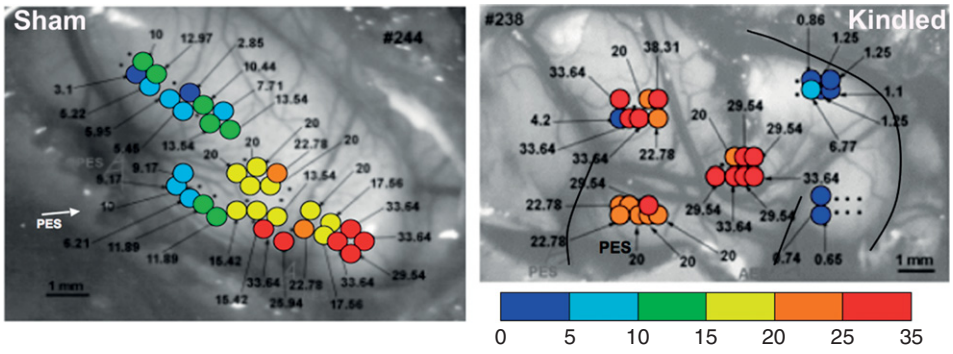


Figure 2 Tonotopic organization of cat primary auditory neocortex. Photographs of the exposed cortical surface depict the map of the characteristic frequency (CF) of each electrode position recorded from the auditory neocortex of Sham and Kindled cat. Indicated are the posterior ectosylvian sulcus (PES) and anterior ectosylvian sulcus (AES). Anterior is toward the right-hand side of each figure. The CFs are color coded according to the color bar shown. Cats normally have a gradual progression from low CFs (down to 2.5 kHz, lower CFs are found in the banks of the PES) near or just beyond the PES to CFs up to 35 kHz near the AES as shown in the Sham. In the example from a Kindled cat, a clear low- to high-frequency gradient is absent and clustering of similar CFs over a large area is found.

enhanced glutamate transmission in the reorganization of maps is suggested by the observation that NMDA antagonists block neocortical motor map plasticity induced by passive limb movements. Repeated seizures have been shown to enhance glutamatergic transmission by enhancing NMDA receptor activation, recruiting previously dormant NMDA receptors, increasing glutamate agonist potency, and increasing presynaptic glutamate release. Kindling has also been associated with a loss of GABAergic inhibition, and GABA antagonists have been shown to facilitate the induction of long-term potentiation in the motor neocortex and to induce expansion of movement representations. Thus, both the relative suppression of GABAergic transmission and enhancement of glutamatergic currents may underlie the reorganized map in rats and cats that have had repeated focal seizures.

Future Directions

The implications of seizure-induced alterations of motor and sensory neocortical organization, at the behavioral level, are altered motor function and sensory perception. Indeed, there are reports of these manifestations in people with epileptic foci in those two areas. Experimentally, we have reported altered skilled motor behavior in rats that have had focal seizures. Our evidence suggests that very strong horizontal synaptic connections within the neocortex likely underlie these neural and neural network properties. We and others have also reported that these connections may be supported by increased numbers of the highly efficacious perforated synapses that have larger synaptic contacts and more abundant NMDA and AMPA channels. A rationally designed treatment may be efficacious if it were to reverse this enhanced horizontal connectivity. Thus, pharmacological agents, and electrical or magnetic stimulation, that reverse glutamate receptor upregulation should be effective. We have recent data that show that low-frequency electrical stimulation can, to some degree, reverse seizure-induced map expansion, possibly by reversing enhanced horizontal connectivity. Another treatment alterative is sensorimotor enrichment or behavioral therapy. Sensory and motor areas are highly plastic, and there is good reason to believe that exposure to broad arrays of sounds, or training on skilled motor tasks, may reverse the seizure-induced pathological reorganization that occurs in auditory neocortex and motor areas respectively.

Seizures are hypersynchronous and hyperexcitatory oscillatory discharges that can occur in the vertebrate central nervous system as the result of a wide variety of intrinsic (i.e., ion channel mutations, neurotransmitter imbalance) and extrinsic (i.e., drugs, infections) factors. Seizures appear to induce a Hebb-like increase in synaptic efficacy: cells that fire together during seizures subsequently 'wire together.' The functional changes summarized here that occur with seizures likely can be accounted for by a corollary to Hebb's rule: cells that are wired together, fire together *and* recruit more cells into the response. Treatments that reverse seizure-induced plastic changes should be effective at restoring sensory and motor map topography and function, and thus reduce the interictal behavioral comorbidities that occur with epilepsy.

Acknowledgements

Sincere thanks to colleagues and collaborators Richard Dyck, Jos Eggermont, Corey Flynn, Amy Henderson, Luke Henry, Jeff Kleim, Bryan Kolb, Dan McIntyre, Marie Monfils, Pamela Valentine, Francine van Rooyen, and Nicole Young.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Consequences of Seizures Early in Life: Insights from the Kainic Acid Model; Cognitive Effects of Seizures in Animal Models; **Models**: Characteristics and Mechanisms of Kindling; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures; **Psychiatric Symptoms**: Interictal Behavioral Comorbidities in a Model of Epilepsy; **Treatments (non-Antiepileptic Drugs)**: Electrical Stimulation.

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Seizure-Related Changes in Dendritic Spine Structure

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Introduction

Epilepsy is one of the most prevalent neurological diseases, affecting about 1% of the population worldwide. In addition to the immediate disabling effects of the seizures themselves, patients with epilepsy frequently suffer from a range of chronic neurological, psychological, and behavioral problems. In particular, epilepsy is often associated with severe cognitive deficits, including mental retardation, learning disabilities, and memory problems, which are often progressive. While the causes of neurocognitive dysfunction in epilepsy are multifactorial and likely involve a complex interaction between biological, environmental, and psychosocial factors, accumulating evidence indicates that seizures themselves may directly cause brain injury and thus contribute to the development and progression of neurocognitive deficits of epilepsy patients.

Seizures can potentially produce brain injury via diverse cellular and molecular mechanisms. The most obvious form of seizure-induced brain injury is neuronal death. Both clinical studies and animal models strongly suggest that neuronal death occurs as a direct result of seizures, at least under some conditions, such as following prolonged seizures (status epilepticus). However, despite the relatively high incidence of neurological and behavioral deficits in epilepsy, many patients with epilepsy have no clear evidence of neuronal death – at least based on neuroimaging, neuropathology, or evaluation of biochemical markers of neuronal injury. Thus, in addition to overt neuronal death, there likely exist more subtle, 'non-lethal' mechanisms of seizure-induced brain injury, affecting neuronal structure and function and accounting for cognitive deficits in epilepsy. Since higher cognitive processes involve highly patterned communication between neurons in complex cortical networks, seizure-induced alterations in the cellular and molecular components of synaptic transmission between neurons could have a profound negative impact on cognition and behavior.

Dendritic spines, tiny protrusions emanating from the dendrites of many neurons, receive the majority of excitatory glutamatergic synaptic inputs to cortical neurons and have been strongly implicated in mechanisms of synaptic plasticity and learning. As such, dendritic spines represent a potential target for pathological modulation by seizures; seizure-related changes in these spines may mediate detrimental effects on neurocognitive function. Indeed, one might logically predict that the synchronous discharging of thousands of neurons in an epileptic focus that occurs repetitively during a seizure, the resulting massive release of glutamate, and the associated intense synaptic activation and depolarization of postsynaptic cells would produce numerous biochemical and structural changes to spines. In fact, numerous studies have provided evidence that dendritic structure is often abnormal following acute seizures or in chronically epileptic brains. Further investigation of seizure-related changes in dendritic spine structure should provide important insights into mechanisms of seizure-induced brain injury and may lead to novel therapeutic approaches for preventing brain injury and associated neurocognitive deficits.

Background

A variety of studies involving both human tissue and animal models suggests that epilepsy does result in alterations in various components of dendritic ultrastructure. However, most of this previous work has used fixed tissue specimens, and thus have several limitations in determining causal relationships and functional implications of these seizure-related dendritic changes. Furthermore, little is known about the specific cellular and molecular mechanisms mediating these effects of seizures on dendritic structure.

Dendritic Spine Changes in Human Epilepsy

Studies of dendritic spines in human epilepsy are essentially limited to pathological analysis of brain tissue, usually obtained during surgical treatment of patients with

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Seizure-Related Changes in Dendritic Spine Structure

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Dendritic Spine Changes in Human Epilepsy

Studies of dendritic spines in human epilepsy are essentially limited to pathological analysis of brain tissue, usually obtained during surgical treatment of patients with

intractable epilepsy. Such pathological specimens from the region of the epileptic focus, in neocortex or hippocampus, have revealed various dendritic abnormalities but most commonly show a loss of dendritic spines. A loss of spines has been reported primarily in hippocampal pyramidal neurons and dentate granule cells in patients with temporal lobe epilepsy, and this loss may occur either in isolation or in association with varicose swelling of the dendrites. Similar findings of spine loss and dendritic swelling have been documented in pyramidal neurons of neocortex distant from the primary site of epileptogenesis. Other less common dendritic abnormalities have been described in both neocortical and hippocampal epileptic tissue, including changes in dendritic length, shape, and branching patterns, as well as a focal increase in dendritic spine number.

A loss of dendritic spines, particularly in hippocampus, represents a logical mechanism to explain learning and memory deficits in epilepsy patients. Studies of the physiological properties of neurons in the resected brain tissue from epilepsy patients have identified correlations between loss of dendritic spines and responses to glutamate, suggesting that the spine loss has important effects on synaptic physiology. However, owing to the intrinsic limitations of clinical-pathological studies, it is difficult to directly link spine changes to behavioral or cognitive problems in epilepsy patients, thus making the functional implications and consequences of dendritic spine abnormalities in human epilepsy unclear. Another limitation of human studies is the inherent problem of determining the specific causes, and mechanisms, of the spine changes. Dendritic abnormalities documented in pathological specimens resected from patients with intractable epilepsy could be due to direct effects of seizures, but could also relate to other variables, such as the underlying neurological insult (e.g., an insult originally causing the epilepsy), the use of antiepileptic drugs, or other environmental factors. Thus, well-controlled animal studies may be better suited for determining the importance of different factors and mechanisms in mediating seizure-related spine changes.

Dendritic Spine Changes in Animal Models of Epilepsy

Animal models of epilepsy can complement and extend the clinical studies of dendritic spines by controlling for many of the confounding factors that complicate interpretation of human data and by facilitating more mechanistic investigations. The direct effect of seizures on dendritic spines has been tested in different animal models. Similar to the clinical studies, the most consistent finding following seizures in animal models has been a loss of spines, often associated with varicose swelling of the dendrites. In the rat pilocarpine model of temporal lobe epilepsy, acute seizures result in a marked reduction in spine density in dentate gyrus within 24 h. Dendritic spines in hippocampus are also decreased in rats with chronic epilepsy induced by tetanus toxin, as well as in other hippocampal and neocortical seizure models. In contrast, at least one study has reported a localized increase in dendritic spines in hippocampus following limbic seizures.

Given the controlled nature and timing of the animal studies, these experimental models strongly suggest that seizures themselves can directly lead to dendritic changes. However, because all these previous studies also relied on conventional histological analyses of fixed tissue from different subjects at different time points, it is still difficult to distinguish between a direct, specific effect of seizures and indirect, coincidental or compensatory processes. Furthermore, pathological studies only give isolated 'snapshots' in time and may miss more acute, dynamic changes that may occur on a more rapid time scale. To address these limitations, studies that can visualize the same dendrites serially over time in living tissue should be able to more directly and comprehensively assess the effects of seizures on spines. Remarkably, pioneering time-lapse live imaging studies have revealed that dendritic spines normally possess a great degree of motility and plasticity, on a rapid time scale of seconds, that was not previously anticipated based on fixed-tissue analysis. Thus, while previous fixed tissue experiments assayed spine changes only hours to days after seizures, modern time-lapse imaging techniques can test for very rapid effects of seizures on spines. We and others have recently used multi-photon microscopy to examine the direct, acute effects of seizures on dendritic spines imaged in vivo in several different rodent seizure models. Although the acute effects of seizures on dendritic structure may vary depending on conditions and seizure model, we have documented stereotypical, evolving changes in dendrites over a rapid time scale following seizures. We have also begun to determine the molecular mechanisms mediating this form of dendritic injury, focusing on regulation of the actin cytoskeleton.

Methods

We utilized modern cellular imaging techniques to assess seizure-induced morphological changes on dendrites and dendritic spines in living mice. Compared with conventional histological studies, these newer imaging methods, particularly multiphoton microscopy, allow repetitive, time-lapse imaging of dendritic spines within the living brain in vivo, so that the same dendritic spines can be directly monitored before, during, and after physiological or pathological perturbations over a rapid time scale of seconds to minutes, as well as for longer time periods.

Transgenic mice expressing enhanced green fluorescent protein (GFP) in subpopulations of pyramidal neurons in neocortex and hippocampus (*thy1* promoter, line GFP-M) have been used for all imaging experiments. Under anesthesia, a craniotomy window was produced surgically over frontal neocortex with a coverslip, which was sealed with dental acrylic. In some experiments, a cannula for drug application directly to the cortex, and neighboring EEG wire electrodes, were placed along the craniotomy window. Images of dendrites and dendritic spines of GFP-expressing neocortical pyramidal neurons were obtained through the cranial window with a Zeiss LSM 510 multiphoton microscope. A Coherent Titanium-Sapphire pulsed infrared laser was used to stimulate GFP at 900 nm. Low-magnification images, ~50–100 μm below the neocortical surface, were first obtained to identify regions with GFP-expressing dendrites. At higher magnification (5× digital zoom), Z-stacks of 6–10 images separated by 1 μm steps were taken of dendrites and accompanying spines.

In control experiments, the same dendrites and spines could be identified and followed serially under continuous anesthesia or while intermittently allowing the animal to recover from anesthesia in between images, over hours to days. For seizure experiments, control images of dendrites were taken under anesthesia prior to seizure induction and then the same dendrites were followed serially after seizure induction by various pharmacological agents. By these methods, we have examined the direct effects of two seizure models on dendrites: focal neocortical seizures induced by 4-aminopyridine (4-AP) applied locally to cortex, and secondarily generalized seizure induced by systemic kainate. Seizures were monitored by video-EEG or standard behavioral observation and staging.

We have also used standard histological, immunohistochemical, and molecular biological techniques to investigate the potential molecular mechanisms of seizure-induced dendritic changes. In particular, we have employed the rhodamine-phalloidin labeling method, which selectively assays changes in filamentous actin, to determine the effects of seizures on actin dynamics. In addition, we have utilized Western blotting to assess the effects of seizures on the expression of a variety of cell signaling molecules and actin-binding proteins that can regulate actin dynamics and potentially mediate structural changes in dendritic spines.

Recent Results

Focal 4-Aminopyridine Neocortical Seizures Increase Risk of Dendritic Injury under Limited Conditions

Using a model of neocortical partial seizures, initial studies examined the acute effects of focal seizures induced by 4-AP on dendritic structure of neocortical pyramidal neurons in vivo. The first set of experiments was performed under continuous isoflurane anesthesia. Time-lapse multiphoton imaging of GFP-positive dendrites of neocortical pyramidal neurons was performed through the same cranial window before and for several hours after local administration of ACSF or 25 mM 4-AP to neocortex. In ACSF-injected mice, no seizure activity was detected by EEG and the dendritic spines remained remarkably stable for the several hour period. Local infusion of 4-AP through the cranial window quickly induced repetitive electrographic seizures (without a clinical correlate) lasting several hours under anesthesia, but had no obvious effect on dendrites or spines. Quantitative analysis demonstrated that >95% of dendritic spines remained stable over several hours under both control and seizure conditions, with minimal loss of existing spines or gain of new spines. There was no significant difference between control and seizure conditions in spine turnover.

Since anesthetics have been reported to inhibit dendritic spine motility, we performed a second set of experiments to eliminate the potential protective effect of anesthesia during seizures. Similar methods were used as previously, except that after baseline control images of dendrites were obtained and 4-AP (or ACSF) was injected under isoflurane anesthesia, the mice were allowed to recover from anesthesia and have EEG-confirmed clinical seizures for 30 min in the absence of anesthesia. The mice were then reanesthetized, and using blood vessel landmarks as a reference, the same dendrites were reimaged for several hours to determine the acute effects of the preceding seizures. A dose-dependent response was observed in both clinical seizure severity and dendritic injury. With ACSF injection, no seizures were elicited and no significant spine changes were detected, comparing serial imaging before and for several hours after ACSF injection (**Figure 1(a)**). Lower doses (250 μM) of 4-AP induced stage 1–3 seizures with staring, head bobbing, and mild forearm clonus, but also had minimal effects on dendrites. Higher doses (1–2.5 mM) of 4-AP reliably resulted in stage 4 seizures with rearing and forearm clonus, but no falling or convulsions, and 25 mM 4-AP usually caused generalized convulsive activity (stage 5). After 30 min of stage 4 or 5 seizure activity, secondary dendrites from some animals exhibited acute swelling and beading, with associated loss of spines that was often irreversible over a several hour period (**Figure 1(b)**). However, there is an important caveat: The dendritic injury usually was more pronounced within the original field of imaging, compared with an adjacent (naïve) site outside the initial imaging field. This result suggests a possible synergistic effect of phototoxic damage (from the initial laser exposure) with severe seizures. As dendritic injury was not observed in ACSF-injected mice or

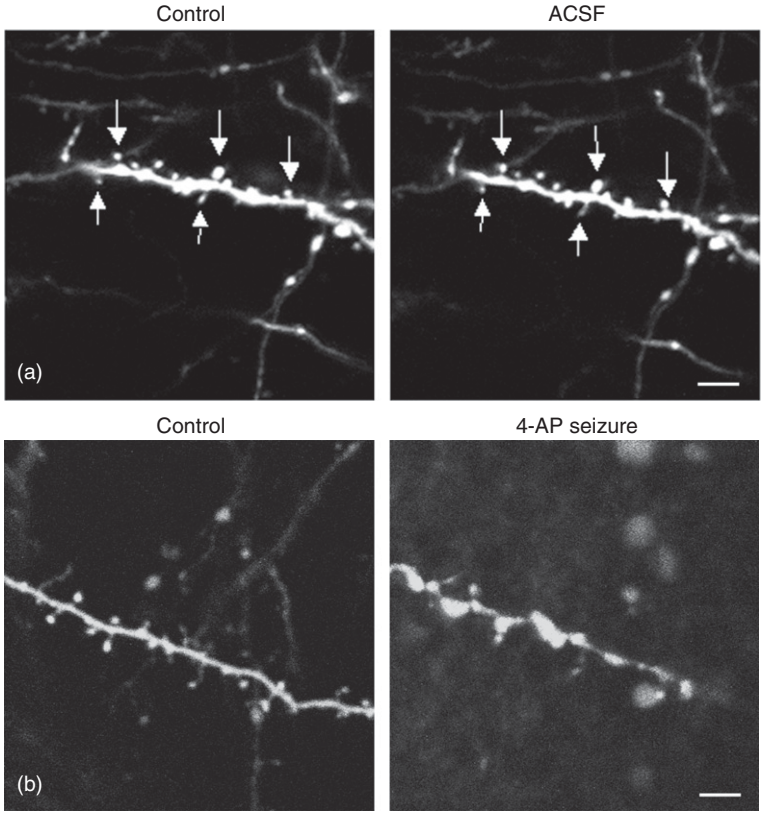


Figure 1 Seizures cause acute dendritic beading and spine loss. (a) Time-lapse in vivo multiphoton imaging of a neocortical dendrite of a control mouse performed serially before and after ACSF injection shows no significant changes in dendritic structure and spine number/position. Arrows mark examples of individual spines, demonstrating the stability of spines over time. (b) 4-AP induced seizures in unanesthetized mice cause acute dendritic beading and loss of spines within one hour of seizure termination. Scale bars = 5 μm for all images. Reproduced from Rensing *et al*. (2005) In vivo imaging of dendritic spines during electrographic seizures. *Annals of Neurology* 58: 888–898, with permission from Wiley.

mice with low stage seizures using identical imaging methods, these findings indicate that some neocortical seizures may increase the risk of dendritic injury when combined with other pathological stimuli. Although laser phototoxicity obviously represents an artificial stimulus, other pathophysiological conditions – such as hypoxia, hypoglycemia or hyperthermia – could be associated with seizures and may act synergistically to cause dendritic injury.

Secondary Generalized Kainate Seizures Cause Robust Dendritic Injury

Since the acute effects of focal neocortical 4-AP seizures on dendrites were relatively limited, and appeared to be contingent upon another pathological factor, we have explored other seizure models that may cause more robust dendritic injury. It is possible that seizure models involving other, more generalized, seizure types or utilizing different mechanisms of seizure induction may have stronger effects on dendrites. Thus, we have performed additional experiments in a different seizure model: the systemic kainate model. More severe kainate seizures, which originate in the hippocampus but then secondarily generalize throughout neocortex, may cause more extensive cortical activation than focal 4-AP seizures and involve direct glutamatergic stimulation. After obtaining control images under anesthesia, mice were injected with 30 mg kg^{-1} kainate i.p and allowed to recover from anesthesia. Following 30 min of severe behavioral stage seizures, the mice were reanethesized and the same dendrites were reimaged. Similar to the results with focal 4-AP injection, more severe behavioral stage (stage 4–5) kainate seizures caused an acute dendritic swelling, beading, and loss of spines. However, in contrast to the 4-AP seizures, the dendritic injury was seen equally in naïve regions of neocortex (i.e., areas that had not been previously imaged and so not exposed to potentially toxic effects of the laser), indicating that there was no synergistic effect with laser phototoxicity. Furthermore, the live time-lapse imaging revealed a rapid, dynamic evolution of dendritic abnormalities, with the dendritic beading often resolving within a couple hours, but spine loss persisting for much longer. Thus, these studies demonstrate more definitively that some seizures can cause direct injury

to dendrites, that the injury may evolve over a rapid time scale, and that different seizure types may have different effects.

Acute Seizure-Induced Dendritic Injury May Be Mediated by Calcineurin-Dependent Activation of Cofilin and Depolymerization of F-Actin

In addition to demonstrating acute dendritic injury in different seizure models, we have also started to investigate potential molecular mechanisms and signaling pathways mediating the effects of seizures on dendritic morphology, focusing on the regulation of the actin cytoskeleton of dendrites (**Figure 2**). Actin is a major structural protein that can exist in a monomer form (G-actin) or a polymerized filamentous form (F-actin); it is highly concentrated in dendritic spines, forming complex filamentous networks that provide structural support for dendrites and dendritic spines. Since physiological activity may modulate actin networks to cause changes in dendritic structure and function, we have examined the effect of both focal 4-AP neocortical seizures and systemic kainate seizures on F-actin levels, as assayed by rhodamine-phalloidin labeling. We found that both seizure types led to a significant decrease in F-actin in relevant cortical areas, indicating that seizures induce a depolymerization of actin filaments.

F-actin can be depolymerized by the regulatory actin-binding protein, cofilin. As cofilin is inactivated by phosphorylation, dephosphorylation of cofilin at the Ser3 residue leads to cofilin activation, which can trigger depolymerization of F-actin (**Figure 2**). Thus, we tested whether seizure-induced depolymerization of F-actin is related to a decrease in the phosphorylated form of cofilin (p-cofilin). By Western blot analysis, both 4-AP and kainate seizures had no effect on total actin or cofilin levels, but caused a significant decrease in p-cofilin expression in cortex. These studies indicate that seizures induce a rapid activation of cofilin and corresponding depolymerization of actin filaments in dendrites, which may, at least in part, account for the acute structural effects of seizures on dendrites.

We have also performed preliminary experiments to test the potential role of up-stream, activity-dependent signaling pathways in mediating seizure-induced regulation of the actin networks and dendritic structure. Cofilin and actin dynamics can be regulated by a number of upstream intracellular signaling elements, including a variety of phosphatases and kinases. In particular, the calcium-dependent phosphatase, calcineurin, is activated by seizures and may mediate regulation of cofilin activity by calcium (**Figure 2**). We utilized a calcineurin inhibitor to test whether calcineurin may be involved in the effects of kainate seizures on cofilin activation and dendritic morphology. Pretreatment with the calcineurin inhibitor, FK506, significantly blocked the seizure-induced decrease in p-cofilin in both hippocampus and neocortex. In addition, FK506 at least partially antagonized the effects of kainate seizures on dendritic beading and spine loss observed in the in vivo imaging studies. These results suggest that calcineurin may play a role in mediating the effects of kainate seizures on actin dynamics and spine morphology, and demonstrate that calcineurin inhibitors may have therapeutic potential in limiting seizure-induced dendritic injury.

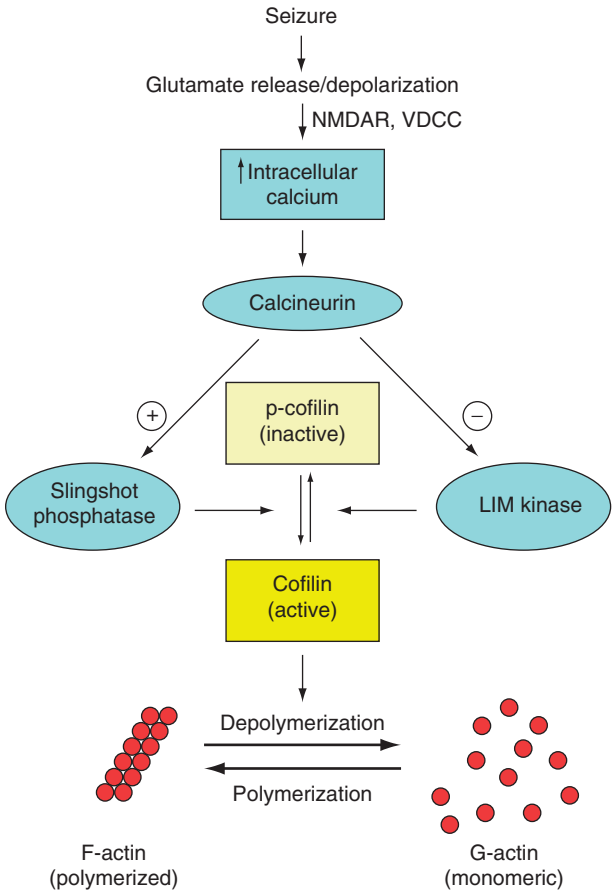


Figure 2 Putative signaling pathways and molecular mechanisms mediating seizure-induced dendritic injury. Seizures cause excessive glutamate receptor activation and neuronal depolarization, resulting in calcium influx into dendrites via NMDA receptors (NMDAR) and voltage-dependent calcium channels (VDCC). Elevated intracellular calcium can trigger a cascade of calcium-dependent enzymes, such as CaM kinase and calcineurin, causing complementary regulation of downstream opposing phosphatases (Slingshot) and kinases (LIM kinase) due to changes in phosphorylation states. Finally, activation (dephosphorylation) of cofilin (or actin depolymerizing factor) causes depolymerization of F-actin, which may lead to break-down of the normal actin cytoskeleton of dendrites.

Future Goals

Live in vivo time-lapse imaging methods have shown that seizures can directly cause dendritic injury and revealed a rapid evolution of acute dendritic changes that could not be detected by previous conventional fixed tissue histological studies. In addition, some of the molecular mechanisms and pathways mediating these seizure-induced changes in dendritic structure have begun to be elucidated. However, much additional work is needed to characterize seizure-induced dendritic injury and determine underlying mechanisms in more detail. First, there is some variability in the properties of dendritic abnormalities observed in different seizures models. Future studies should help determine what network, cellular, or molecular factors are most critical in determining the observed differences in propensity for dendritic injury (e.g., from kainate seizures versus the focal 4-AP model). Similarly, future studies will need to elucidate the potential synergistic roles of seizure-associated systemic factors – such as hypoxia, hypoglycemia, and hyperthermia – in promoting dendritic injury. Second, while previous fixed tissue experiments support the finding of a chronic seizure-induced spine loss, future time-lapse in vivo imaging studies over days to weeks should be performed to extend and verify the long-term time course of the observed acute, evolving dendritic changes. Finally, while work to date strongly implicates the involvement of the actin cytoskeleton, more detailed mechanistic studies are needed to further clarify and delineate the critical molecular pathways and mechanisms mediating seizure-induced dendritic injury. In particular, the role of other regulatory enzymes (e.g., LIM kinase, Slingshot, Rho-GTPases) and actin-binding proteins can be studied.

Of direct clinical relevance, further understanding of the molecular mechanisms mediating seizure-induced spine changes may finally lead to new therapeutic strategies for either treating seizures or preventing neurocognitive deficits in epilepsy. While most drugs for epilepsy have targeted neurotransmitter receptors and ion channels, an innovative therapeutic approach would be to modulate the structural components of synaptic plasticity, such as dendritic spine motility, in an activity-dependent fashion. As suggested by our preliminary data, inhibition of activity-dependent intracellular signaling elements, such as calcineurin, may limit seizure-induced dendritic injury. A more selective stabilization of the dendritic actin cytoskeleton during seizures, by drugs that directly regulate actin polymerization, could potentially help prevent seizure-induced spine changes and reduce associated cognitive deficits. Thus, better insights into the mechanisms of spine modulation by seizures could have significant impact in reducing the long-term negative consequences of epilepsy.

Acknowledgements

This work was supported by the National Institutes of Health (K02NS045583) and Citizens United for Research in Epilepsy (CURE)

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Behavioral Comorbidities in Children with Epilepsy; **Glia/Astrocytes**: Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; **Imaging**: Optical Monitoring of Circuitry Activity In vitro; **Networks**: Determinants of Neuronal Network Activity in Cultured Hippocampus; **Plasticity**: Dendritic Abnormalities in Epilepsy; The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis.

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Synaptic Potentiation and Inhibition after Partial Seizures

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Introduction

There are two major models of neural plasticity induced by brain stimulation – kindling and long-term potentiation (LTP). The kindling model involves brief but repeated stimulations that evoke electrographic seizures or afterdischarges (ADs), as was first reported by Goddard *et al.* in the late 1960s. Repeated stimulations (kindling) are accompanied by prolongation of the AD duration and increase in severity of the behavioral seizures. Although first proposed as a model of learning and memory, kindling is more appropriately described as a model relevant to epilepsy; indeed, the kindling process of increasing seizure duration and severity may be considered a process of epileptogenesis.

LTP is a long-lasting enhancement of synaptic transmission, first reported by Bliss and Lomo in the early 1970s for the synapse between the medial perforant path (MPP) and the dentate gyrus (DG). LTP is suggested to be a cellular correlate of learning and memory, and many glutamatergic synapses are capable of LTP. The cellular mechanisms and behavioral significance of LTP have been intensively studied. Ca^{2+} influx into the postsynaptic cell is essential to activate various kinases, resulting in long-lasting postsynaptic increase in the number and sensitivity of α-amino-3-hydroxyl-5-methyl-isoxazole-4-proprionate (AMPA) receptors, and/or an increase in presynaptic release of glutamate. Coincidence of pre- and postsynaptic activity is important for a type of LTP (Hebbian LTP) that depends on Ca^{2+} influx through *N*-methyl-D-aspartate (NMDA) receptors. In addition, LTP may be mediated by Ca^{2+} influx through voltage-dependent Ca^{2+} channels or other Ca^{2+}-permeable channels and on Ca^{2+}-induced Ca^{2+} release. Long-term depression (LTD) is the converse of LTP, and it may be induced with low-frequency stimulation that causes a smaller Ca^{2+} influx than that during LTP induction.

Stimulations that evoke LTP, without evoking ADs, contribute little to the kindling process (i.e., to increasing AD duration and seizure severity) (**Table 1**). Further, LTP stimulations do not induce the clear behavioral changes in animals seen with kindling (e.g., changes in emotional and psychiatric symptoms) (**Table 1**). However, short-term neural plasticity (e.g., changes that last for several milliseconds to minutes after stimulation, as induced by posttetanic potentiation or paired-pulse facilitation, may help to augment and propagate a seizure.

The term 'seizure-induced potentiation' (SIP) will be used here to describe the synaptic potentiation that is induced by a seizure or an AD, as distinct from LTP that is induced by stimulation without evoking an AD. Two models of partial epilepsy, kindling and status epilepticus (SE), are considered in this article.

Background

SIP often occurs during the process of kindling. During kindling by stimulation of the MPP, SIP was found after the first AD, but incremental SIP was small with subsequent ADs. However, SIP was not found during kindling of some pathways (e.g., at the lateral entorhinal cortex (EC)-to-DG

Table 1 Summary of phenomena induced by repeated afterdischarges or long-term potentiation in the hippocampus or amygdala

Phenomenon	*ADs*	*LTP*
AD duration	increase	NC
Seizure severity	increase	NC
AD threshold (amygdala)	decrease	NC
Facilitates kindling	sometimes	no
Spontaneous interictal spikes	increase	minimal change
Monosynaptic potentiation	yes	yes
Trans-synaptic hippocampal potentiation	yes	yes, less persistent
CA1 inhibition ($GABA_A$)	decrease	NC/increase
Psychiatric symptoms	increase	NC
Emotional behaviors	increase	NC
Spatial maze performance deficit	sometimes	sometimes
Retention in radial arm maze	decrease	NC

ADs are induced by tetanic stimulation; LTP is elicited without an AD.
ADs, afterdischarges; LTP, long-term potentiation; NC, no change.

synapse). Changes in epileptiform activity, such as increase in AD severity and duration and increase in spontaneous interictal spikes, are reported to have a different pharmacology from LTP or SIP. Blocking NMDA receptors blocks SIP of the MPP–DG synapse; kindling is slowed but not abolished. Similarly, antagonizing NMDA receptors blocks an AD-induced potentiation of basal dendritic synaptic transmission in CA1 (**Figure 1(a)**). However, NMDA receptor blockade does not block the AD-induced interictal spikes in CA1 (**Figure 1(b)**). Many interictal spikes resemble large basal dendritic field excitatory postsynaptic potentials (fEPSPs), with a negative wave at the basal dendrites and a positive wave at the cell body layer and apical dendrites. However, SIP is not essential for the interictal spikes.

Considerable evidence supports an alteration of inhibition after partial seizures. $GABA_A$ receptors in the DG are persistently altered following SE and kindling. After seizures, mini- or evoked $GABA_A$-mediated inhibitory postsynaptic currents (IPSCs) are increased in DG granule cells. These IPSCs, in tissue from animal models or from temporal lobe epilepsy patients, are more sensitive to blockade by Zn^{2+} and insensitive to the benzodiazepam type1-specific agonist zolpidem, possibly because of a relative decrease in α1-subunit expression. GABAergic interneurons may be functionally disconnected from their excitatory inputs ('dormant basket cells'), which may explain functional disinhibition despite preservation of GABAergic neurons. However, specific types of interneurons may be lost, as suggested by the differential loss of inhibition at dendritic and not somatic synapses. Also, not all brain areas show loss of inhibition; indeed, after hippocampal kindling, inhibition is increased in the DG, but decreased in CA1 and CA3.

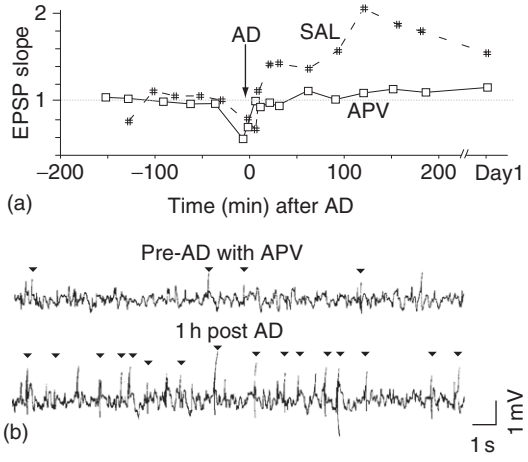


Figure 1 Potentiation of field excitatory postsynaptic potentials (fEPSPs) and interictal spikes in CA1 after an afterdischarge (AD) evoked by tetanization of the basal dendritic CA1 afferents in freely behaving rats. (a) Slope of the basal dendritic fEPS, normalized by the average fEPSP slope before each AD, showed a long-lasting potentiation after an initial depression, when saline (SAL) was infused intracerebroventricularly (i.c.v.); the AD-induced potentiation in the same rat was blocked when NMDA-receptor antagonist APV (D,L-2-amino-5-phosphonovaleric acid) was infused i.c.v., in another experiment several days later. (b) APV, given i.c.v., did not block the increase in frequency of spontaneous interictal spikes (marked by inverted triangles) induced by a hippocampal AD. APV alone did not change the frequency of interictal spikes, unpublished figure from study of Leung (1994). (a) From Leung LS and Shen B (1993) Long-term potentiation in hippocampal CA1: Effects of afterdischarges, NMDA antagonists and anticonvulsants. *Experimental Neurology* 119: 206.

Synaptic plasticity may be altered after partial seizures. The magnitude of LTP in the hippocampus and amygdala is diminished in animals after amygdala kindling and in the human DG near the primary focus of temporal lobe epilepsy. The capacity for synaptic plasticity, or the plasticity of synaptic plasticity, is called 'metaplasticity.' A synapse may show altered synaptic plasticity (LTP or LTD) without manifesting a change in synaptic strength. Synaptic metaplasticity may depend on all the factors that affect synaptic plasticity, including NMDA receptors, Ca^{2+} channels, metabotropic glutamate receptors, inhibition, and kinase function (protein kinase C, protein kinase A or Ca^{2+}/calmodulin kinase). Since partial seizures cause massive pre- and postsynaptic activities that affect the signaling pathways for synaptic plasticity, the effects of partial seizures can be regarded as 'metaplasticity.' An important major rationale for studying the altered capacity for synaptic plasticity after partial seizures is that it may be a mechanism of postictal and interictal cognitive and behavioral deficits.

Methods

We studied changes in glutamatergic and GABAergic transmission after hippocampal kindling. Adult male rats were implanted chronically with indwelling electrodes for stimulation and recording. Electrophysiological criteria were used to guide the recording electrodes to straddle the CA1 or the DG cell layer. Stimulating electrodes were placed in the MPP, and in CA1 to stimulate the basal or apical dendritic afferents to CA1. Freely moving rats were recorded at least 4 days after surgery. Before and after each AD, fEPSPs were recorded in CA1 and the DG following single or paired-pulse stimulation of the MPP, or of the basal and apical dendritic afferents to CA1. ADs during kindling were evoked by high-frequency stimulation in CA1 at hourly intervals, 5 times a day. Results described here followed partial hippocampal kindling, using 10–15 ADs.

In a different approach, in vitro transverse hippocampal slices were prepared at various times (1 day to 6 weeks) after CA1 kindling. fEPSPs were recorded in CA1 and DG. A theta-frequency primed burst (PB) stimulation of CA1 stratum radiatum (SR) was used to induce LTP. One PB consisted of 2 priming pulses at 100 Hz

followed 170 ms later by a train of 8 pulses at 100 Hz; the PBs were repeated 8 times at 10-s intervals. In other experiments, the monosynaptic IPSC in CA1 was evoked in the presence of ionotropic glutamatergic antagonists, following paired-pulse stimulation of the local interneurons in SR (apical dendritic layer).

Hippocampal electrophysiology was also examined in urethane-anesthetized rats at various times after a SE induced by intracerebroventricular (i.c.v.) injection of kainic acid (KA) in adult male rats. Laminar profile of the field potentials in CA1 and DG was recorded simultaneously by a 16-channel silicon probe, or sequentially mapped by a micropipette at 50-μm depth intervals. Current source density was analyzed from the depth distribution of field potentials at one instant of time. A current sink is a macroscopic measure of net inward current, and current source is a measure of net outward current, in a fixed volume. In a cortical structure such as the hippocampus, the time course and location of dendritic excitation are revealed by current source density analysis.

Results

Kindling Results in Potentiation of Multisynaptic Pathways

Robust SIP of the basal dendritic responses occurred during partial hippocampal kindling. The basal dendritic fEPSP in CA1 was increased after the 1st AD, and appeared saturated after the 5th and 10th AD (**Figure 2**). No behavioral convulsion was evoked, but the AD duration increased with the number of ADs evoked, indicating the process of kindling. The MPP-to-DG synapse, which was activated trans-synaptically by commissural (CA3 axon) stimulation, showed a robust and long-lasting potentiation (SIP). SIP of the MPP–DG synapse was shown by the increase of the population spike (extracellular compound action potential) generated in the DG (PS1 and PS2 in **Figure 2(b)**). The increase in the DG population spikes and MPP-evoked multisynaptic response in CA1 (Sch1 and Sch2 in response to LMPP stimulation in **Figure 2(b)**) persisted at least 27 days after kindling. In contrast, SIP at the basal dendritic CA1 synapse typically lasted only ~5 days after kindling. SIP at the apical dendritic CA1 synapse (not shown) may be small but long lasting (>27 days after kindling). The cross temporo-ammonic response (RMPP-evoked responses in **Figure 2(b)**) was minimally altered during and after kindling.

In summary, hippocampal kindling induced SIP in a multisynaptic circuit, 'propagating' to several synapses beyond the directly activated synapse. The persistently potentiated MPP-to-DG synapse was at least two synapses away from the directly activated CA3-to-CA1 synapse (**Figure 2(a)**). During a hippocampal AD, the synchronous firing of principal cells likely recruits neurons several synapses away. The frequency of synchronous firing during an AD is typically 2–15 Hz, which is capable of inducing LTP or recruiting other neurons by frequency potentiation. A temporal lobe AD is known to increase metabolic activity in many areas of the brain, including the thalamus, substantia nigra, and nucleus accumbens.

Excitation, Inhibition, and LTP In Vitro after Kindling

When hippocampal slices from partially kindled rats were recorded in vitro, on one day or 3 weeks after kindling, there was no significant difference in the single-pulse-evoked fEPSP in CA1, CA3, and DG in vitro. However, paired-pulse facilitation of the fEPSP, and in particular of the population spike, was increased in CA1 and CA3, but decreased in the DG in kindled rats than in control rats.

When monosynaptic $GABA_A$-mediated IPSCs were recorded, paired-pulse depression (PPD) of the IPSCs was strong in control rats, but much smaller in kindled rats (**Figure 3(a)**). PPD of the IPSCs is mediated by $GABA_B$ receptors on GABAergic terminals (autoreceptors), and it is blocked by a $GABA_B$ receptor antagonist. This result suggested that kindled rats show a decrease in $GABA_B$ autoreceptor efficacy. The amplitude of the single-pulse IPSC was not significantly different between kindled and control rats, but the decay of the IPSC was faster in neurons from kindled rats than those from control rats (**Figure 3(a)**).

When LTP was induced by theta-frequency PBs in vitro, slices from partially kindled rats showed decreased LTP magnitudes as compared with control rats (**Figure 3(b)**). In the presence of a $GABA_B$ receptor blocker, PB-induced LTP was suppressed in both control and kindled rats. The decrease in efficacy of the $GABA_B$ autoreceptors was suggested to account for the smaller PB LTP in kindled rats than in control rats.

Palizvan *et al.* recently reported another change in LTP associated with kindling. They showed that the mechanism of nonprimed high-frequency LTP is different before and after kindling. LTP in CA1 is normally dependent on NMDA receptors, but it becomes increasingly dependent on voltage-dependent Ca^{2+} channels after pentylenetetrazol-induced kindling.

Multisynaptic Alterations of Entorhinal-Hippocampal Circuit after Kainic Acid SE

Similar to kindling (**Figure 2**), multisynaptic changes were found in the EC–hippocampus circuit (**Figure 4(a)**) in vivo after SE induced by KA. Synaptic excitation was assessed by mapping a depth profile of the field potentials in rats under urethane anesthesia, and current source density analysis was used to reveal the extracellular current sink (inward current) at particular layers of the hippocampus. Increase in single-pulse excitatory response was found for the MPP-to-DG synapse and for the monosynaptic

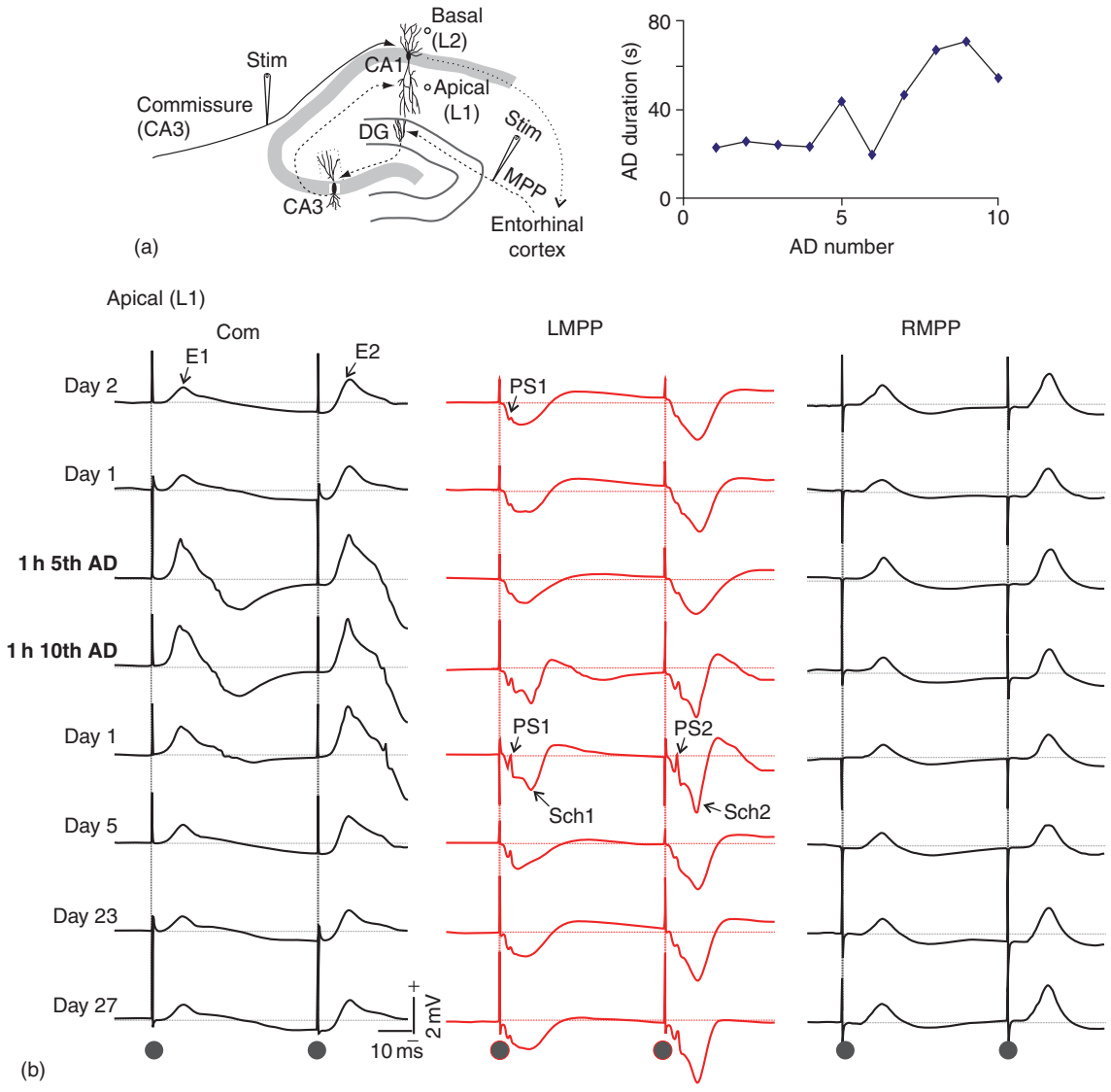


Figure 2 A schematic diagram showing stimulation and recording configuration, and increase in afterdischarge (AD) duration during partial kindling of the right CA1 in a representative rat. fEPSPs were recorded in left CA1 following paired-pulse stimulation of right CA1 (commissural), left and right medial perforant path (MPP) derived from the entorhinal cortex. L1 electrode shows a large increase of fEPSP in left CA1 during and within 5 days after kindling (10 ADs). Population spikes (PS1 and PS2 after 1st and 2nd pulse respectively) and polysynaptic CA1 responses (Sch2 after 2nd LMPP pulse) were enhanced until day 27 after kindling. Cross temporo-ammonic (RMPP) response was largely unchanged. Unpublished data from the study of Leung and Shen (2006).

excitation of MPP at the distal dendrites of CA1. However, single-pulse CA3-to-CA1 transmission was decreased in KA-treated rats than in control rats, likely because of CA3 cell death. In addition, mossy-fiber excitation of the inner molecular layer of the DG in vivo was decreased in KA-treated rats than in control rats, possibly due to increased inhibition in the DG (despite mossy-fiber sprouting).

Robust changes were found for paired-pulse responses in KA-treated rats than in control rats, in both the DG and CA1. Inhibition was measured by the population spike response to paired-pulse stimulation. The population spikes evoked by the 1st and 2nd stimuli of MPP were measured as PS1 and PS2, respectively (**Figure 4(b)**); the PS2/PS1 ratio at 30–100-ms interpulse interval was significantly smaller in KA-treated rats than in control rats. Interestingly, a small i.c.v. dose of $GABA_A$ receptor antagonist bicuculline also *decreased* the PS2/PS1 ratio in control rats (**Figure 4(c)** top trace). The same bicuculline dose given to KA-treated rats induced spike bursts in the DG after MPP or CA3 stimulation (**Figure 4(c)**). Thus, the enhanced inhibition in the DG was 'fragile,' and this fragility may be associated with the alteration of $GABA_A$ receptors after SE.

Paired-pulse inhibition in CA1 was decreased after KA treatment. This change is shown by the increased PS2/PS1 ratio in KA-treated rats than in control rats (**Figure 4(b)**). In addition, an extracellular (population) backpropagating

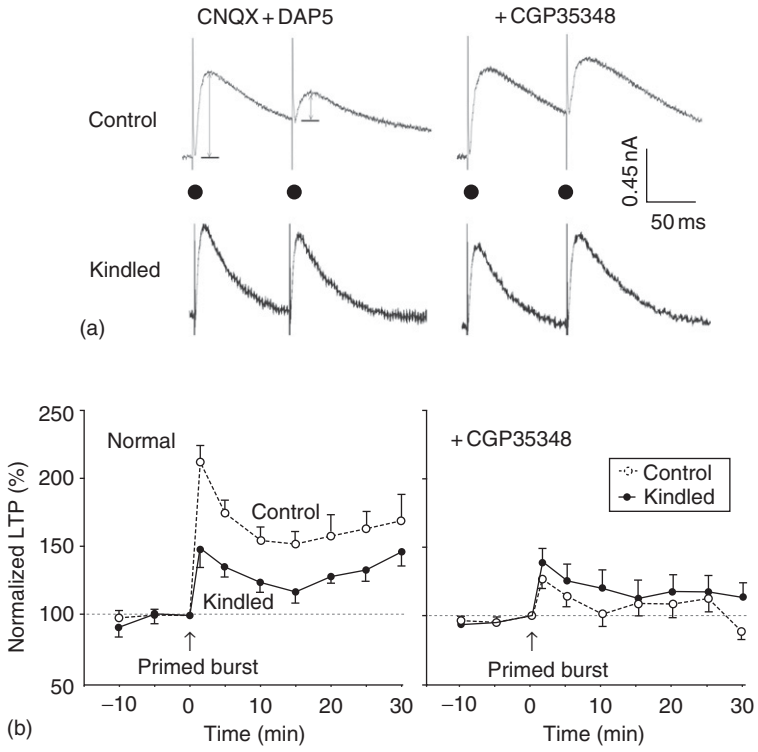


Figure 3 In vitro hippocampal slice recorded one day after partial hippocampal kindling (15 ADs). (a) Monosynaptic IPSCs recorded in CA1 pyramidal cells following paired-pulse local stimulation in the presence of ionotropic glutamate antagonists, without (left) and with (right panel) addition of a $GABA_B$ receptor antagonist CGP35348 (1 mM). A decrease in $GABA_B$ autoreceptor function in the kindled rat was indicated by the decreased paired-pulse depression of the IPSCs. Adapted from Wu C and Leung LS (1997) Partial hippocampal kindling decreases efficacy of presynaptic $GABA_B$ autoreceptors in CA1. *Journal of Neuroscience* 17: 9261–9269. (b) Apical dendritic fEPSPs recorded at the apical dendrites of CA1 showed LTP after theta-frequency primed bursts. LTP was larger in control slices than in kindled slices, but no difference was found when LTP was induced in the presence of CGP35348. Data from Leung LS and Wu C (2003) Kindling suppresses primed-burst-induced long-term potentiation in hippocampal CA1. *Neuroreport* 14: 211–214.

dendritic spike was shown to invade the distal dendrites of CA1 pyramidal cells in KA-treated rats but not in control rats, likely because of a loss of distal dendritic inhibition. In control rats, the CA3-evoked population spike in CA1 propagated to the middle part of SR, similar to the first-pulse evoked spike (* in **Figure 4(d)**). After KA treatment, the population spike evoked by the second-pulse invaded further into the distal dendrites (** in **Figure 4(d)**), and a 'late' spike started from the distal dendritic layers of CA1. The late spike was activated by polysynaptic excitation – CA3 excited the EC that excited CA1. A backpropagating distal dendritic spike in CA1 in SE rats has also been reported in vitro.

Seizure-Induced Potentiation and Behavioral Changes

SIP may change the synaptic strengths of behaviorally related circuits, via trans-synaptic mechanisms, and these synaptic changes may cause postictal and chronic behavioral changes (**Table 1**). One example is that kindling of the hippocampus induces schizophrenia-like behavioral changes, such as hyperactivity, loss of sensorimotor gating, and abnormalities in social interactions. These changes may in part be mediated by the hippocampal projection to the nucleus accumbers and ventral tegmental area. Affective defensive behaviors in cats were attributed to potentiation of excitatory transmission from the amygdala to ventromedial hypothalamus/periaqueductal gray after partial amygdala kindling. Partial hippocampal kindling (repeated ADs) in CA1 induces a deficit in retention of a spatial task, and the duration of retention deficit correlates with the duration of altered synaptic transmission in the hippocampus.

Future Goals

Understanding of the mechanisms of SIP is incomplete. Mechanisms of LTP are unlikely to account for all aspects of SIP. Since seizure or repeated seizures is capable of altering cellular biochemistry and molecular biology of neurons and glialcells in ways beyond LTP, it is expected that SIP will involve multiple receptors and signaling pathways, but these signaling pathways remain to be studied in detail.

Some comparisons between seizure (kindling) and LTP may be inappropriate, because a seizure (and

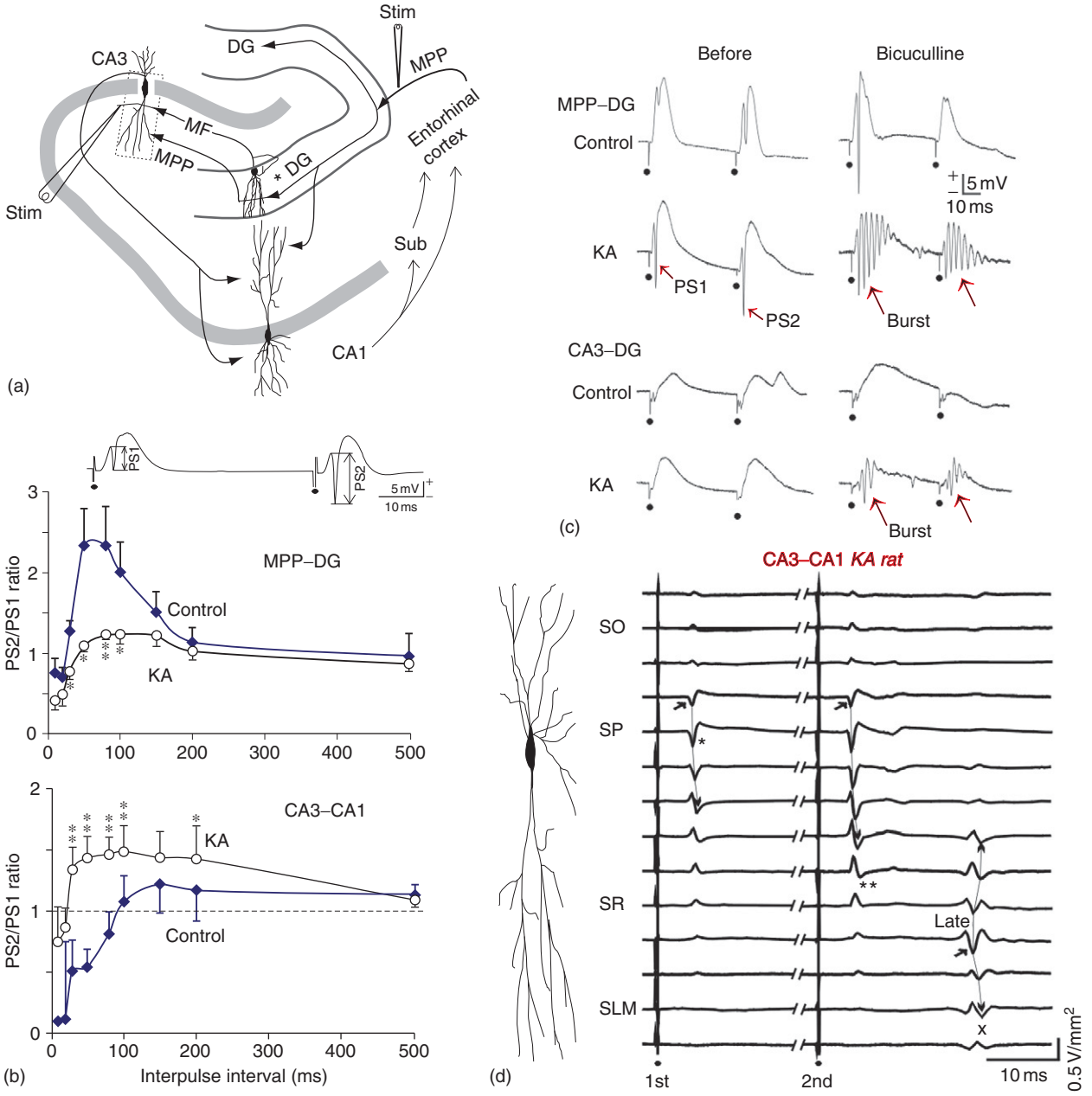


Figure 4 Changes in the entorhinal cortex–hippocampal circuit after kainic acid (KA) or control treatment. (a) Schematic diagram of circuit involving dentate gyrus (DG), CA3, CA1, subiculum (Sub), MPP, and mossy fibers (MF). (b) Paired-pulse facilitation in CA1 (after CA3 stimulation) and paired-pulse depression in DG (after MPP stimulation) are shown in KA-treated rats than in control rats (group difference, $^{*}p < 0.05$, $^{**}p < 0.01$). (c) Evoked field potentials recorded at the DG cell layer, before and after injecting bicuculline i.c.v. Control rat showed paired-pulse depression after bicuculline stimulation, while KA-treated rat showed bursting (arrow) after MPP or CA3 stimulation. (d) Current source density in CA1 after paired-pulse (50-ms interpulse interval) CA3 stimulation in a KA-treated rat. The 1st pulse evoked an early population spike (*) that started in stratum oriens (SO) near stratum pyramidale (SP) and propagated to stratum radiatum (SR). A population spike of ~20-ms latency (labeled 'late') was initiated by the 2nd pulse at distal SR (arrow) and propagated proximally and distally to stratum lacunosum-moleculare (SLM). (a) and (c): Reproduced from Wu K and Leung LS (2001) *Neuroscience* 104: 379–396; (b) and (d): Reproduced from Wu K and Leung LS (2003) Increased dendritic excitability in hippocampal CA1 in vivo in the kainic acid model of temporal lobe epilepsy: A study using current source density analysis. *Neuroscience* 116: 599–616, with permission from International Brain Research Organization.

epileptogenesis) requires many synapses while LTP studies are typically restricted to single synapse. Thus, the absence or blockade of LTP or SIP at a single synapse does not exclude the participation of LTP or SIP at other synapses in distributed and multisynaptic neural circuits. In addition, involvement of NMDA receptor-independent LTP in kindling and epileptogenesis has not been thoroughly tested.

Short-term neural plasticity that lasts several milliseconds or minutes after stimulation may help to augment and propagate neural activity, including epileptiform activity, across synapses. The causes and effects of short-term potentiation after seizures have not been fully addressed. The results reviewed in this article indicate that paired-pulse excitatory and inhibitory responses are changed after kindling and SE, and these changes may increase frequency potentiation during normal and abnormal activity, and perhaps increase seizure susceptibility. Conversely, short-term and LTD may have therapeutic significance since they may suppress seizure generation and propagation, but investigations of how these processes may affect seizure generation are preliminary.

Seizure-induced alteration in synaptic metaplasticity may contribute to cognitive and behavioral deficits, since hippocampal LTP may be a mechanism of spatial navigation and declarative memory. The mechanisms of metaplasticity remain to be studied, and so far, the relation between LTP and behavioral deficits after seizures is tentative and purely correlational. The ability of partial seizures to alter excitatory and inhibitory synapses in multisynaptic circuits suggests that neural network changes may underlie the behavioral deficits and seizure susceptibility that are characteristic of partial epilepsy.

See also: **GABA**: Pathophysiology of Status Epilepticus; **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Plasticity**: LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures.

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Synaptic Reorganization in the Hippocampal Dentate Gyrus

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Introduction

The hippocampus is one of the most plastic neural structures, and that plasticity is thought to be particularly important in epileptogenic processes. A major subject of analysis with respect to hippocampal plasticity is the sprouting of dentate granule cell axon collaterals, primarily to the region of their own dendrites within the dentate gyrus (DG) – the mossy fiber sprouting (MFS) that occurs in both human mesial temporal lobe sclerosis and in

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several animal models of temporal lobe epilepsy. It has been proposed that MFS, both in humans and experimental animals, could be responsible for epileptogenesis in temporal lobe epilepsy. The aberrant reorganization of the dentate mossy fiber system has been touted as one of the leading bases for hyperexcitability that underlies hippocampal seizures. It is becoming clear, however, that MFS is only one side of a more involved tale. Regardless of its relevance to epilepsy, the robustness of MFS as a plastic phenomena allows us to investigate issues that might be relevant to synaptic reorganization in general, whether adaptive or not.

Background

In a significant number of patients with temporal lobe epilepsy, several types of studies (imaging, electrophysiological, and psychological) suggest a seizure focus in the hippocampus (in at least one side of the brain). Diagnosis of this localization often leads to surgery, and adequate removal of the structures underlying the irritative focus can cure the epileptic condition. This apparently direct cause-and-effect relationship indicates the need for the investigation of the features present in the resected brain structures that underlie the epilepsy. Early pathologists, studying tissue from autopsies, described a hardening of the mesial portions of the temporal lobe and coined the term 'mesial temporal sclerosis.' The medial temporal lobe consists of the hippocampal region (CA fields, DG, and subicular complex) and the adjacent perirhinal, entorhinal, and parahippocampal cortices. Analysis of this brain area in patients with epilepsy and in animal models of epilepsy, often reveals marked atrophy and cell loss. More importantly, in the late 1980s and early 1990s, several authors described an impressive synaptic plasticity in this region, the MFS.

Connectivity

Mossy fibers are the axons of dentate granule cells in the hippocampal region. In the normal hippocampus, these axons of granule cells elongate within the dentate hilus and stratum lucidum, and innervate hilar cells and CA3 pyramidal cells. In temporal lobe epilepsy, however, mossy fibers 'sprout' out of the dentate hilus, and cross the granular cell layer into the inner molecular layer. These sprouting axon collaterals form new – aberrant – synaptic connections with the dendrites of granule cells in the inner third of the molecular layer or with other cellular elements in the dendritic fields of CA2 and CA1. As a result, some of the granule cells become monosynaptically interconnected. Mossy fibers not only sprout into the molecular layer just above the granular layer (giving rise to supragranular MFS) but also within the CA3 stratum oriens region and into CA2 and CA1, giving rise to MFS onto pyramidal cells. The best studied of these phenomena, in terms of a potential epileptogenic mechanism, is the supragranular MFS.

The organized laminar nature of the inputs that contact normal dentate granule cell dendrites is maintained in the epileptic tissue. Consistent with this generality, mossy fibers sprouted to the DG have a projection field that is restricted to the inner molecular layer. The factors underlying the selectivity of this aberrant projection have been one of the subjects of interest in our laboratory.

Functional Relevance

Investigators first demonstrated that in slices from control rats, antidromic stimulation of the mossy fibers elicited a single population spike in the granular layer of the fascia dentata. They then showed that, in contrast, when slices from kainic acid-treated epileptic rats were similarly tested, antidromic stimulation elicited multiple population spikes. The study showed that abnormal responses to antidromic stimulation correlated with the loss of CA4 (i.e., hilar) neurons and the growth of supragranular mossy fiber collaterals in the same animals. The authors suggested that aberrant supragranular mossy fiber collaterals form a functional recurrent excitatory circuit that could facilitate seizure activity. Futher, loss of hilar neurons was seen as a key, indissociable element of supragranular MFS.

Failed Attempts to Block MFS

Given its position as a likely culprit in temporal lobe epilepsy, a number of experimental strategies were devised to suppress or modulate supragranular MFS, and thus directly assess the role of sprouting in epileptogenesis. Infusion of antibodies against NGF in animals subjected to kainate- or pilocarpine-induced status epilepticus (SE), while effective in modulating the sprouting of cholinergic fibers to the DG inner molecular layer, failed to influence supragranular MFS. In a subsequent series of experiments using knockout mice or the intracerebral infusion of neurotrophic factors, the contribution of tyrosine kinase B (trkB), trkA, and trkC receptors, c-Fos, neurotrophin 3 (NT3), and brain derived neurotrophic factor (BDNF) for MFS were evaluated by several groups. While all of these neurotrophins were found to be relevant (i.e., able to either impair or promote MFS in animal models of epilepsy), none of those experiments was able to completely abolish MFS.

An important source of evidence for the link between supragranular MFS and epilepsy was observed (drawn from both kainate and pilocarpine models) that the more intense the SE and more widespread the cell loss, the greater was the ensuing supragranular MFS and greater the likelihood of spontaneous recurring seizures. In some

of these studies, the authors reported a lack of supragranular MFS; however, this observation should be viewed with caution given that in those experiments the investigators suppressed or greatly attenuated the lesional event (e.g., SE). As expected, animals of a nonepileptic background that do not undergo sufficient hyperexcitability (i.e., SE) and related hilar cell death, will not develop any of the plastic changes associated with epilepsy.

Methods

When we initiated our studies, some of the evidence mentioned earlier was already available. We, as most authors in the field, hypothesized that effective blockade of supragranular MFS would be associated with a substantial reduction in hyperexcitability, and possibly in attenuation or suppression of the subsequent development of spontaneous seizures. To increase the effectiveness of such a blockade, we considered the use of a protein synthesis inhibitor in close association with the induction of SE. Given the profound changes likely to be associated with such a treatment (i.e., in terms of the activation of both transcription and translation), we opted for a broadly acting agent to achieve our goals. We chose cycloheximide. To eliminate the possibility that the obtained results were due to some effects of cycloheximide other than to its well-established actions on transcription and translation, we also tested anisomycin, an antibiotic that is a potent inhibitor of protein synthesis.

The choice of animal model was also based on the already available evidence. The pilocarpine model of SE had the advantage of inducing the most robust supragranular MFS, as well as providing a high frequency of later spontaneous seizures. Disadvantages of the model included the fact that seizures could arise form several different sources, especially given the systemic nature of the administration of pilocarpine and the associated widespread brain damage. To address this problem, we also tested the effects of cycloheximide in the small dose intrahippocampal model of kainic acid. Finally to further broaden the scope of the findings with cycloheximide, we evaluated its ability to affect supragranular MFS in mice subjected to pilocarpine-induced SE.

The main resource used in the evaluation of the effects of cycloheximide and anisomycin (in both pilocarpine- or kainate-induced SE animals) was a histological technique to evaluate the presence of mossy fibers. Timm staining detects the high content of zinc in the mossy fiber terminals, yielding a high contrast picture of the mossy fiber axons and terminals, and thus a reliable way to detect changes in this axonal system. In all of our studies, we used smaller concentration of sodium sulfide (0.1% as opposed to the usual 1%) than typically employed, thus providing a greater specificity for the detection of mossy fibers. Given that Timm staining requires a 'development' process analogous to that used in film photography, the intensity of staining and thus sensitivity of the method can be adjusted as desired. Additional techniques used in our study included observation of behavioral spontaneous seizures, standard behavioral tests of cognitive and emotional function, electroencephalographic (EEG) recordings for the assessment of SE and for the detection of spontaneous seizures, slice electrophysiology for the functional evaluation of the reorganized dentate circuitry, staining for injured cells, evaluation of the mitotic rate of dentate granule cells, and immunohistochemical approaches for the detection of specific neuronal populations.

Results

We initially demonstrated that administration of cycloheximide, in close proximity to the induction of pilocarpine SE, blocked supragranular MFS in rats – but did not prevent the subsequent development of spontaneous seizures or affect their frequency. Those results provided, for the first time, direct evidence against a causal role for supragranular MFS in temporal lobe epileptogenesis. One possible explanation for this dissociation is that seizure generation in the pilocarpine and kainate models of epilepsy could be driven by extrahippocampal sources. Indeed, the relative contributions of thalamus, amygdala, and piriform cortex in the initiation of spontaneous seizures in these models have been indicated in a number of studies.

To address this issue, we next used the small dose intrahippocampal application of kainic acid. Here again, cycloheximide was able to completely block supragranular MFS in most animals, without altering the frequency and intensity of spontaneous ictal and interictal EEG events. We concluded that also in the intrahippocampal small dose kainate model, where spontaneous seizures were more likely to initiate in the injected hippocampus, supragranular MFS is not necessary for the occurrence of ictal events.

Given that most of our analysis in the initial experiments evaluated animals 2 months after the initial precipitating injury, we then sought to investigate how supragranular MFS would be affected by the spontaneous seizures. Some publications on this subject have shown that animals with more frequent seizures have a more intense supragranular MFS, thus suggesting that this form of synaptic reorganization is somehow associated with a greater propensity to seize (even if it does not determine whether or not seizures emerge). Other investigators have argued that the density of supragranular MFS is not associated with the total number of lifetime seizures or the seizure frequency (in experimental or human temporal lobe epilepsy), and many authors no

longer consider MFS as a necessary or sufficient event in temporal lobe epilepsy. However, in the human condition we have no control on the background status of the tissue. And in most experimental studies, SE was required for the emergence of seizures – where there is no question that supragranular MFS is largely the reflection of a preceding SE episode. By producing an experimental condition in which no supragranular MFS was derived from the initial SE episode, we were able to investigate the relative contribution of subsequent spontaneous seizures to this synaptic reorganization. We concluded that supragranular MFS did not show any association with the frequency of spontaneous seizures, and seemed to depend more critically on time after the initial injury. Indeed, under this condition, supragranular MFS eventually developed in all animals, even though at a much slower pace; further, it did not seem to be more robust in animals with greater frequency of recurrent spontaneous seizures.

The description of granule cell dispersion in human tissue from patients with temporal lobe epilepsy gave rise to the suggestion that developmental changes could be at the base of the subsequent epilepsy in those patients. The demonstration, first in the pilocarpine rat model and later in kainate-treated mice, that adult rodents could develop granule cell dispersion indicated that these cytoarchitetonic changes could also be the consequence of the initial precipitating episode of SE. The subsequent demonstration that neurogenesis of dentate granule cells was increased after SE introduced an additional possibility – that hippocampal network plasticity associated with epileptogenesis could arise from aberrant connections formed by newly born dentate granule cells. We, therefore, investigated whether cycloheximide would affect the onset of supragranular MFS by means of blocking granule cell neurogenesis. Much to our surprise, we observed that cell proliferation in the DG is dramatically increased in epileptic animals treated with cycloheximide. Induction of SE in animals injected with pilocarpine alone led to 2–7-fold increases in the mitotic rate of dentate granule cells, as compared to 5–30-fold increases for pilocarpine/cycloheximide-treated animals. We suggested that such increased mitotic rates were associated with a protection by cycloheximide of a vulnerable precursor cell population that would otherwise degenerate after pilocarpine-induced SE. We further suggested that MFS and neurogenesis of granule cells are not necessarily linked to one another. This dissociation is consistent with the finding that irradiation of animals subjected to pilocarpine-induced SE essentially abolished dentate granule cell neurogenesis, but failed to prevent mossy fiber reorganization in the dentate molecular layer.

Whole cell patch-clamp recordings in hippocampal slices had shown that the amplitude and frequency of spontaneous excitatory postsynaptic currents in granule cells increased with time after kainate treatment. This increased excitatory synaptic input was correlated with the intensity of the Timm stain in the inner molecular layer. When inhibition was reduced with bicuculline in slices from kainate-treated animals, flash photolysis of caged glutamate applied in the granule cell layer evoked epileptiform bursts of action potentials (an effect not seen in granule cells from control rats). These data supported the hypothesis that kainate-induced supragranular MFS results in the progressive formation of aberrant excitatory connections between granule cells. To evaluate the changes in the electrophysiological profile that would result from cycloheximide treatment, in vitro electrophysiology (extracellular recordings) was performed 2 months after pilocarpine-induced SE. Evoked potentials in slices from pilocarpine- and pilocarpine/cycloheximide-treated rats displayed small-amplitude polyspiking activity (epileptiform responses) in CA1 and an apparently normal isolated population spike in DG. More importantly, the DG in slices from pilocarpine and pilocarpine/cycloheximide animals did not differ with respect to electrophysiologic abnormalities, even under high K^+ or high K^+/bicuculline. Analysis of the neo-Timm staining, as expected, revealed strong supragranular MFS in pilocarpine-injected rats and significantly less staining in pilocarpine/cycloheximide rats. Thus, occurrence of abnormal stimulus responses, and high K^+- or high K^+/bicuculline-induced epileptiform activities, did not depend on the degree of supragranular MFS.

One long standing question regarding the development of MFS in animal models of SE is whether sprouted mossy fibers would add new synapses to the dendrites in the molecular layer – or whether these sprouting fibers simply substituted for vacated synapses (due to hilar cell degeneration). Indeed most studies on the subject suggested the loss of hilar mossy cells, normally a major hilar target of granule cell mossy fibers, as an important factor in initiating supragranular MFS. Pilocarpine-induced SE in the presence of cycloheximide resulted in a reduced number of injured hilar cells compared to rats not pretreated with cycloheximide. Presumed mossy cells, identified by calcitonin gene related peptide (CGRP) immunohistochemistry, were not significantly reduced in either groups 60 days after SE (given the seeming inconsistency of this finding with a preponderance of evidence indicating a loss of mossy cells SE, we assume that CGRP might constitute a marker of a sub-population of mossy cells.). Whereas controls had a strong band of CGRP-positive fibers (putative mossy cell axons) and no neo-Timm stained fibers in the inner molecular layer, pilocarpine-treated rats had no CGRP fibers and strong neo-Timm staining. Cycloheximide–pilocarpine-treated animals, in contrast, had CGRP and neo-Timm staining similar to controls. We concluded that cycloheximide could protect hilar CGRP-positive cells during SE; by allowing those cells to retain their normal axonal projection, MFS was prevented – a scenario consistent with the 'irritable' mossy cell

hypothesis that relies on the survival of mossy cells for network hyperexcitability. We suggested that cycloheximide prevents SE-induced supragranular MFS by preventing the loss of hilar CGRP-positive cells (putative mossy cells).

The fact that cycloheximide protected hilar cells and further increased cell proliferation in the DG after SE created the setting for a hypothesis that integrates current observations. Investigators have shown that some newly born granule cells develop in abnormal locations within the hilus. These ectopic granule cells demonstrate regular bursts of action potentials that are synchronized with CA3 pyramidal cell burst discharges and the bursts of hilar neurons (including mossy cells). In addition to the formation of ectopic hilar granule cells, several articles characterized the formation of hilar basal dendrites on granule cells in epileptic animals. Both features certainly allow for aberrant synapses in the hilus, either by ectopic or irritable cells or in aberrant basal dendrites, which may account for changes in hyperexcitability – regardless of the presence of supragranular MFS.

Future Directions

We have not yet examined whether cycloheximide affects the development of ectopic dentate granule cells. Such an investigation should include not only the histological characterization of these cells, but also a description of their electrophysiological properties. It is also important to evaluate whether cycloheximide affects the formation of aberrant basal dendrites on granule cells.

Still unresolved are the specific aspects of synaptic reorganization that underlie the epileptogenic process. The global changes that ensue after SE – and our ability to block specific aspects of these changes – will remain a hot topic of investigation. The current emphasis on trkB receptors has, to date, not yet yielded any major insights, but it is clear that neurotrophic factors and their receptors will remain major targets of research in this area.

Detailed investigation of synaptic changes associated with seizure-like plasticity is likely to require the use of organotypic cell culture in addition to studies in intact animals. Some of the differences between rodents and primates, in terms of neuronal connectivity or neuronal response to injury, may require studies in nonhuman primates. Also, greater attention should be devoted to exploring seizure-related plasticity in other portions of the hippocampal region such as the pyramidal CA1 and entorhinal cortex, and thalamus. Finally, research using computational models of the reorganized circuits and ionic channels is also likely to provide important information.

Regardless of its importance to epileptic (or epileptogenic) processes, supragranular MFS will remain an important topic of research, especially given the enormous amount of information that has already been generated and the wealth of knowledge now available about hippocampal connectivity. Rather than being a maladaptive change, this form of 'aberrant' synaptic plasticity may reflect a critical step in the ongoing process of recovery from injury in damaged tissue.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury and Progressive Cell Damage; **Neurogenesis**: Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; **Plasticity**: Axon Sprouting in Epilepsy; Circuitry Reorganization, Regeneration, and Sprouting; LTP and Kindling: Phenomena of Activity-Dependent Plasticity Influencing Memory and Epilepsy; Possible Role of Synaptic Reorganization in Epileptogenesis after Experimental Status Epilepticus; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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POST-TRAUMATIC EPILEPSY

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The Causes of Epilepsy after Traumatic Brain Injury

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Introduction

The Center for Disease Control (CDC) has estimated that each year 1.5 million people suffer a traumatic brain injury (TBI) in the United States, resulting in almost 100 000 patients per year with long-term disabilities and annual costs to the economy of over $50 billion. TBI and its sequelae have been referred to as an 'invisible epidemic' in which patients suffer devastating life-long cognitive, sensory, emotional, and motor deficits.

Head trauma and brain injury are important factors in epilepsy etiology, contributing about 4% of newly diagnosed cases in the United States. However, the underlying mechanisms remain unknown. The percentage of all victims of a serious head injury that go on to develop posttraumatic epilepsy (PTE) has been estimated at 10–34%, depending on the severity of the injury. One of the strongest predictors of the likelihood of PTE is a penetration of the dura. A unique characteristic of PTE is the variable delay, or latent period, between the trauma itself and the development of seizures – a delay which can last from weeks to years. This latent period is of considerable biological interest. What slow processes are triggered during injury that do not cause seizures until much later? The latent period provides a unique therapeutic opportunity. If we knew what was happening during the latent period, then therapies might be designed that stop this process and prevent a patient at risk from ever becoming epileptic.

The consequences of TBI are as diverse as its causes. Acute aspects of TBI include loss of blood, hemorrhaging, thrombosis, infection, inflammation, intracranial hypertension, and edema. Although these acute complications can often be managed with timely clinical care, long-term consequences, such as PTE, frequently leave TBI victims disabled. To devise effective therapeutic strategies for minimizing the long-term adverse consequences of TBI, we should understand their biological basis.

Background

Fundamental Elements of TBI

Which features of TBI need to be modeled to understand the genesis of PTE? Multiple processes undoubtedly act together to alter the normal balance of synaptic excitation and inhibition and render the brain incapable of functioning normally after injury. Given the overwhelming complexity of the injured brain, it is helpful to consider separately several of the fundamental consequences of lesions that are common to many forms of TBI and are likely to

internal lysyl residues. The reagents are isobaric, with a different distribution of isotopes between a reporter and a balance group. Thus, the labeled peptides obtained after modification are identical in MS mode. Upon fragmentation in MS/MS mode, the labeled peptides produce strong, diagnostic, low-mass MS/MS signature ions. Relative areas of these reporter peaks indicate the contribution of each sample to total peptide present, allowing for quantification of up to eight different samples simultaneously. MALDI–TOF/MS (matrix-assisted laser desorbtion ionization–time of flight–mass spectrometry) analysis gives low femtomole to high attomole sensitivity in both MS and MS/MS modes. It should be noted that the iTRAQ approach has been used to demonstrate relatively modest increases in phosphorylation state of CaM kinase II on depolarization of purified rat brain synaptosomes. This demonstration clearly shows the power of the iTRAQ approach in kindling, especially for detecting potential alterations in depolarization dependent protein modifications.

As a final comment, it must be noted that it is essential to confirm any putative kindling-related changes seen with proteomic analyses by traditional quantification methods such as quantitative immunoblotting. We have used this approach quite successfully to study specific members of the secretory protein family. The ultimate goal of these types of studies is to identify changes in the levels of protein at the synapse, reflected by corresponding changes in gene expression, that dictate the state of elevated neuronal activity leading to seizure activity. It is hoped that determining these changes, and the nature of the molecular events and signaling pathways responsible for them, will provide new targets for therapeutic intervention to treat even the most persistent and pernicious seizure conditions in humans.

See also: **Epileptogenesis**: Surrogate Markers for Epileptogenesis; **Neurotransmitter Function**: Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Seizures**: Seizure-Induced Gene Expression.

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The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis

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Introduction

Acquired epilepsy (AE), the development of spontaneous, recurrent seizures following a neurological insult, constitutes approximately half of all cases of epilepsy. The majority of cases of AE are produced by one of the three injuries to the brain: status epilepticus (SE), stroke, and traumatic brain injury (TBI). Although the precipitating events causing these injuries are distinct from one another, it has been shown that each can lead to the development of AE. This process wherein healthy brain tissue is transformed into a hyperexcitable network of

neurons manifesting spontaneous recurrent epileptiform discharges (SREDs) is called epileptogenesis. Identification of common molecular pathways that lead to development of AE following neuronal injury would lend insight into the pathophysiology of epileptogenesis and provide novel molecular targets that would aid in the development of newer approaches to prevent epilepsy.

Background

Calcium (Ca^{2+}) is not only a divalent charge carrier but is also an important second messenger. Intracellular calcium concentration ($[Ca^{2+}]_i$) is normally maintained at approximately 100 nM by a complex network of intracellular Ca^{2+} regulators and Ca^{2+} efflux pathways. This concentration is significantly lower than the 1–2-mM concentration observed in the extracellular space. This concentration gradient between extracellular and intracellular spaces provides a potent driving force for Ca^{2+} entry into the cell; further, since the intracellular concentration in normally so low, even minute changes in intracellular Ca^{2+} concentration provide a sufficiently large change to trigger downstream signaling. Brief elevations in $[Ca^{2+}]_i$ (**Figure 1**, top arrow) are required for transmitter release at synapses and for gene expression, and represent the normal physiological stimuli that occur for the maintenance of the basic neuronal functions. These transient elevations in $[Ca^{2+}]_i$ are also necessary for the cellular correlates (e.g., long-term potentiation, LTP) of higher brain functions such as learning and memory consolidation.

Elevated $[Ca^{2+}]_i$ and altered Ca^{2+} homeostatic mechanisms have been demonstrated following severe brain injuries such as SE, stroke, and TBI. The severity of injury and the resulting Ca^{2+} influx dictate the fate of neurons (**Figure 1**); this Ca^{2+} influx and injury are mediated largely by elevations in the excitatory neurotransmitter, glutamate. Under normal conditions, glutamate is highly regulated, with extracellular concentrations measured between 1 and 5 μM. The excess glutamate, a hallmark of SE, stroke, and TBI, causes the overstimulation of glutamate receptors, including the *N*-methyl-D-aspartate receptor (NMDA-R) that is coupled to a Ca^{2+} permeable channel (**Figure 2**). Hence, increased levels of glutamate can lead to the excess movement of Ca^{2+} across the cell membrane into the cell. While glutamate acts at several receptors, it appears that the NMDA receptor contributes most significantly to the neuronal pathology and Ca^{2+} changes observed post-injury, confirmed by the ability of NMDA antagonists to decrease the volume of ischemic infarct in whole animal models and offer neuroprotection in cell culture models of excitotoxicity.

Together, the degree of glutamate-stimulation and the subsequent Ca^{2+} influx contribute to the level of injury sustained by the neurons. Severely injured cells exhibit an irreversible Ca^{2+} overload that leads to cell death (**Figure 1**, bottom arrow). In all of the major injuries associated with AE, cell death is often an associated manifestation in the epileptic brain. Since dead neurons do not seize, it is the surviving neurons that contribute to the development of the epileptic phenotype. The surviving neurons – presumably those that are less severely injured – sustain reversible elevations in $[Ca^{2+}]_i$ that are maintained over days or weeks by altered homeostatic mechanisms. These increases mediate changes in synaptic plasticity, gene expression, and neuronal function (**Figure 1**, middle arrow). These neurons become the substrate for epileptogenesis, and also the target for future therapies to prevent AE. The ability to target these intracellular changes and reduce the intracellular Ca^{2+} load may prevent the development of the epileptic phenotype following SE, stroke, and TBI.

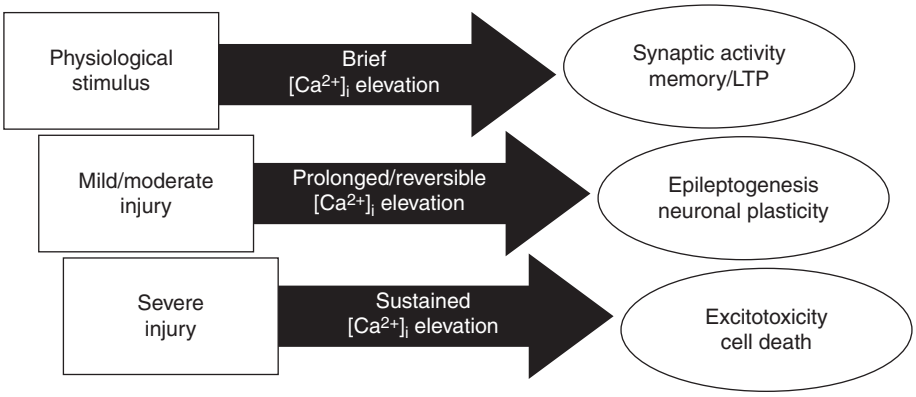


Figure 1 Effect of elevated $[Ca^{2+}]_i$ on neurons. Normal stimuli cause transient elevations in $[Ca^{2+}]_i$ that control synaptic activity and are responsible for learning and memory (top arrow). Mild to moderate injuries may cause prolonged elevations in $[Ca^{2+}]_i$ that are ultimately reversible (middle arrow). Such elevations are not substantial enough to initiate cell death but are able to cause plasticity changes in neurons, leading to epileptogenesis. Finally, severe injury causes sustained, massive increases in $[Ca^{2+}]_i$ that lead to cell death (bottom arrow).

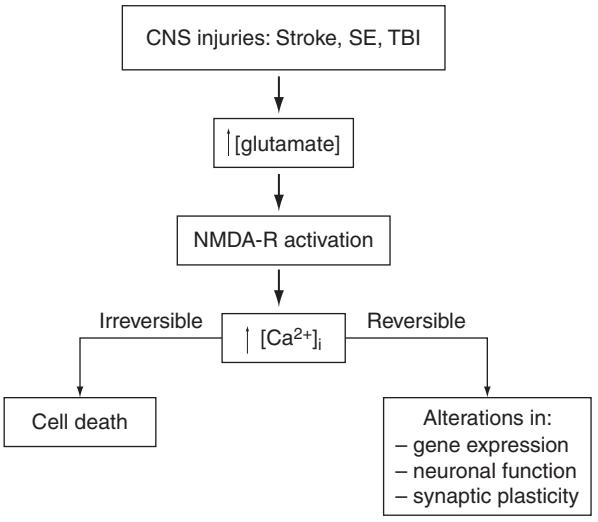


Figure 2 SE, stroke, and TBI cause death or plasticity changes in neurons. CNS injuries such as stroke, SE, and TBI can lead to one of two fates for affected neurons. First, all injuries cause increases in glutamate concentration, which cause the over-stimulation of glutamatergic receptors, including NMDA receptors (NMDA-R). The activation of these receptors trigger an influx of Ca^{2+} across the cell membrane and cause a Ca^{2+} overload. This elevation in $[Ca^{2+}]_i$ can be irreversible and overwhelm cellular processes, resulting in cell death. In contrast, neurons that are less severely injured exhibit a sustained but reversible increase in $[Ca^{2+}]_i$ that leads to alterations in gene expression, neuronal function, and synaptic plasticity through second messenger systems.

Methods

We have utilized several different in vivo and in vitro models to study AE and to characterize Ca^{2+} changes in SE-induced AE. For in vitro studies, hippocampal neurons are treated with a solution containing no added Mg^{2+} (low-Mg^{2+}) for 3 h during which time cells exhibit high frequency spiking analogous to SE. Following the return of Mg^{2+} to the solution (physiological basal recorded solution, pBRS), the networked neurons continue to exhibit synchronous spontaneous recurrent epileptiform discharges (SREDs) for their life in culture. For studies in the intact brain, we use the pilocarpine model. Injection of the cholinergic agonist pilocarpine induces SE in rats and following a latent period, these animals develop recurrent seizures.

To study stroke-induced AE in an in vitro setting, we developed a model in which subexcitotoxic concentrations of glutamate were added to neuronal cultures. This concentration was maintained on the cells for 30–45 min and induced transient depolarizations of the membrane potential and increases in $[Ca^{2+}]_i$, consistent with what is observed in the ischemic penumbra of in vivo models of stroke. SREDs were observed in the surviving neurons 24 h following this treatment. For the in vivo studies, the middle cerebral artery occlusion model was used. We also employed the decapitation ischemia model wherein ischemia was produced in vivo and Ca^{2+} imaging and electrophysiological experiments were then performed on acutely dissociated hippocampal neurons from the ischemia-injured brain slices.

Our in vivo rat model for TBI utilizes a central or lateral fluid percussion injury that leads to hyperexcitability of the hippocampal dentate gyrus and spontaneous recurrent seizures. For in vitro studies of TBI, neurons are cultured on a flexible silastic membrane substrate; cells are mechanically injured by stretching the membrane in a controlled manner. The majority of work using in vitro model has been performed at acute time points; therefore, it remains to be determined whether these models accurately mimic chronic hyperexcitability and epileptiform activity.

Following experimental stroke, SE, or TBI, animals are monitored for the development of AE using video/EEG to detect behavioral and electrographic seizures. Electrodes are implanted following recovery from injury and animals are subjected to a minimum of 4 days of monitoring (at various time points post-injury) to determine the development of epilepsy.

In the whole animal, after determining whether an animal has developed chronic epilepsy, Ca^{2+} dynamics are evaluated in vitro. We acutely isolate neurons by triturating hippocampal slices from the animals of interest, and plate them on poly-L-lysine and Cell-Tak® coated glass bottom chambers for Ca^{2+} imaging or on 35-mm dishes for electrophysiology.

Changes in $[Ca^{2+}]_i$ are evaluated using fluorescent calcium indicators – Fura-2, Fura-ff, and Indo-1. Ratio images are acquired using alternating excitation wavelengths and are corrected for background. Data analyses are performed using standard methods and procedures.

Results

The results of our studies reflect the changes in $[Ca^{2+}]_i$ levels and alterations in Ca^{2+} homeostatic mechanisms, observed acutely and at different times following SE, stroke, or TBI. $[Ca^{2+}]_i$ was assessed at several time points – during injury, during epileptogenesis (i.e., in 'latent' periods), and finally during the chronic phase of AE. Following the injury, neurons were also evaluated for their ability to restore Ca^{2+} homeostasis following a brief excitatory stimulation with glutamate, as altered homeostatic mechanisms may account for dysregulated $[Ca^{2+}]_i$.

In vitro SE Causes Increased $[Ca^{2+}]_i$

To model SE in an in vitro system, cultured hippocampal neurons were treated with low-Mg^{2+} solution for 3 h.

Whole-cell current clamp techniques revealed that the low-Mg^{2+}-treated cells displayed high frequency spiking that exceeded 3 Hz. Clinical SE is characterized by spike frequencies greater than 3 Hz on an EEG; thus, the low-Mg^{2+}-induced high frequency spiking in the hippocampal neurons meets the electrophysiological definition of clinical SE. Control neurons showed only the occasional firing of action potentials (**Figure 3(a)**). During the 3 h in low-Mg^{2+} solution, $[Ca^{2+}]_i$ increased almost sevenfold compared to the levels observed in control neurons (**Figure 3(b)**). Moreover, when the neurons were briefly stimulated with 50 μM glutamate immediately following the restoration of Mg^{2+}, they demonstrated marked alterations in the ability to recover from this excitatory challenge (**Figure 3(c)**). Changes in $[Ca^{2+}]_i$ observed in neurons following treatment with low-Mg^{2+} were evident in pseudocolor images from neurons after in vitro SE. These images from low-Mg^{2+}-treated cells showed increased Fura-2 ratios compared to the control neurons (**Figure 3(d)**). These altered $[Ca^{2+}]_i$ levels observed in neurons following SE lasted for 6–7 h following the restoration of Mg^{2+}, representing the Ca^{2+} plateau following in vitro SE.

In vitro SE Causes AE-like Activity and Prolonged Alterations in $[Ca^{2+}]_i$ and Ca^{2+} Dynamics

To determine whether Ca^{2+} dynamics were altered after the establishment of in vitro epilepsy, cultured hippocampal neurons were observed for electrophysiological activity and changes in fura-2 ratios 24 h post-in vitro SE. Whole-cell current clamp recordings displayed SREDs in low-Mg^{2+}-treated neurons, while the control neurons displayed baseline activity (**Figure 4(a)**). Consistent with clinical epilepsy, in vitro seizure episodes were comprised of paroxysmal

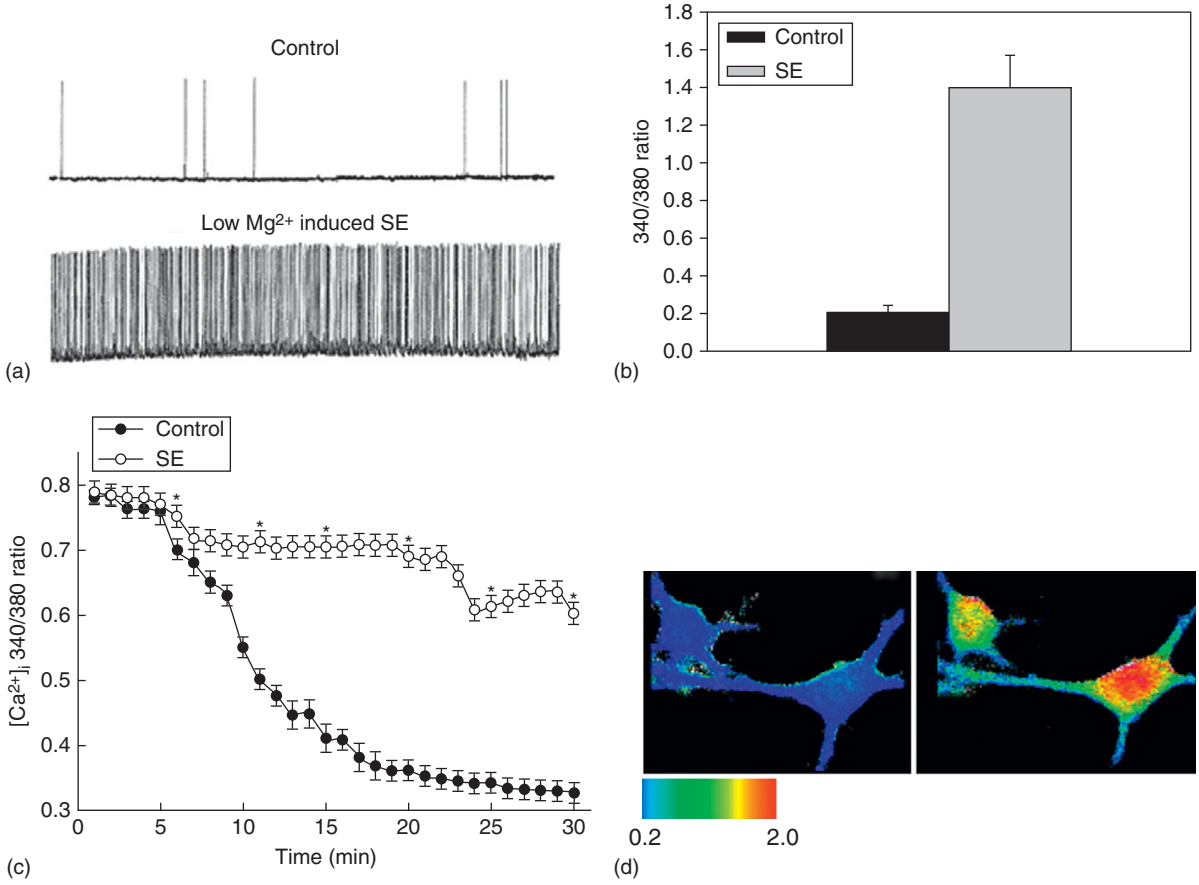


Figure 3 Alterations in $[Ca^{2+}]_i$ and Ca^{2+} homeostasis immediately following 3 h of low-Mg^{2+}-induced in vitro SE. (a) Current–clamp electrophysiological recordings of a representative control neuron and a neuron in low-Mg^{2+}. The control neuron displays occasional action potentials, whereas the neuron in low-Mg^{2+} shows frequent discharges. Resting membrane potential = −62mV; duration of shown trace = 15 min. (b) Bar graph comparing fura-2 340/380 ratios in control neurons versus in vitro SE neurons. The low-Mg^{2+} cells exhibit ratio values of 1.4 ± 0.17 as compared to the control neurons with values of 0.20 ± 0.04. (c) Following glutamate stimulation, elevations in $[Ca^{2+}]_i$ are observed in both control and in vitro SE groups. Control neurons show faster recovery rates than treated neurons. (d) Pseudocolor ratio images of control (left panel) and low-Mg^{2+}-treated (right panel) neurons show increased 340/380 ratio immediately following treatment with low-Mg^{2+}.

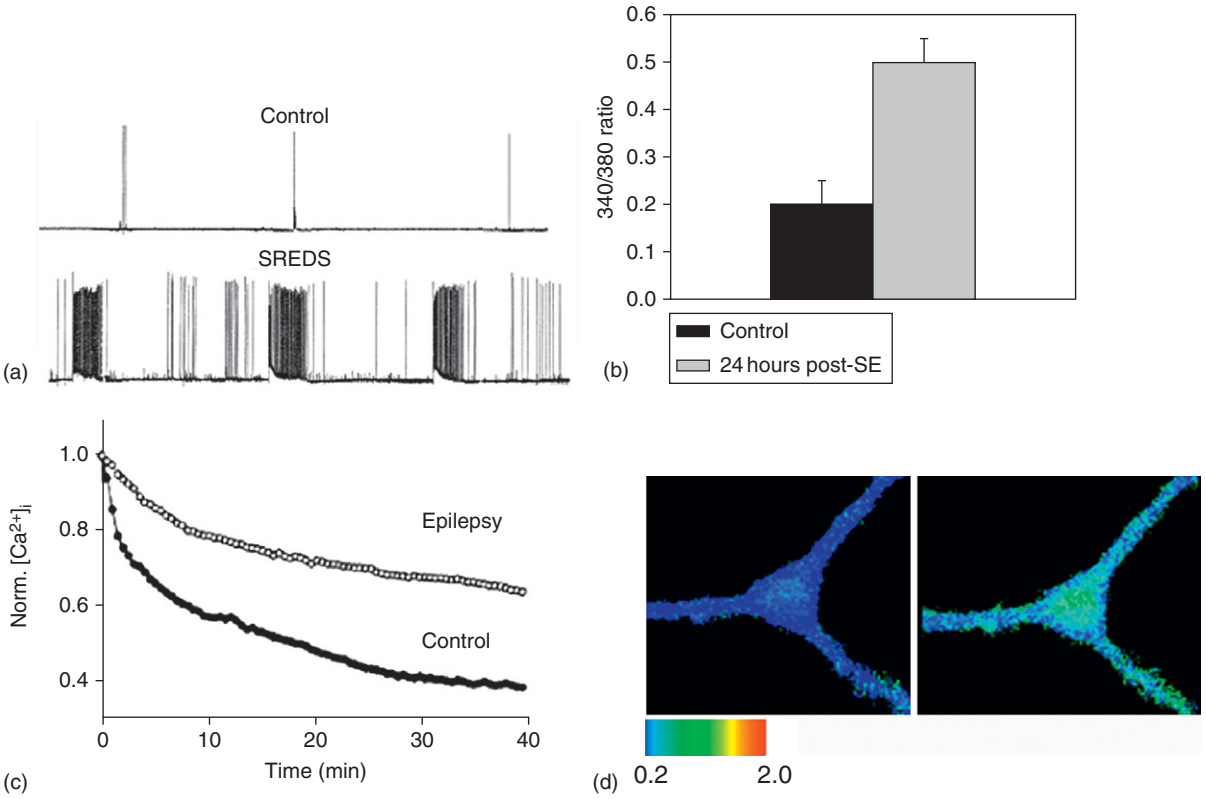


Figure 4 Alterations in $[Ca^{2+}]_i$ and Ca^{2+} homeostasis in neurons expressing spontaneous recurrent epileptiform discharges (SREDs), 24 h post-in vitro SE. (a) Current–clamp technique demonstrate baseline activity in control neurons and SREDs in neurons that were treated in low-Mg^{2+} for 3 h. Resting membrane potential = −61.5 mV; duration of shown trace = 30 min. (b) Compares 340/380 ratio values in neurons, 24 h after either pBRS + $MgCl_2$ or low-Mg^{2+} treatment. Neurons in the low-Mg^{2+}-treated group exhibit mean ratios of 0.5 ± 0.08, significantly higher than the ratio of 0.20 ± 0.05 observed in the control group. (c) Shows recovery of both control and low-Mg^{2+} groups following glutamate stimulation. Peak ratios following the glutamate challenge are normalized to 1.0. SRED neurons show a decreased ability to recover. (d) Pseudocolor ratio images of neurons 24 h after either pBRS + $MgCl_2$ (left panel) or low-Mg^{2+} (right panel) treatment highlight the increases in $[Ca^{2+}]_i$ in epileptic neurons.

depolarization shifts and exhibited similar anticonvulsant sensitivity. Calcium imaging revealed that ratio values for the baseline neuronal $[Ca^{2+}]_i$ levels were elevated 2.5-fold when cells were exposed to the low-Mg^{2+} treatment that caused continuous epileptiform discharges (Figure 4(b)). Moreover, when the neurons were stimulated with 50 µM glutamate, they demonstrated marked alterations in the ability to recover from this excitatory challenge (Figure 4(c)), indicating a diminished ability to buffer the increased Ca^{2+} load when compared to the control (Figure 4(d)).

In vivo SE Causes Increased $[Ca^{2+}]_i$, Altered Ca^{2+} Homeostasis, and a Prolonged-Elevated $[Ca^{2+}]_i$ Plateau

Rats injected with pilocarpine were allowed to seize for 1 h before the termination of seizure activity with diazepam. The electrical activity in the brain was evaluated using electroencephalography, with pilocarpine-treated animals showing activity consistent with clinical SE (Figure 5(a)). Immediately following SE, the rats were sacrificed and hippocampal neurons were acutely isolated. Significant elevations in $[Ca^{2+}]_i$ were observed in hippocampal neurons from the CA1 region following pilocarpine-induced SE, but not in those isolated from vehicle-control animals (Figure 5(b)). When these acutely isolated neurons were stimulated with glutamate (Figure 5(c)), a decrease in the rate of recovery and the ability to restore $[Ca^{2+}]_i$ to baseline levels was evident in the neurons isolated from post-SE animals, whereas control neurons were able to restore the Ca^{2+} load to baseline levels.

We have also demonstrated that the elevated $[Ca^{2+}]_i$ falls slightly over the days and weeks following SE; however, levels never decrease to baseline values observed in controls. This maintenance of a Ca^{2+} 'plateau' following injury contributes to the 'Ca^{2+} hypothesis of epileptogenesis' which suggests that altered Ca^{2+} homeostatic mechanisms and the inability of the cells to maintain normal, resting $[Ca^{2+}]_i$ contribute to the long-term development of AE through Ca^{2+}-triggered second messenger effects.

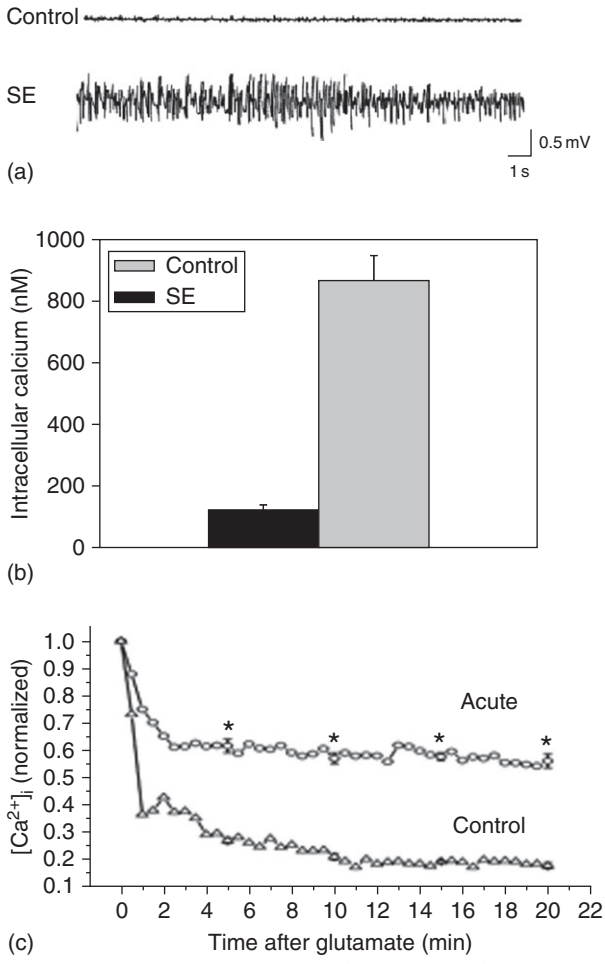


Figure 5 SE causes increased $[Ca^{2+}]_i$, altered Ca^{2+} homeostasis, and a prolonged, elevated $[Ca^{2+}]_i$ plateau in vivo. (a) Representative EEG recording from control (vehicle) rats and rats in SE (pilocarpine-treated). (b) $[Ca^{2+}]_i$ is higher in neurons from animals immediately following SE when compared to animals in the control group – 850 ± 59 and 90 ± 22 nM, respectively. (c) Upon glutamate stimulation, the $[Ca^{2+}]_i$ in control neurons returns to baseline levels within ~10 min but fails to do so in neurons from the SE group.

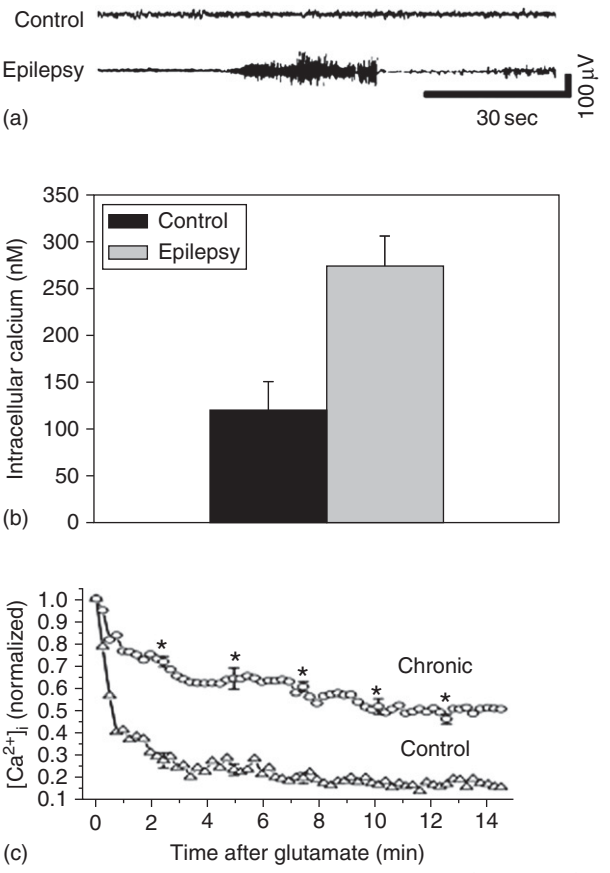


Figure 6 AE is associated with alterations in $[Ca^{2+}]_i$ and Ca^{2+} homeostatic mechanisms 1-year post-in vivo SE. (a) Representative EEG recording from control (vehicle) rats and epileptic rats (following pilocarpine-induced SE). (b) $[Ca^{2+}]_i$ is higher in neurons from epileptic animals (325 ± 35 nM) when compared to those from animals in the control group. (c) Upon glutamate stimulation, the $[Ca^{2+}]_i$ returns to baseline in neurons from the control group, unlike neurons from the epileptic group, which fail to return to baseline.

In vivo SE-Induced AE is Associated with Alterations in $[Ca^{2+}]_i$ and Ca^{2+} Homeostatic Mechanisms

To determine whether these changes were transient or still present during chronic epilepsy, animals were treated with pilocarpine or vehicle (as earlier) and were maintained for one year following SE. Video-EEG monitoring revealed spontaneous, recurrent seizures in the pilocarpine but not vehicle-treated group (Figure 6(a)). Hippocampal neurons isolated from epileptic animals one year post-pilocarpine demonstrated markedly elevated $[Ca^{2+}]_i$ compared to neurons isolated from age-matched animals (Figure 6(b)). The cells were evaluated for the ability to restore baseline Ca^{2+} levels post-glutamate stimulation; similar to our observations in culture models, neurons from epileptic animals demonstrated a decreased ability to buffer a given load of Ca^{2+} at one year post-injury (Figure 6(c)).

Alterations in Ca^{2+} Following Stroke-Induced AE

We used the in vitro glutamate-mediated injury model to mimic ischemic stroke. With this model, we evaluated the development of SREDs. This model results in approximately 10–20% cell death and SREDs in the 80–90% of the neurons that survive the injury. Studies have demonstrated that surviving cultured hippocampal neurons manifested elevated $[Ca^{2+}]_i$ compared to control neurons (Figure 7(a)). Glutamate excitotoxicity thus provides an in vitro model of stroke-induced AE.

In this model, tetrodotoxin (TTX) was used to block epileptiform discharges, to determine whether Ca^{2+} influx was due to the seizurelike activity recorded from these

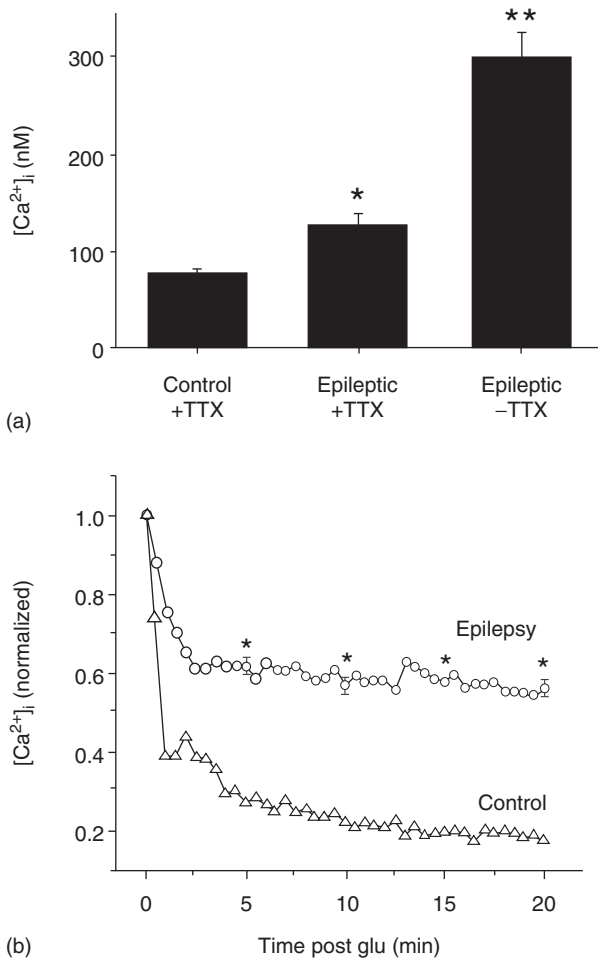


Figure 7 Alterations in $[Ca^{2+}]_i$ and Ca^{2+} homeostatic mechanisms following in vitro stroke. (a) 5 days post-injury with glutamate, chronic elevations in basal $[Ca^{2+}]_i$ were measured. Epileptiform activity contributed in part to these levels; however, blockade of epileptiform activity by 600 nM TTX significantly reduced the long-lasting elevations in basal $[Ca^{2+}]_i$ in epileptic neurons in comparison to epileptic neurons exhibiting SREDs. Basal $[Ca^{2+}]_i$ in epileptic neurons was still significantly elevated compared with control neurons in the presence of TTX. (b) Following glutamate stimulation to assess homeostatic mechanisms, the percent of Ca^{2+} load remaining 20 min post-washout in the epileptic neurons was significantly higher than that observed in control neurons.

neurons. Following treatment with TTX, cells in these cultures still showed elevations in baseline Ca^{2+} (Figure 7(a)). It appears that while a component of the elevated $[Ca^{2+}]_i$ is due to seizure activity, it does not account for the entire Ca^{2+} overload. These observations are similar to those following SE-induced hyperexcitability in vitro. Cultures were also evaluated for their ability to respond to glutamate-stimulated elevations in $[Ca^{2+}]_i$. As demonstrated in the SE model, control neurons restored basal $[Ca^{2+}]_i$ at a higher rate than the SRED-producing neurons following in vitro glutamate exposure (Figure 7(b)). Thus, in culture models of stroke-induced hyperexcitability, we demonstrated that both $[Ca^{2+}]_i$ and Ca^{2+} homeostatic mechanisms are altered following injury for the life of the neurons in culture.

Little work has been done to evaluate changes in Ca^{2+} dynamics employing in vivo models of stroke-induced AE. We have performed both decapitation ischemia and middle cerebral artery occlusion, and have found that both cause acute increases in $[Ca^{2+}]_i$, consistent with what is seen both in models of SE-induced AE and in vitro stroke. Furthermore, preliminary results following middle cerebral artery occlusion show that neuronal Ca^{2+} dynamics are affected by this injury. Whether these alterations in Ca^{2+} dynamics eventually lead to the development of the epileptic phenotype is currently unknown and is being actively investigated.

TBI-Induced Increases in $[Ca^{2+}]_i$ Levels and Ca^{2+} Homeostatic Mechanisms

The TBI model of epilepsy is the least characterized of these models. However, recent studies by Pitkanen's group, in which they carefully examined the experimental conditions and time course of the development of spontaneous recurrent seizures following TBI caused by the fluid percussion in rats, demonstrated (with long-term continuous EEG monitoring) that AE develops in a significant number of rats following TBI. Our laboratory has recently evaluated $[Ca^{2+}]_i$ levels in cells from rats subjected to fluid percussion TBI. We have shown that $[Ca^{2+}]_i$ remains elevated for prolonged periods after the injury in hippocampal neurons from animals post-TBI. More specifically, $[Ca^{2+}]_i$ was elevated at day-1 and week-1, but not month-1, after central fluid percussion injury. These recent observations indicate that the injury produced by TBI initiates the same changes in $[Ca^{2+}]_i$ levels that occur after injury produced by SE or stroke (Figure 8). Further studies are needed to correlate these changes with the development of epileptogenesis.

Alterations in Ca^{2+} homeostatic mechanisms following TBI were evaluated using the standard glutamate-stimulation technique. When challenged with glutamate (50 μM, 4 min), neurons acutely isolated from TBI animals (at 1, 7, and 30 days following injury) demonstrated significant alterations in their ability to recover baseline Ca^{2+} levels. Age-matched control and sham-operated neurons promptly restored elevated $[Ca^{2+}]_i$ following the excitatory stimulus (Figure 8).

Thus, TBI causes long-lasting elevations in hippocampal neuronal $[Ca^{2+}]_i$ levels and alterations in Ca^{2+} homeostasis. These long-lasting changes in $[Ca^{2+}]_i$ levels and Ca^{2+} homeostasis were not the result of dying neurons, as demonstrated through simultaneous assays examining cell death and Ca^{2+} changes. These studies demonstrated that the neurons manifesting elevated $[Ca^{2+}]_i$ levels did not show

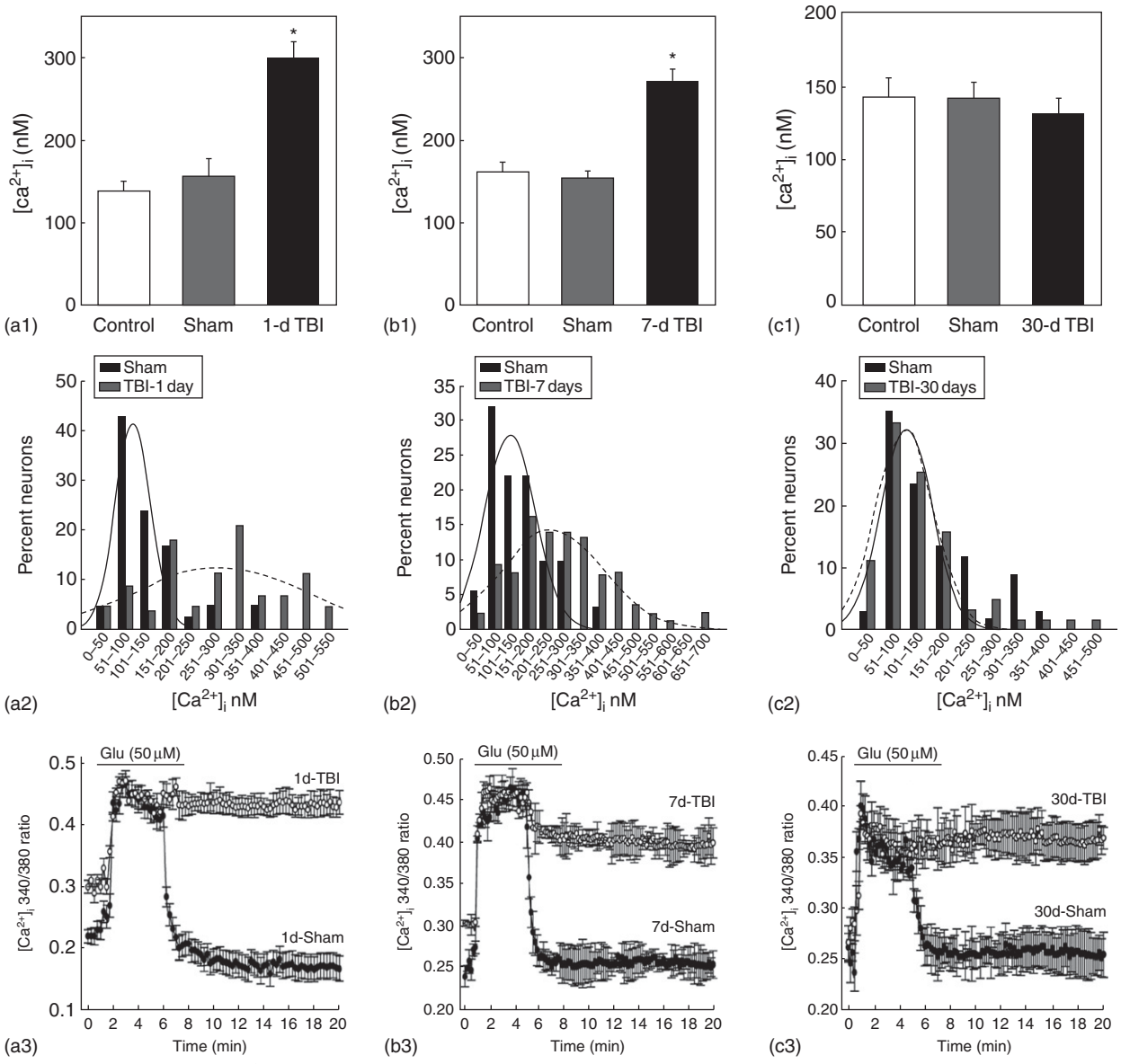


Figure 8 Basal neuronal $[Ca^{2+}]_i$ in TBI injured hippocampal neurons 1,7, and 30 days after moderate central fluid percussion injury. (a1) Acutely isolated CA3 hippocampal neurons 1 day post-injury manifested significant elevations in $[Ca^{2+}]_i$ compared to both age-matched control and sham neurons. (b1) Elevations in basal neuronal $[Ca^{2+}]_i$ persisted 7 days following TBI. Injured neurons manifested significant elevations in $[Ca^{2+}]_i$ compared to both age-matched control and sham neurons. (c1) Basal neuronal $[Ca^{2+}]_i$ normalized 30 days after moderate central fluid percussion injury. Thirty days post-injury, acutely isolated CA3 hippocampal neurons did not manifest significant differences in $[Ca^{2+}]_i$ compared to age-matched control and sham neurons. (Color scheme: white = control, gray = sham and black = TBI).Distribution of $[Ca^{2+}]_i$ levels for sham and TBI hippocampal neurons one, seven and thirty days after moderate central fluid percussion injury. (a2) Sham neurons demonstrated a normal distribution for $[Ca^{2+}]_i$ levels with approximately 88% of neurons exhibiting $[Ca^{2+}]_i$ levels less than 200 nM. In contrast 1-day TBI neurons demonstrated a rightward shift towards higher $[Ca^{2+}]_i$ levels with only 35% neurons exhibiting $[Ca^{2+}]_i$ levels less than 200 nM. (b2) Seven days following TBI, neurons still exhibited a rightward shift towards higher $[Ca^{2+}]_i$ levels compared to sham neurons. (c2) Thirty days following TBI there was no shift towards higher $[Ca^{2+}]_i$ levels. Approximately 75% and 85% of sham and TBI neurons exhibited $[Ca^{2+}]_i$ levels less than 200 nM. The distributions of $[Ca^{2+}]_i$ levels was significantly different at days 1 and 7 post TBI but not at 30 days following TBI.Inability of TBI injured hippocampal neurons to restore basal neuronal $[Ca^{2+}]_i$ following excitatory stimulation. A $[Ca^{2+}]_i$ load was produced by a glutamate exposure (50 μM, 4-min) and the ability to restore preglutamate Ca^{2+} levels was measured using the low affinity Ca^{2+} indicator, Fura-FF. Glutamate stimulation in both sham and TBI dissociated neurons at all the time points following injury produced a Ca^{2+} load as evidenced by sudden increase in Fura-FF ratio. Upon removal of glutamate, sham neurons promptly restored Fura-FF ratio to pre-stimulation levels. However, TBI neurons [a3: 1-day, b3: 7-day, and c3: 30-day] failed to restore Fura-FF ratio to preglutamate levels despite glutamate washout. The ratio remained elevated throughout the experiment indicating a chronic inability of TBI neurons to handle Ca^{2+} loads. All the time points for TBI neurons following the glutamate washout were significantly different compared to sham neurons.

histopathological evidence of apoptosis or acute cell death, indicating that the persistent alteration in Ca^{2+} dynamics is a function of injured but surviving neurons. These long-term alterations in Ca^{2+} dynamics contribute to the pathophysiology of TBI, and may represent a molecular basis for mediating some of the persistent plasticity associated with the chronic disability from TBI, including cognitive disorders and post-traumatic epilepsy.

Future Directions

As diagrammed in Figure 9, stroke, TBI, SE, and other injuries of the central nervous system have the ability to elicit permanent cellular alterations, some of which may result in epilepsy. The period between injury induction and expression of recurrent seizures is called epileptogenesis. It represents a therapeutic window of opportunity wherein the progression of brain tissue from healthy to hyperexcitable can be halted. The development of such therapeutic targets is dependent on our ability to isolate and identify specific changes that allow for such dissimilar injuries to result in one common clinical condition.

The findings that $[Ca^{2+}]_i$ and Ca^{2+} homeostatic mechanisms are significantly altered in both in vitro and in vivo models of AE may prove to be the common thread in the pathogenesis of AE. While we have begun to elucidate which regulatory systems are responsible for both the increased $[Ca^{2+}]_i$ and the decreased ability to buffer this load, it is crucial to further tease out the specific contributions of Ca^{2+} systems. Drugs targeting alterations in Ca^{2+} binding proteins, Ca^{2+}-induced Ca^{2+} release mediated by ryanodine- and IP_3-receptors, sarco-endoplasmic reticulum Ca^{2+}-ATPase, Ca^{2+}/calmodulin-dependent protein kinase II, and myriad Ca^{2+} channels in the plasma membrane may prove to have therapeutic potential. Studies using NMDA antagonists have already demonstrated that blocking the Ca^{2+} overload can be neuroprotective. Clearly, identifying which systems contribute to the changes observed post-injury is necessary for the development of new therapeutic targets. The identification of new targets to prevent the development of epilepsy has tremendous clinical implications. Currently there are no antiepileptogenic drugs to prevent the transformation from a normal to an epileptic phenotype. The development of such a drug, that could be administered shortly after injury to prevent the induction of epilepsy through the buffering of $[Ca^{2+}]_i$ or the restoration of normal, Ca^{2+} homeostasis, may prove to be invaluable.

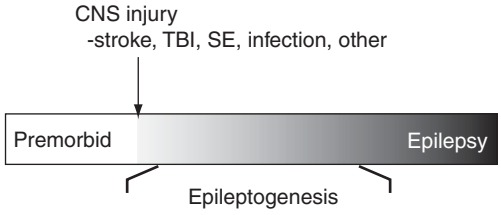


Figure 9 The progression to acquired epilepsy: A possible therapeutic window of opportunity. Injuries to the central nervous system such as stroke, TBI, and SE (down arrow) can elicit permanent changes in neurons, resulting in the development of epilepsy. The period between the inciting injury (down arrow) and the manifestation of recurrent seizures or epilepsy (black) is called epileptogenesis (gray). This gray period may provide a therapeutic window of opportunity wherein the use of pharmacological agents or molecular tools can prevent the transition of healthy brain tissue to a hyperexcitable network manifesting spontaneous recurrent epileptiform discharges.

See also: **Aging**: Poststroke Epilepsy and the Aged Brain; **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; **Non-Synaptic Mechanisms**: Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury.

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The Role of Short Term Synaptic Plasticity in Development and Spread of Seizures

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Introduction

Factors that contribute to the generation and propagation of seizure activity throughout the brain undoubtedly include the way in which neurons interact with one another at the circuit level. More specifically, the balance between excitatory and inhibitory processes within the circuit can govern the level of local network stability. Indeed, experimental manipulations, both in vivo and in vitro, which disrupt inhibition or enhance excitation, have been shown to generate seizures or seizure-like electrographic activity. Thus, the initiation of seizures from the preictal state may depend on a shift in the relative strengths of synaptic excitation and inhibition in a very small set of neural networks. This shift may come about because of changes in the expression of a variety of voltage-gated and ligand-gated channels in a 'damaged' area of brain and/or because of altered circuits.

Although partial seizures are initiated in areas of the brain that act as epileptic foci, seizure activity can readily spread through regions of relatively normal brain, both locally and at a distance. This propagation of seizure activity through regions of normal brain tissue implies that 'normal' synaptic connections and processes are sufficient to support the spread of seizure activity throughout the brain. It has therefore been hypothesized that short-term, frequency-dependent changes in the degree of inhibition and excitation can contribute substantially to both the initiation of seizures and the spread of seizure-like activity in the brain. We will provide a brief overview of the kinds of short-term synaptic plasticity that have been observed at CNS synapses, and that are thought to play a role in the generation and propagation of seizure activity.

Background

From even the earliest studies of chemical neurotransmission at the neuromuscular junction, it became apparent that the amplitudes of postsynaptic responses are quite labile and depend on the firing frequencies of the presynaptic neuron. Therefore, not only can neurons within a circuit convey information strictly via excitation or inhibition, but short-term modifications in the relative strength of the excitation and/or inhibition can provide information and dictate the overall stability of the network.

Facilitation is a common form of synaptic plasticity that has been identified at synapses with a low probability of release. Facilitation is a quickly occurring and quickly dissipating (in the order of hundreds of milliseconds) increase in the amplitude of a postsynaptic potential (PSP) or current (PSC), in response to presynaptic action potentials that occur at high frequency. The residual calcium hypothesis remains the most widely accepted theory for explaining short-term synaptic facilitation. Following an initial action potential, calcium concentrations near neurotransmitter release sites remain elevated. With the occurrence of subsequent action potentials, this residual calcium contributes to an overall larger calcium signal than that which occurred following the first action potential, causing an enhancement of the probability of release of neurotransmitter and a subsequent increase in the amplitude of the postsynaptic response. The residual calcium hypothesis can also adequately explain other common forms of short-term synaptic enhancement, such as augmentation and posttetanic potentiation (PTP). These forms of synaptic plasticity require bursts of action potentials and can last for several seconds (in the case of augmentation) to several minutes (in the case of PTP).

Another hypothesis to explain at least some forms of facilitation relates to accumulation of extracellular K^+. After each action potential, K^+ is extruded from the cell and may accumulate around cell bodies and axons. Small increases in extracellular K^+ may facilitate action potential generation, which can subsequently be reflected in an apparent increased probability of transmitter release. This mechanism may be particularly important in epileptic processes, where injured tissue may be more susceptible to the influences of extracellular K^+ accumulation and where astrocytes (that normally effectively buffer extracellular K^+) may be altered.

Synaptic depression is another prominent form of short-term plasticity that most often occurs at synapses with a high probability of release. Robust depression of PSP(C)s can be observed following just a single presynaptic action potential and can last for several seconds. Strikingly, such depression has been observed at many inhibitory synapses in the cortex and hippocampus. Based on the fact that even mild disruptions of inhibitory

processes elicit seizures, it is compelling to think that such depression at inhibitory synapses can result in a profound reduction of inhibition in cortical and hippocampal circuits, and thus contribute to both ictogenesis and the spread of seizures once seizures develop. While this feature of many inhibitory synapses has been known for some time, the advent of paired recordings of identified neurons (in brain slice preparations) has shown that many excitatory synapses in the cortex also exhibit depression. In a series of elegant experiments, investigators recorded from monosynaptically connected pairs (either pyramidal cell–pyramidal cell excitatory connections, or fast spiking cell–pyramidal cell inhibitory connections) of neurons in slices of the rat visual cortex. Both excitatory synapses between the recorded pyramidal cells and inhibitory synapses between the fast-spiking interneurons and pyramidal cells showed depression in response to trains of action potentials. Indeed, over the course of long stimulation trains, the EPSCs diminished steadily until the majority of responses consisted of failures of transmission. Although the amplitude of the IPSCs depressed faster than the EPSCs, IPSCs reached a steady-state plateau very rapidly; in contrast to EPSCs, the probability of failures was substantially lower and IPSCs were measurable throughout the train of action potentials. It is hypothesized that such differential depression among recurrent excitatory and inhibitory connections serves to dynamically stabilize cortical networks, which might otherwise be prone to instability and hyperexcitability. It remains to be determined if such a homeostatic property of short-term plasticity is preserved in the epileptic brain.

As is the case for synaptic enhancement, most forms of short-term depression appear to be due primarily to presynaptic mechanisms such as depletion of a readily releasable pool of transmitter vesicles. In addition, other presynaptic mechanisms have also been shown to contribute to short-term synaptic depression. For example, activation of presynaptic receptors that modulate release (such as $GABA_B$ receptors, adenosine receptors, or kainate receptors) can play a role in the activity-induced depression observed at some synapses. Indeed, this mechanism of action appears to complicate the effects of one of the newer anticonvulsant drugs. This drug was designed to inhibit GABA uptake at synapses. Although the drug did work to suppress partial seizures, it also appeared to enhance generalized seizures in some individuals – an effect attributed to the fact that the increased GABA in the synaptic cleft was able to activate presynaptic $GABA_B$ receptors and diminish inhibition at some synapses in the thalamus.

Other postsynaptic mechanisms may also contribute to the types of plasticity observed. For example, at most excitatory synapses, postsynaptic AMPA receptors desensitize rapidly, thus contributing to the depression observed. In the hippocampus, it has been shown that the reversal potential for $GABA_A$ receptor-mediated IPSPs in inhibitory interneurons of the stratum oriens can change rapidly during a train of action potentials elicited at high frequencies. This rapid change in driving force for Cl^- results in a unique form of short-term plasticity at these synapses, as both presynaptic and postsynaptic mechanisms are simultaneously involved.

Methods

To understand short-term synaptic plasticity, our laboratories have primarily used the whole cell patch clamp technique in a number of different preparations, including dissociated cell cultures, organotypic hippocampal slice culture preparations, and acute brain slices containing the entorhinal cortex and hippocampus. These tools have been used to evaluate and determine underlying mechanisms of short-term synaptic plasticity at a number of different types of identified synapses. We use dual patch clamp recording techniques to directly monitor the electrical activity of both the presynaptic and postsynaptic neurons in a synaptically coupled pair of cells. In addition, when finding pairs of synaptically coupled neurons is not possible, we utilize extracellular minimal stimulation techniques to evaluate short-term plasticity. Finally, we have begun to explore plasticity in preparations obtained from animal models of temporal lobe epilepsy. For details concerning the methods employed to prepare, maintain, and record from the different preparations, please consult Further Reading.

Results

Much of our work on short-term plasticity was originally performed in low-density primary cultures of either hippocampus or cortex; Figure 1 illustrates the basic approach. A pair of neurons is patch clamped; action potentials are elicited in the presynaptic cell, and either excitatory or inhibitory postsynaptic currents (EPSCs or IPSCs, respectively) are recorded in the postsynaptic cell

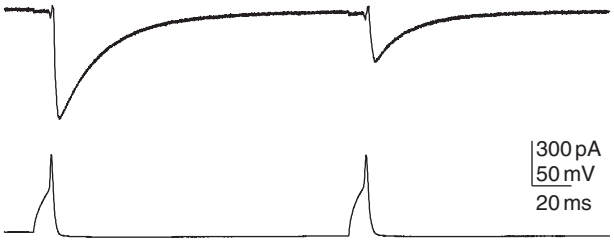


Figure 1 Paired pulse depression is observed between monosynaptically connected neurons in a low-density, dissociated hippocampal culture. Action potentials elicited in the presynaptic cell (lower trace) reliably evoke IPSCs in the postsynaptic cell. Note that the second IPSC is greatly attenuated compared with that of the first.

(depending on the neurotransmitter being released by the presynaptic cell). In these dissociated cell cultures, the predominant excitatory neurotransmitter is glutamate, whereas the predominant inhibitory neurotransmitter is GABA. An advantage of the whole cell patch clamp technique is that the reversal potential for PSCs can be determined by the investigator, and depends on the internal solutions used in the recording pipettes. In these studies, it is critical to determine that the paired cells establish monosynaptic – and not polysynaptic – interactions. Toward that end, we have modified our culture techniques to allow development of neurons at very low density, such that there are only two (or at most, a very few) neurons in a microscopic field. In addition, physiological criteria for monosynaptic connectivity include a constant latency for the PSC, a monotonic rise to peak in the PSC, and the ability of PSCs to occur with fidelity during high-frequency trains of action potentials. These experiments have led to many insights into the basic principles of short-term plasticity, as discussed earlier. In addition, these experiments allow us to ask detailed questions about the presynaptic or postsynaptic location of action of novel compounds that might be effective for decreasing excitability and treating seizures (a topic that is beyond the scope of the present article).

Figure 1 is an example of commonly observed paired pulse depression at a $GABA_A$-mediated inhibitory synapse recorded from pairs of neurons in a low-density primary culture of dissociated hippocampal neurons. Whereas low-frequency stimulation results in stable IPSC amplitudes, action potentials delivered within several hundred milliseconds of each other resulted in substantial postsynaptic IPSC depression. Our work has demonstrated that this depression is primarily due to a depletion of the readily releasable pool of vesicles. Depression at other synapses, in other systems, may be due to activation of G-protein-coupled autoreceptors that are activated as a consequence of neurotransmitter being released following the first action potential.

Our laboratories, and others, have also utilized the organotypic hippocampal slice culture preparation to examine short-term plasticity in a system with considerably more intact circuitry. Figure 2 is an example demonstrating target selective plasticity of CA3 pyramidal cells. When pyramidal cells in this preparation synapse onto other pyramidal cells, no dramatic depression of EPSCs is observed when action potentials occur at high frequency. In contrast, EPSCs recorded in the postsynaptic interneurons (receiving input from CA3 pyramidal cells) exhibited profound depression. Such target selective plasticity is not uncommon throughout the CNS. Therefore, it is critical that as synaptic properties are incorporated into computational models of network activity in physiological and pathological conditions, there must be careful evaluation of individual synapses within the circuit.

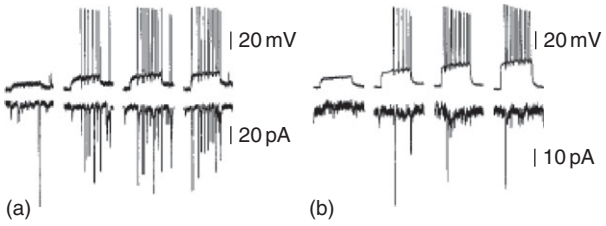


Figure 2 Responses to trains of action potentials (500-ms current injections of increasing amplitudes) in (a) PYR:PYR pair and (b) PYR:INT pair. Note the reliable following and lack of major depression in the PYR:PYR pair, while the PYR:INT pair shows extensive IPSC dropout or decrement during each train, reprinted with permission from Aaron and Dichter, 2001.

An additional complication in assessing short-term plasticity at various synapses is the not uncommon observation that a given synapse may simultaneously exhibit different forms of plasticity. For example, in cerebellar slices, and in cultured hippocampal neurons, repetitive EPSCs depress with high-frequency stimulation, whereas asynchronous release of miniature EPSCs (mEPSCs) dramatically increases over the same time scale. The enhancement of asynchronous release is calcium dependent, as perfusion with a membrane-permeant calcium chelator with slow on-rate kinetics, EGTA-AM, can prevent the facilitation of mEPSCs while having no overt effect of the depression of the evoked EPSC. It is unclear what the barrage of mEPSCs means functionally for the postsynaptic cell; however, it is likely that such asynchronous release of neurotransmitter would contribute greatly to the overall level of excitability of the postsynaptic cell.

Synaptic plasticity that might also be involved in ictogenesis may involve long-term forms. Although we propose that short-term plasticity plays a significant role in the transition from the interictal to ictal state, there is evidence to suggest that other mechanisms of synaptic strengthening, such as long-term potentiation (LTP), may also be involved. This contribution may be especially important during the 'buildup' of activity prior to the actual seizure. Furthermore, low-frequency stimulation of the kind typically used to induce long-term depression (LTD) can dramatically reduce the propensity of brain slices to burst, and continuous sinusoidal electrical stimulation has recently been shown to be an effective mechanism to suppress epileptiform activity in the in vitro rat hippocampal preparation. Therefore, although short-term plasticity might enable seizure initiation and spread, it may follow on the heels of strengthening certain feedforward excitatory pathways by the development of longer lasting forms of potentiation within the circuits.

As suggested earlier, a major technical breakthrough in the study of synaptic transmission in the central nervous system has been the development of methods to simultaneously record from identified, monosynaptically

connected neurons in both culture systems and in brain slices. Experiments in these preparations have yielded a wealth of important findings concerning basic mechanisms of synaptic transmission, and the ability to record from synaptically connected neurons within the context of the neural circuit has proven invaluable in determining a wide variety of 'rules,' which govern synaptic function, at least within the circuitry of the hippocampus and cortex. Furthermore, these types of experiments have begun to unravel important homeostatic mechanisms governing frequency-dependent activity in the mammalian brain. These 'synaptic rules' appear to stabilize intrinsically excitable neural circuits, which might otherwise be predisposed toward uncontrolled activity. Such studies have given rise to important concepts about synaptic regulation, such as synaptic scaling, spike timing-dependent plasticity (STDP), synaptic redistribution, inverse homosynaptic versus heterosynaptic plasticity, and maintenance of constant firing rates by changes in ion channel expression function and expression.

Future Goals

Great strides in our understanding of synaptic activity have been made with our increased ability to record from pairs of identified neurons within the context of neuronal circuitry in brain slices. Furthermore, by combining experimental studies with computational approaches, improving EEG monitoring technologies within different animal models of epilepsy, and enhancing our cellular monitoring/recording techniques, we should be able to assess in much greater detail the neural circuits involved in seizure generation and propagation. Questions concerning the homeostatic strategies of synaptic transmission in pathological conditions should provide an in-depth understanding of the processes underlying seizure initiation and propagation. This mechanistic approach to analyzing the events leading up to seizure generation will no doubt subsequently provide new approaches for the control of seizures.

While the experiments discussed here have been performed in tissue from nonepileptic animals, it is imperative that the field investigates changes in short- and long-term synaptic plasticity in tissue obtained from epileptic animals or in tissue resected from patients with epilepsy. This goal is especially important for analyzing partial seizures that arise from the temporal lobe, the most prevalent and troublesome form of intractable epilepsy seen in adults. In such tissues, brain injury can initiate several potentially epileptogenic reactions: many types of cells die throughout the limbic system, including large populations of inhibitory interneurons; sprouting of remaining axons results in functional changes in neural circuitry, possibly introducing aberrant forms of recurrent excitation, and multiple changes are observed in gene expression (for ion channels, neurotransmitter receptors, and other important regulatory proteins). Understanding changes in the rules governing synaptic plasticity in the various forms of epilepsy should set the stage for innovative therapeutic approaches for the control of seizures, and perhaps even illuminate strategies that might modify the process of epileptogenesis in patients at risk following an insult to the CNS.

See also: **Models**: The use of Cell Culture Models to Study Mechanisms Related to Epilepsy and Antiepileptic Drugs; **Neurotransmitter Function**: Presynaptic Neurotransmission: Alterations in Exocytotic/Secretory Machinery in Kindling; **Plasticity**: The Role of Calcium in Mediating Neuronal Plasticity in Epileptogenesis; Synaptic Potentiation and Inhibition after Partial Seizures.

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Posttraumatic Epilepsy Caused by Fluid Percussion Injury

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Introduction

Western societies are plagued by acquired forms of epilepsy. Among these, posttraumatic epilepsy (PTE) is the most common. Furthermore, PTE often manifests itself as complex partial seizures, which are often resistant to treatment with antiepileptic drugs (AEDs). Therefore, PTE results in a significant social burden and has been the subject of large research efforts in the basic, translational, and clinical realms. The socio-economic value of developing drugs that prevent epilepsy following insult to the central nervous system, or fully control seizures now considered to be pharmacoresistant, would be enormous. However, several obstacles currently hamper the achievement of better treatments:

1. The cellular mechanisms necessary for clinical ictogenesis and epileptogenesis after head injury are still not known. Many different pro-epileptic mechanisms have been proposed to cause (or have a role in) PTE, including loss of inhibition, intrinsic membrane depolarization of hilar neurons, mossy cell hyperexcitability, enhanced excitatory synaptic connection, saturation of synaptic long term potentiation, and impaired K^+ buffering by posttraumatic glia. However, head injury probably results in countless pro-epileptic changes in the brain, and the clinical relevance and the relative importance of any of the investigated mechanisms – in the onset of human PTE or its pharmacoresistance – have yet to be defined; indeed, no direct cause–effect relationship between any of these pro-epileptic changes and the precipitation or maintenance of clinically relevant chronic spontaneous recurrent partial seizures (CSRPSs) has ever been established. Therefore, we currently do not know what the best therapeutic targets might be.
2. Human PTE often manifests with multiple epileptic foci (or epileptogenic zones), which greatly complicates the treatment of the pharmacoresistant patient. Therefore, animal models that faithfully reproduce this level of complexity should be employed to investigate mechanisms and treatments of disease progression that might be relevant to the human condition. Conversely, the hippocampus has been the almost unique center of attention for much of experimental epilepsy research (based on one-focus models). While the hippocampus certainly has a role in human mesial-temporal lobe epilepsy, head injury patients often develop neocortical foci, suffer from progressive temporal cortex pathology, and may not be seizure-free after hippocampectomy. Therefore, understanding the mechanisms by which multiple neocortical and subcortical epileptic foci arise, interact, and progress following head injury is crucial for the development of novel treatments. Animal models that just focus exclusively on hippocampal mechanisms may be greatly limiting our capability to understand clinical epileptogenesis.
3. Current efforts to discover better treatments rely on two sub-optimal approaches.

   The first approach consists of screening substances with animal models of acute seizures or epilepsy that typically involve application of electrical (kindling, electroshock) or exogenous chemical stimuli (pilocarpine, kainate, pentylenetetrazole), which are quite unlike the insults that produce clinical PTE. Further, the mechanisms of epileptogenesis in these models are unclear, and have never been shown to correspond to those responsible for human epileptogenesis. In addition, the lack of temporal definition of chemical insults and the occurrence of acute seizures which commonly appear during the induction of epileptogenesis, make it difficult to distinguish antiepileptic, neuroprotective and antiepileptogenic effects of the agent screened.

   The second approach is to test a substance in clinical trials on patients who suffered injuries that put them at risk for acquiring epilepsy. In the hope that either acute seizure control could prevent a hypothetical seizure-induced kindling-like phenomenon in humans, or that neuroprotection could be equated with antiepileptogenesis – both unproven concepts – several classical AEDs and neuroprotective agents have been studied. In lengthy and expensive clinical trials on posttraumatic patients, these agents have been shown not to be antiepileptogenic in humans. These clinical trials extended over decades and costed tens of millions of dollars. There remain many questions about why these drugs failed to exert antiepileptogenicity – e.g., because of an intrinsic lack of activity or because of the doses and windows in which they were tested. Whatever be the answers to these questions, there is increasing concern that epilepsy models used to screen antiepileptogenic drugs may not optimally target clinically relevant epileptogenic mechanisms, and therefore may be unsuited to find the first clinically effective antiepileptogenics, or even better AEDs.

Overcoming all these obstacles requires the development of improved and clinically relevant animal models of epileptogenesis and pharmacoresistant epilepsy. That is not to say that classic epilepsy models are not useful. Indeed, work on these models has resulted in the identification of several effective AEDs. However, to take the next step in epilepsy research – to control the large fraction of complex partial seizures that are currently pharmacoresistant, or have lower side effects – novel models are needed that probe the neuropathophysiological mechanisms of human epilepsy, and allow for preclinical tests and optimization of clinical trial design.

Background

In an effort to fill the gap, two laboratories have worked independently to develop a novel in vivo model of PTE in the rat in which CSRPS appear after a single clinically relevant insult that mimics concussive closed head injury – the fluid percussion injury (FPI). This model represents a significant departure from previous models of acquired epilepsy because it presents unique similarities to the human post-traumatic epileptic condition (Table 1), and all the features tested thus far satisfy the conditions identified as desirable in an "ideal" animal model for the study of acquired epileptogenesis:

- The initiating insult, a transient compression of the *dura mater* without brain penetration, is mechanically very similar to human cases of concussive closed head injury. The FPI-induced epilepsy is not dependent on the presence of chronic stimuli (the clinical relevance of which is unproven) – which provide the bases for neurotoxicant- and kindling-based models, and/or are of questionable clinical relevance.
- FPI-induced epilepsy presents with: (1) focal seizures, as in human cases of acquired epilepsy, (2) pathology consistent with human epilepsy, (3) a 'seizure-free' latent period between the initiating injury and the onset of the epileptic condition, (4) CSRPSs following this latent period, and (5) temporal progression in seizure frequency and duration. Whether this form of PTE is resistant to classic AEDs (another feature of the ideal animal model of PTE) is currently under investigation. Finally, FPI-induced PTE presents progressive mesial temporal lobe seizures and pathology similar to human mesio-temporal lobe epilepsy. In this regard, FPI-epilepsy is not a *model* of head injury-induced epilepsy – it is real head-injury induced epilepsy in the rat, and it models human PTE to the extent a rat brain may model the human. In FPI, a fluid pressure wave models the blunt impact induced by a solid usually seen in humans. Because of these similarities, we consider FPI-epilepsy to provide the best effort on a "similarity scale" of current models of acquired epilepsy and believe that it is highly likely that the epileptogenic mechanisms recruited by FPI are those involved in human post-traumatic epileptogenesis.

Table 1 Known similarities between FPI-induced PTE in the rat and human PTE

1	The traumatic brain injury model is mechanically very similar to human cases of closed head injury.
2	Chronic spontaneous recurrent seizures appear after a single event of traumatic brain injury.
3	Seizure free latent period between the initiating injury and the onset of the epileptic condition.
4	Seizures are focal, with or without secondary bilateral spread.
5	The ictal behavior is consistent with human complex partial seizures.
6	Neocortical and hippocampal epileptic foci.
7	Progressive temporal lobe sclerosis in a subgroup of epileptic individuals, and not in others.
8	Time-dependent changes in epileptic syndrome.
9	Cases of remission.
10	Heterogeneity of posttraumatic electro-clinical syndrome.

Methods

There are different variations of FPI employed in traumatic brain injury research. The most important variants involve the anesthetic employed, the location of the injury, and the size of the craniotomy through which the injury is delivered. In our laboratory we implemented specific variations to render FPI more suitable for epilepsy research. Briefly, our animals are anesthetized with 4% halothane in an anesthesia box and then placed on a heated pad to maintain core temperature at 37 °C. Rats are endotracheally intubated and then they are mechanically ventilated with ~1.5% halothane and 30% oxygen/air mixture. Lidocaine (1%) plus bupivacaine (0.25%) are infiltrated subcutaneously at the proximal ventral region of the tail and midline of the cranial vertex. After the head of the rat is fixed in the stereotaxic frame, the skull is exposed, under aseptic conditions, by a longitudinal incision from occiput to the forehead. A 3-mm burr hole is drilled parasaggitally, taking maximal care not to damage the underlying dura either by compression or heat. A Leur–Lok cannula with an inner diameter of 2.5 mm is secured (with methyl methacrylate) to the skull over the craniotomy. The injury cannula is filled with sterile isotonic saline and connected to the FPI device. The animal is disconnected from the ventilator, and a single pressure pulse (2–4 atm, ~8 ms) is delivered into the closed cranial cavity to induce the injury. In response to this insult, the animal stops breathing for up to 10 s and then either spontaneously recovers, or it is put back on the ventilator. The FPI injury does not penetrate the dura. The animal is weaned from the ventilator and extubated. The wound site is closed with wound clips and gentamycin is applied topically on the wound.

We prefer performing the procedure with volatile anesthetics because rats are known to suffer from respiratory arrest following either FPI or cortical impact, when the pressure shock reaches the pons. If the animals are intubated, then they can be rescued by ventilation. Conversely, with anesthesia by i.p. injection, animals are not ventilated and the injury results in much higher mortality because of respiratory arrest. It should be noted that severe head injury patients would be rescued by intensive care and would enter the pool of patients suffering from PTE. Therefore, volatile anesthetics allow for a more clinically relevant application of the model, and for lower mortality of the animals. Also, we prefer to use a smaller craniotomy (3 mm vs 4.8 mm) because it allows for a defined neocortical focus, limits the diffuse component of the FPI, and therefore, also limits the direct injury to subcortical structures such as hippocampus. This strategy has allowed us to observe a clearly distinguished time course of epileptogenesis in neocortex versus hippocampus after FPI which may have clinical therapeutic relevance.

An important technical issue of FPI-induced epilepsy is worth mentioning here. Drilling a skull craniotomy is a significant surgery that, if not performed with the utmost care and skill, can result in cortical damage and later focal EEG discharges. Cortical damage can result either by compression of the brain during drilling, or by heat produced by friction of the drill bit carving the bone. To reduce the chances of damage we continuously cool the bone during drilling with room-temperature sterile saline. In addition, to prevent brain compression and laceration, we never drill down to the dura. Rather, we carve a crown of bone and lift it up with forceps. Similar precautions must be used when implanting epidural electrodes. We always obtain pathological data at the end of each experiment to verify that animals entering the studies have not suffered from damage induced by electrode implantation or craniotomy – e.g., as indicated by a focus of glial reactivity right under the location of the electrodes. These controls are time consuming and require attention to details. However, omitting them is risky and may explain why some investigators see short focal seizures in their control rats – electrode-induced cortical damage.

Recent Results

FPI-induced epilepsy is a progressive disorder (**Figure 1**). Seizure activity appears within 2 weeks after injury and worsens over time, as reflected in frequency, duration, and behavioral correlates. FPI-induced epilepsy manifests with 3 types of cortical electrical discharges. Grade 1 electrical seizures consist of focal epileptic ECoG events, detected only by peri-lesional electrodes, which do not appear to spread ipsi- or contralaterally (**Figure 1a**). Grade 2 seizures are first detected by the perilesional electrodes, and then spread to the ipsi-and/or contralateral cortex (**Figure 1b**). Grade 3 seizures appear bilateral at their cortical onset, often involving all electrodes simultaneously. The observation that grade 1 and 2 seizures are first detected by the perilesional electrodes, together with the finding of focal neocortical hyperexcitability in vitro, suggests that the neocortex near the site of FPI develops the earliest epileptic focus.

Seizures in the first weeks postinjury are difficult to detect by simple behavioral observation because they are associated with only subtle behavioral output (i.e., are most commonly associated with behavioral arrest). To best assess the behavioral correlates of CRSPSs, we rank (off-line) the concurrent behavior of epileptic ECoG events according to a scale we designed: 0 = no behavioral change (i.e., subclinical to the best of our observational capability); 1 = freeze-like pause in behavior without impaired posture; 2 = freezelike pause in behavior without impaired posture and accompanied by facial automatisms (twitching of vibrissae, sniffing, eye blinking, or jaw automatisms); 3 = head nodding; 4 = body myoclonus; 5 = loss of posture, atonia; 6 = loss of posture followed by motor manifestations (facial automatisms, contralateral limb dystonia); 7 = tonic–clonic convulsion; and 8 = partial statuslike electroclinical syndrome. Typically, 2–3 weeks after FPI, epileptic rats show freezelike pauses in their behavior, with or without facial automatisms, in synchrony with all three grades of epileptic ECoG events. Behavioral correlates of seizures 4–7 months postFPI more commonly include clonus or loss of posture (**Figure 1d**). A partial seizure at this time postinjury typically consists of crawling behavior (at the bottom of the cage) in a stereotyped fashion for few seconds before the onset of bilateral cortical discharge. The fact that the clinical seizure precedes cortical epileptiform activity suggests a subcortical origin for grade 3 seizures.

Another consistent feature of FPI-epilepsy is a time-dependent change in the frequency (**Figure 1e**), severity (**Figure 1f**), and type (**Figure 1g**) of seizures, similar to certain forms of acquired human epilepsy. Furthermore, there is a distinctive time-dependence in the relative proportion of seizure types (**Figure 1e**). We consistently find that 2–3weeks postinjury, grade 1 seizures are by far the most common seizure type, which is in agreement with a neocortical origin of FPI-induced epilepsy at this time-point. However, their proportion progressively decreases over time. Grade 2 seizures are rare at early times after injury, but peak at about 14–15 weeks postinjury. Grade 3 seizures are also rare in the early weeks postinjury, but progressively increase over time, becoming the most common seizure type by 4months postinjury. The worsening in severity of FPI-epilepsy can therefore be appreciated in the increase in seizure frequency, duration, and probability of bilateral spread of seizures over time postinjury.

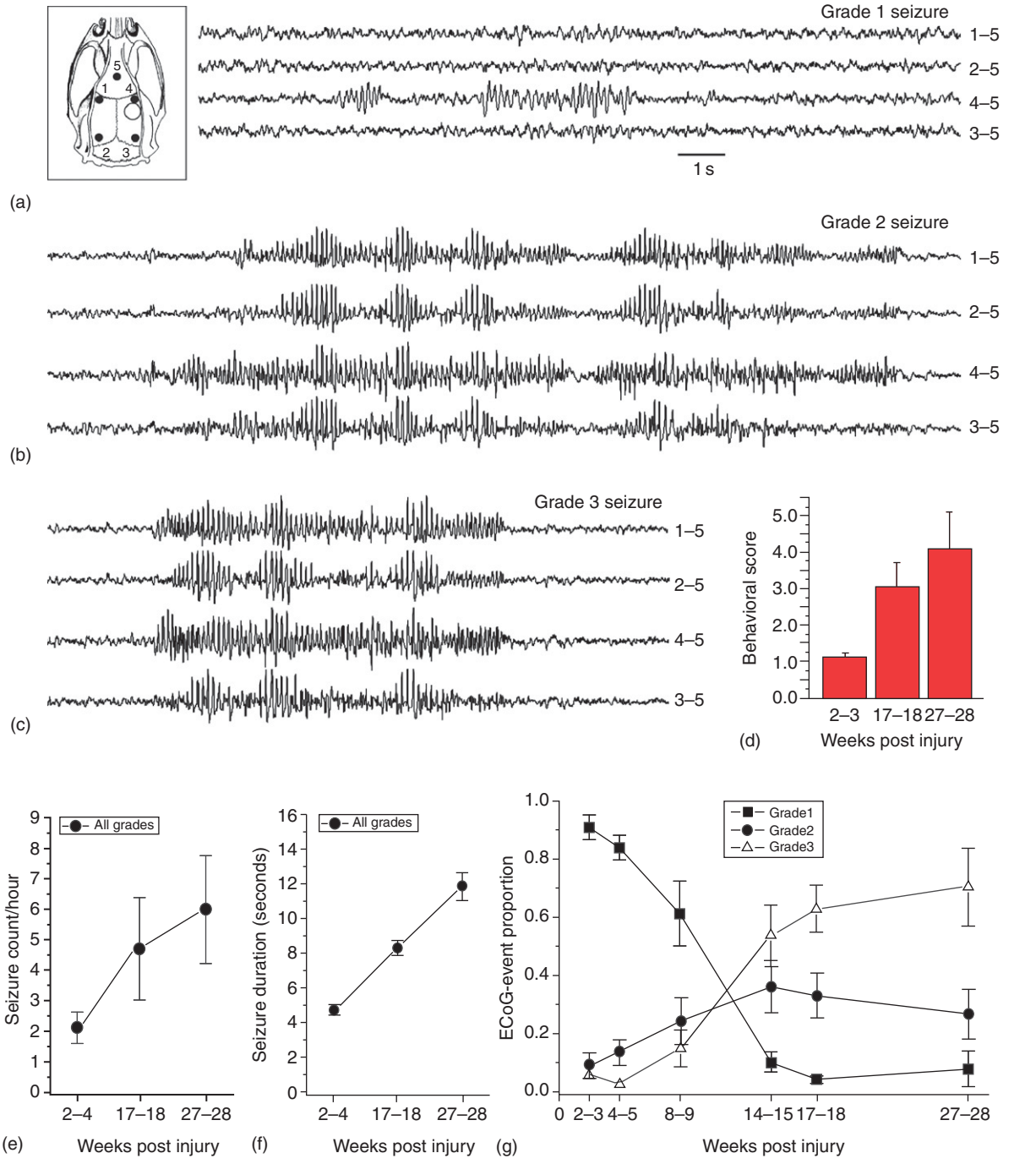


Figure 1 Types and temporal evolution of chronic spontaneous recurrent partial seizures induced by rostral parasaggital FPI. Panels A though C represent continuous ECoG recording in wake animals. (a) Representative grade 1 seizure detected exclusively by the peri-lesion electrode 2 weeks post-FPI. The Inset depicts the locations of the five epidural electrodes (filled circles) and of the injury site (hollow circle) in respect to the rat skull. (b) A representative grade 2 seizure first detected by the peri-lesion electrode and then by multiple channels 27 weeks post-FPI. (c) A representative grade 3 seizure detected simultaneously by multiple channels 15 weeks post-FPI. The numbers next to each ECoG trace indicate the electrodes by which the trace is recorded and its reference. (d) Temporal progression of the behavioral correlate of seizures. See behavioral scale in text. (e) The frequency of FPI-induced seizures is time dependent. (f) Also the duration of FPI-induced seizures increases over time post-injury. (g) 2–3 weeks post-injury, grade 1 seizures are the most common seizure type, but their proportion progressively decreases over time. Conversely, grade 2 and especially grade 3 seizures are rare in the early weeks post-injury, but progressively increased over time becoming the most common seizure type 7 months post-injury. These data show that the probability of seizures spreading bilaterally increases over time and that epilepsy worsen over time post injury. Data are shown as mean ± S.E.M. (d–g) are modified with permission from D'Ambrosio *et al*. (Brain 2005, 128: 174–188).

Future Goals

The Model Needs Optimization and Standardization

The two groups working with this model have reported remarkably similar findings – in particular, the observation of CRSPSs and their progressive worsening over time. However, they also observed significant differences. One group observed progressive neocortical and hippocampal electrical and behavioral partial seizures without tonic–clonic convulsions, while the other observed only hippocampal seizures accompanied by tonic–clonic convulsions. The different electrical and behavioral correlates of PTE reported by the two laboratories probably reflect differences in their experimental approach, including the genetic background of rat colonies, the age of the animal at the time of the injury, the location/extent/degree of injury, and anesthesia employed. Indeed, while one group employed rostral-parasaggital FPI performed through a 3-mm craniotomy on 1-month-old rats, the other employed caudal-parasaggital FPI performed through a 4.8-mm craniotomy on 2.5-month-old rats. These differences require further study and suggest the need for standardization of the model before it can be used confidently as a preclinical investigative tool. However, these differences also greatly enhance the potential value of, and the enthusiasm for, the FPI-induced epilepsy model. Indeed, human PTE is also very heterogeneous, manifesting itself as complex partial seizures with a variety of behavioral and electrical correlates that likely depend on the type, degree, and location of the injury; the age of the patients at the time of injury; and their genetic backgrounds.

Models of FPI-induced epilepsy have only recently been developed, and therefore, we expect that a certain period of time will be needed for protocols to be optimized. For example, in our initial studies we induced FPI by delivering injuries at 3.5–4 atm, which resulted in 33% acute mortality. That was in itself not objectionable, because it compared favorably with the mortality obtained with the pilocarpine protocol used by many laboratories studying epileptogenesis. However, after further tests, we determined that injuring the animals at exactly 3.25 ± 0.1 atm resulted in only 11% acute mortality, and resulted in comparable seizure frequency. Two other variables that need to be studied are the location and degree of injury on the type and severity of FPI-induced epilepsy.

FPI-Epilepsy Should be Adapted to the Mouse

The development of a murine model of genuine CSRPSs would be of tremendous help in epilepsy research. The use of genetically engineered mice would allow the investigation of genetic factors – such as apolipoprotein E epsilon-4 allele, haptoglobin Hp2–2 allele, or IL-1β (511) allele 2 – that predispose the posttraumatic brain to the later development of PTE. In addition, it would allow investigators to further assess the relevance of specific cellular mechanisms of epileptogenesis and seizure progression. For example, recent elegant work has shown that conditional deletion of TrkB, the receptor for brain-derived neurotrophic factor, prevents epileptogenesis in the kindling model. However, it is not known what role TrkB might play in more relevant models of human PTE such as FPI-induced epilepsy.

Reliable Automatic Seizure Detection is Currently Lacking

Another area that requires further development to make the best use of FPI-epilepsy – and of similar models of epilepsy that could be used for drug discovery – is computerized EEG analysis for automatic seizure detection. Animals must be monitored for prolonged periods of time, and the problem of mining for seizures in such large datasets is considerable. This issue has generally not been critical in past studies of epilepsy-related mechanisms in animal models, as investigators have been primarily focused on evaluating drug effects relative to acute and/or behavioral correlates of severe seizures. These effects are easier to identify/measure because they tend to be stereotyped and obvious in a video played at fast-forward speed. However, detection of seizures in the EEG, and especially counting seizures that may have subtle behavioral correlate (i.e., behavioral arrest) is much more difficult. In the absence of software to automate such work, seizures must be found by manual analysis of the ECoG, which is exceedingly time consuming and expensive. In our laboratory, primary manual analysis of 24 h of continuous rat ECoG takes approximately 1.5 h.

Conclusions

The recent discovery that FPI, a relevant model of concussive closed head injury, induces PTE in the rat opens new research horizons in the study of clinically relevant mechanisms of acquired epileptogenesis. FPI is the model of acquired epilepsy that provides the greatest similarity to the human condition. As such, it is the model that offers the greatest opportunity for identifying mechanisms of posttraumatic epileptogenesis that are clinically relevant. We believe that it will be an important aid in the search for new antiepileptic drugs and for the first antiepileptogenic agents as it probes mechanisms of epileptogenesis that are likely to be closely related to epileptogenic mechanisms in humans. However, as always occurs when a new model is first introduced, an extensive characterization of its features is necessary to optimize its use. Furthermore, development of algorithms and software for computerized seizure detection will greatly facilitate the

analysis of data obtained with the FPI-induced epilepsy model, as well as with any other model of CRSPSs.

Acknowledgements

The development of the FPI-model of PTE has been possible thanks to the financial support of the NIH/NINDS (grants NS053928, NS40823), the Epilepsy Therapy Development project, the CURE epilepsy foundation and ARMY, the University of Washington (grant RRF2985) and the Department of Neurological Surgery.

See also: **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury; Posttraumatic Models; Traumatic Hemorrhage and Iron-Induced Epileptogenesis.

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Posttraumatic Models

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Introduction

Traumatic brain injury (TBI), according to the World Health Organization, will surpass many diseases as the major cause of death and disability by the year 2020. With an estimated 10 million people affected annually by TBI, the burden of mortality and morbidity that this condition imposes on society makes TBI a serious public health and medical problem. TBI accounts for 20% of symptomatic epilepsy in the general population and 5% of all epilepsy. Posttraumatic epilepsy refers to the occurrence of late seizures (1 week or longer after the injury), and the latency from injury to appearance of epilepsy may be many months or years.

Clinicians involved in the regular care of patients with epilepsy know that posttraumatic epilepsy is an extremely disabling condition. These patients are frequently pharmacoresistant and are often not good surgical candidates. Traditionally, TBI patients have been treated with a prophylactic anticonvulsant – that is, an antiepileptic drug (AED) – with the expectation that treatment will reduce the incidence of posttraumatic epilepsy. The results of trials with AEDs as antiepileptogenic agents have been very disappointing. Nevertheless, the long latency period between the initial insult and the occurrence of chronic spontaneous seizures raises the possibility of designing preventive – that is, antiepileptogenic – therapies for susceptible individuals after serious head trauma.

The challenge of translating discoveries at the cellular and molecular level to an effective antiepileptogenic therapy requires an appropriate animal model. Also, studies on posttraumatic epileptogenesis would greatly benefit from surrogate markers that may serve to denote risk for future epilepsy. Such markers would be useful for the timely identification of key points in the epileptogenic process that would be amenable to intervention. Targets for the prevention of epileptogenesis most likely change as the process proceeds, so that appropriate targets at one

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The challenge of translating discoveries at the cellular and molecular level to an effective antiepileptogenic therapy requires an appropriate animal model. Also, studies on posttraumatic epileptogenesis would greatly benefit from surrogate markers that may serve to denote risk for future epilepsy. Such markers would be useful for the timely identification of key points in the epileptogenic process that would be amenable to intervention. Targets for the prevention of epileptogenesis most likely change as the process proceeds, so that appropriate targets at one

point may not be relevant at another. Many other issues need to be addressed, including such questions as: What are the risk factors for eventual development of posttraumatic epilepsy? When are these risk factors sufficiently clear and of high enough predictive value to warrant treatment with potentially deleterious drugs? What are the critical and realistic time points for these risk factors to be detected clinically? The importance of preclinical studies in animal models of PTE, to address these questions, cannot be overestimated. Useful laboratory data must be based, however, on the development and study of clinically relevant animals models – so that anatomic, biochemical, cellular, and molecular components of the posttraumatic epileptogenic process can be understood.

Background

Clinical research to date has defined a clear relationship between the severity of TBI and the development of posttraumatic epilepsy. Severe brain injuries with focal intracranial lesions, skull fractures, or prolonged alteration in consciousness are the most important risk factors for the development of posttraumatic seizures. Other important factors include bilateral contusions, subdural hematoma with surgical evacuation and acute intracerebral hematoma, multiple intracranial procedures, and midline shift (over 5 mm). Focal seizures are the most common type diagnosed. According to human studies, up to 56% of patients with PTE present with temporal lobe epilepsy. Seizures arise from frontal lobes in 36% of patients, while cases of parietal and occipital seizure focus are relatively rare (5% and 3%, respectively).

The multiple pathological consequences of TBI include primary damage from direct shearing, laceration or contusion of brain tissue by bone fragments or foreign bodies, hemorrhage, and diffuse axonal and vascular injury. Also, secondary damage may be caused by hypoxia-ischemia, edema, and breakdown of the blood–brain barrier. Epileptogenesis develops simultaneously with the progress of secondary injury and posttraumatic recovery. From the point of view of developing clinically relevant models, disease heterogeneity presents a real challenge; indeed, simulating all aspects of human TBI with any one single animal model may be an impractical goal. Given the great variation in the nature of TBI, it is not surprising that a number of different models – ranging from mechanical trauma to individual neurons in culture to acceleration–deceleration injury in nonhuman primates – have been developed to study basic mechanisms of head injury. Several animal models have been used to elucidate the structural, chemical, and physiologic changes that are associated with TBI and that may contribute to seizures and epilepsy.

On the basis of our current understanding of the factors that contribute to the development of epilepsy, the following characteristics have been suggested for an ideal animal model: (1) mechanisms consistent with those of human epilepsy, (2) existence of latent period between the initiating insult and expression of behavioral seizures, (3) spontaneous seizures as a final result of the functional and structural reorganization that occurs during the latent period. The remainder of this article reviews the three TBI models in which chronic epilepsy, or permanent hyperexcitability, has been consistently reported after the initiating brain insult (**Table 1**).

Methodology and Summary of Results

Hemorrhage-Iron Deposition (A Model of PTE Caused by Blood–Brain Barrier Damage Associated with TBI and Extravasation of Blood with Iron Deposition)

Hypothesis of action

Head injury with contusion, cortical laceration or intracerebral hematoma formation results in extravasation of

Table 1 Occurrence and characteristics of posttraumatic epilepsy in different animal models as compared to humans

	Hemorrhage - iron deposition		*Chronic partial cortical isolation*		*Fluid percussion brain injury*		
	Cortical	*Amygdalar*	*Rat*	*Cat*	*Lateral*	*Parasaggital*	*Human PTE*
Latency period	48 h–5 d	5 d	10–14 d*	~3 h	1–11 mo	1–2 wk	within 2 years in 86% of patients
Spontaneous seizures (% of cases)	94%	92%	n/a	40–90%	43–50%	100%	up to 53%
Behavioral appearance	partial	generalized	partial	partial generalized	partial and sec. generalized	partial (?)	partial and sec. generalized
Seizure frequency	n/a	n/a	n/a	n/a	0.3 sz/day	up to 7 sz/h	n/a
Seizure duration	~20 sec	~120 sec	~10 sec	mean 17 sec	mean 113 sec	~10 sec	n/a

*According to the experiments performed in neocortical slices

blood and breakdown of red blood cells and haemoglobin. Hemolysis and deposition of iron compounds (such as hemosiderin) within brain tissue is a common finding in cortical tissue samples of patients with drug-refractory PTE and a cortical seizure focus. Moreover, according to human imaging studies, the occurrence of PTE is significantly correlated with the evidence of acute intracerebral hematoma, and hemorrhagic contusions with a gliotic wall incompletely surrounding hemosiderin dregs present a risk factor for PTE.

Subpial or amygdalar injection of ferrous/ferric chloride is suggested to mimic the harmful effects of iron after parenchymal hemorrhage. Iron liberated from haemoglobin, and haemoglobin itself, are associated with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS, especially ·OH, induce peroxidation of unsaturated fatty acids that are components of neuronal membranes, and also cause alterations in excitatory and inhibitory neurotransmitters, presumably leading to epileptogenicity.

Cortical iron injection

In a model of PTE developed initially by Willmore and colleagues, a Ø1 mm burr hole was made in the calvarium over the left sensorimotor cortex of a rat (1.5 mm rostral and 3.5 lateral to bregma). A 30-gauge needle attached to a microinjection syringe was inserted into the cortex 1.2 mm below the exposed dura, and 5 µl of aqueous solution containing 100 mM $FeCl_2$ or $FeCl_3$ was injected (over a 5 min period). In cats, 10 µl of aqueous solution of iron salts was injected into the left postcruciate gyrus or left posterior sigmoid gyrus. Cortical electroencephalographic recordings were made daily for 1 week and weekly thereafter for 3 months.

Findings

Isolated spikes appeared in EEG almost immediately after the iron injection, and by 24 h the majority of animals displayed isolated spike- or sharp-wave discharges or sustained bursts of focal spike-wave activity ipsilateral to injection site. Between 48 h and 5 days after injection, 96% of iron-injected animals had frequent and sustained bursts of high amplitude polyspike discharges on the ECoG. Interestingly, 22% of control animals (injected with 0.9% NaCl acidified to pH 1.5 with hydrochloric acid to equal the pH of the solutions of iron salts) also exhibited focal ECoG spike activity during the first 14 days. In iron-injected animals, epileptiform activity originated on the injection side and spread contralaterally. Occasional behavioral convulsions and electrographic seizures were reported at later time-points.

To determine whether this epileptiform activity lasts beyond a 3 months time-point, (i.e., can be considered chronic iron-induced epilepsy), a more extensive ECoG study was performed by Moriwaki and colleagues with the same model. Three-hour long recordings were started immediately after the completion of surgical procedures, and thereafter repeated 3 times a week for 12 months. In the period starting immediately after injection and lasting for the next 30 days, isolated spikes were recorded that increased in frequency. After 30 days, spike-and-wave complexes (200–1000 µV, 7–10 Hz) appeared, and this activity was sustained until the end of experiment in 28–38% of injected animals, accompanied with head nodding and vibrissae tremors. No full-blown electrographic seizures have been reported.

Later the procedure was modified by Ueda, Willmore and colleagues, and amygdalar $FeCl_3$ injections were used to create lymbic-type seizures in rats. Animals were injected in the right amygdaloid body (3.3 mm posterior, 5.0 mm lateral to bregma, and 8.5 mm below the skull surface) with a silica tube connected to the microinjection pump. The silica tube was positioned inside a 22-gauge guide canula, and 1–1.5 µl of 100 mM $FeCl_3$ was injected over a 1.5 min period. Following the injection, EEG recording and behavioral observations were obtained daily for 10 days and then every 5 days for the 30 days of the experiment. Acute injection of $FeCl_3$ into the amygdala caused a gradual increase in spikes over 20 min, followed by normalization of EEG pattern by the end of the 30 min recording. From 7 to 30 days after injection, spontaneous stage 4 (rearing and forelimb clonus, according to Racine) behavioral and electrographic seizures were observed in 92% of animals. Seizures started from the amygdalar regions (basolateral nucleus of the left amygdaloid body) and rapidly propagated to the hippocampal electrodes (**Figure 1(a)**).

Histologic analysis 6 weeks after cortical iron injection revealed a narrow 0.5 mm cavity at the site of injection, extending through all layers of the isocortex, with ferric-cyanate complexes in macrophages within the lesion. Neuronal loss and astrogliosis were observed within the depth of the injection site, and pyramidal neurons in lamina V beneath the injection site stained with iron-cyanate particles. Neurons adjacent to the lesion cavity showed deformity of dendrites, dendritic nodularity and loss of spines. After $FeCl_3$ injection in amygdala, histopathologic changes were reflected by mossy fiber sprouting and cell loss in the hilus and CA1 region of the hippocampus of animals with spontaneous seizures. Aspartic acid, glutamic acid and γ-aminobutyric acid levels were reported to decrease transiently after the injection of ferric chloride in the rat brain. The in vivo antioxidant capability of the dorsal hippocampus was reported to decrease significantly bilaterally in animals with $FeCl_3$-induced seizures. The expression of hippocampal GABA transporter protein GAT-1 and GAT-3 (which remove GABA from the synaptic cleft and terminate the synaptic effect of this neurotransmitter) increased bilaterally at 5–15 days after injection; GAT-1 returned to control levels by 30 days and GAT-3 remained elevated up

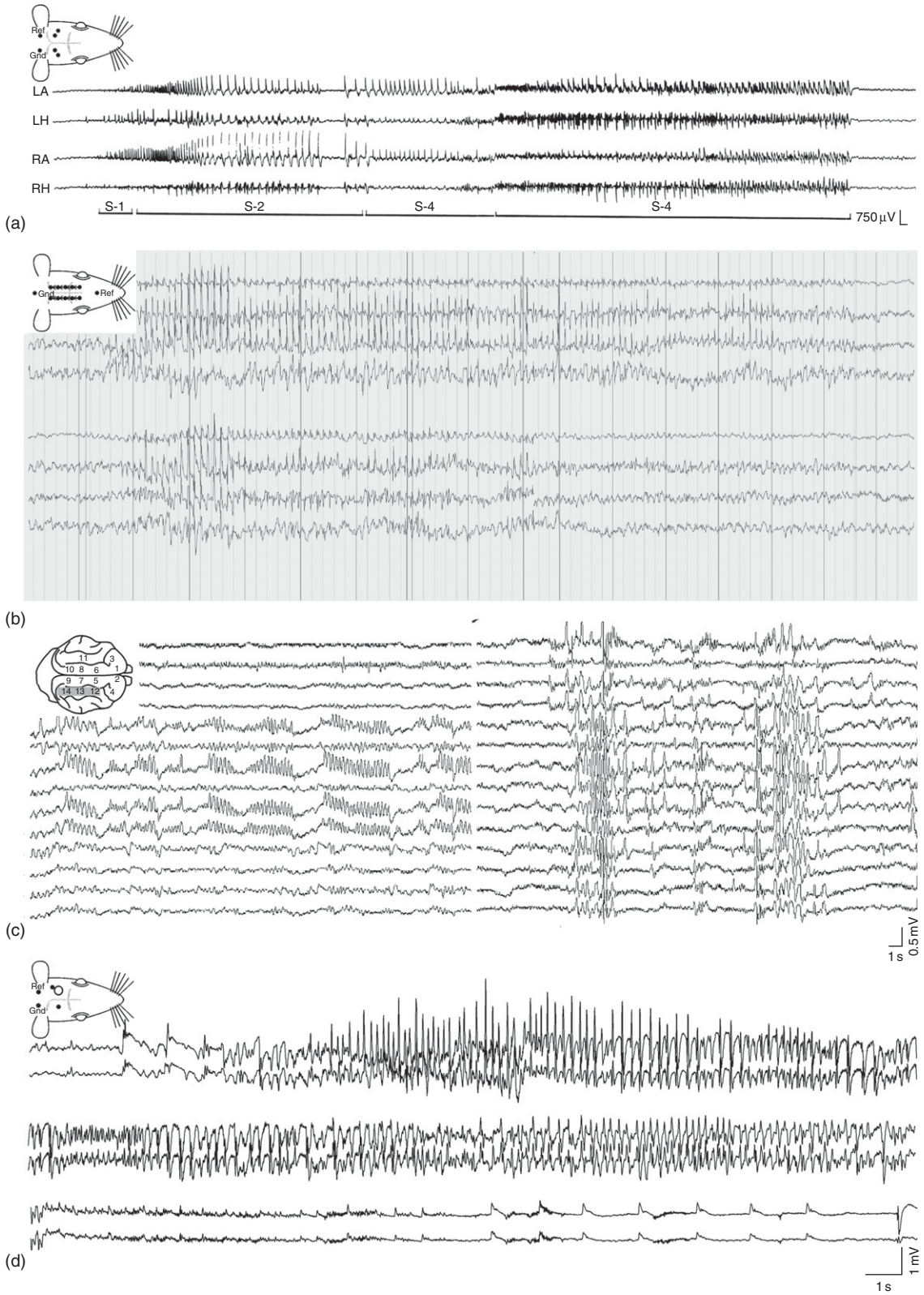


Figure 1 Representative EEG fragments showing ictal episodes in each of the models of post-traumatic epilepsy. (a) Amygdalar ferric chloride injection. Spontaneous electrographic seizure in an 8-week old Sprague–Dawley rat recorded 15 days after the injury. Electrographic ictal event lasted 120 s and was accompanied by a generalized behavioral seizure, progressing from score 1 (S1) to score 4 (S4), according to Racine scale). Recording electrodes bilaterally in the amygdala (LA–RA) and hippocampus (LH–RH). (b) Chronic partial cortical isolation in rat. Seizure in a 4-month old Sprague–Dawley rat recorded ~3 months after surgery. Seizure

to 60 days. The glutamate–aspartate transporter protein GLAST, which limits excitation initiated by synaptic release of glutamate, was upregulated in the hippocampus 5 days after iron injection, but decreased chronically at 15–60 days post-injection, when animals experienced spontaneous seizures. The authors suggested that the failure to remove synaptic glutamate may underlie the process of posttraumatic epileptogenesis. The immunoreactivity of another glutamate transporter, EAAC-1, peaked at day 5 after the iron injection and remained elevated from 15 to 30 days.

Several antiepileptic drugs have been tested over the years (1984–2007) in the iron-injection model. Valproate treatment administered for 14 days after amygdalar iron injection caused the downregulation of the two GABA transporters, GAT-1 and GAT-2, in the hippocampus, as well as a marked increase in the production of GLAST. Similarly, clobazam, levetiracetam, and zonisamide treatment administered for 14 days after iron injection suppressed glutamate neurotransmission by upregulating the levels of glutamate transporter proteins, enhanced GABAergic inhibition by downregulation of GABA transporters, and suppressed spontaneous seizures in a significant number of injected animals. Pretreatment with α-tocopherol in combination with selenium, 90 mg kg^{-1} methylprednisolone and natural antioxidant TJ-960 prevented histopathologic changes caused by iron injection and suppressed epileptiform discharges in EEG.

Caveats

Compared to human PTE, with a latency period lasting from months to years, in iron-injected animals the epileptiform activity developed soon after injection, without a clearly defined latency period. Ictal activity developed in 92–96% of animals, as opposed to a maximum 53% of human subjects with severe head injury. Further, according to clinical imaging studies (GE T2*-weighted MRI) in patients with isolated hemosiderin deposits as the sole MRI finding, occurrence of PTE is very low. EEG monitoring during the extended period of follow-up would be useful to detect and quantify the occurrence of chronic spontaneous seizures beyond the subacute (2 weeks) period.

Cortical Undercut (A Model of PTE Caused by Penetrating Cortical Injury)

Hypothesis of action

Axotomy of cortical outputs of neurons, as well as their cortical and subcortical inputs, disruption of intracortical inhibitory and excitatory circuitry, and neurodegeneration occur as a result of a direct cortical injury produced by a stab wound. These features are proposed to be the anatomic substrates that underlie the development of hyperexcitability in the cortical undercut model.

Data from human cortical slabs isolated at the time of surgery have demonstrated that the deafferented cortical tissue can display paroxysmal high-voltage activity shortly after isolation. A computational model of posttraumatic epileptogenesis in isolated cortical islands concluded that paroxysmal discharges are due to changes in intrinsic properties of pyramidal cells and enhanced NMDA synaptic conductances, without reduced (or even in the face of increased) inhibition.

Partial cortical isolation in rats

The procedure designed by Graber and Prince is currently performed in the rats 1–5 weeks of age. A rectangular (4 × 8 mm) craniectomy is made over one hemisphere with a medial edge ≥1 mm away from the midline, to avoid damage to the superior sagittal sinus. Underlying dura, pial vessels, and cortical surface are kept intact. Almost any cortical region can be chosen for partial isolation, but sensorimotor cortex is most commonly selected one. For this procedure, the bone window is centered over the coronal suture. A 30-gauge needle is bent to 90° at 2–3 mm from the tip; the needle is inserted through the dura and under the pia, so that the bent distal end of the needle is parallel to the pial surface. The needle is then pushed down through all cortical layers (2 mm), rotated 180° and carefully removed through the point of insertion.

Findings

Digital video-EEG recordings show brief (10 s) 10 Hz spike-wave ictal discharges arising from the posterior part of the partially isolated cortex (**Figure 1(b)**), accompanied by freezing, slight rising of the head, followed by head-drop and return to normal exploratory activity.

lasted ~10 s and was accompanied by arrest of movement and eye blinking followed by the head drop at the end of the ictal activity. Generalized motor seizures have not been reported in animals with undercuts, supposedly because most of the connections to other brain areas are limited by the lesion. (c) Cortical undercut in cat. Ictal events recorded over the cortical surface at day 5 and day 45 during the waking state. Recordings in a cat with deafferented left suprasylvian gyrus (depicted as a grey box on the schematic drawing) indicate that paroxysmal activity is present initially in the cortical areas adjacent to the deafferented cortex, and shows a tendency toward generalization at later time-points. Electrographic ictal events were associated with ocular movements at the beginning and end of the discharge. Recording electrodes bilaterally over surpasylvian gyrus, marginal gyrus. (d) Lateral fluid percussion. Spontaneous electrographic seizure recorded in a Sprague–Dawley rat 9 months after the injury. Electrographic ictal activity lasted 49 s and was accompanied by generalized score 4 (modified Racine scale) seizure, i.e., forelimb clonus and rearing. Recording electrodes in the ventral hippocampus ipsilateral to injury and contralateral parietal cortex. The insert in the left upper corner of each panel shows location of the electrodes. Courtesy of Dr. Willmore and Dr. Ueda (a), Dr. Prince (b), Dr. Timofeev (c).

Generalized motor seizures or full-blown electrographic seizures have not been described in this partial cortical isolation model in rats. Electrophysiological characterization of brain slices prepared from the injured region has shown hyperexcitability 10–14 days following the isolation and not earlier. Hyperexcitability lasted up to 122 days. Transcortical rather than undercut lesions seem to be critical for the development of hyperexcitability. The combination of these two lesions, however, produces slices that are more excitable than those with tanscortical lesions alone. Epileptiform potentials arose from layer V. Furthermore, layer V pyramidal neurons displayed increased input resistance and membrane time constants, and appeared to be the source of sprouted axons that were associated with an increased number of excitatory connections. Targets of sprouted axons included both other pyramidal cells in layer V and inhibitory interneurons. Immunocytochemical labeling for parvalbumin and calbindin, (calcium binding proteins present in subgroups of GABAergic neurons), as well as for GABA itself (or neuropeptide Y) has demonstrated an increase in the number of immunoreactive inhibitory interneurons. Layer V pyramidal neurons have shown a decreased capacity to maintain normal Cl^- gradients during intense firing, due to downregulation of KCC2 transporter; this change would decrease the efficacy of GABA inhibition during epileptiform activity. Neurofilament immunoreactivity was highly increased in both pyramidal neurons and interneurons, consistent with substantial reorganization induced by injury; c-fos immunoreactivity also increased, likely indicating early postinjury molecular alterations. Investigators have shown that hyperexcitability could be blocked when tetrodotoxin (TTX), a voltage-dependent sodium channel blocker, was placed subdurally over the lesion immediately following injury in vivo. TTX treatment started at the time of injury (or with a maximum 3-day delay), and lasting for at least 3 days, was effective against the subsequent development of hyperxcitability after the silent period. Treatments of shorter duration were ineffective, indicating the presence of a critical period for development of epileptognesis. Finally, it was shown that uptake of glutamine (immediate precursor of synaptically released glutamate) was increased in the slices from injured cortex compared to control slices, and this difference was caused by increased expression of the system A transporters SNAT1 and SNAT2; these results suggest that glutamine metabolism and system A transport would be potential targets for pharmacological intervention in posttraumatic epilepsy.

Caveats

Further investigations with a long term video-EEG monitoring would help to elucidate whether the development of hyperexcitability in partially isolated cortex is later followed by the occurrence of chronic spontaneous seizures – a hallmark of posttraumatic epilepsy.

Partial cortical deafferentation in cats

In the procedure designed by Steriade, Timofeev, and colleagues, the undercut cortex is produced by large white matter transections below the suprasylvian gyrus (posterior–anterior, 13–15 mm; medio-lateral, 3–4 mm; depth 3–4 mm). A knife is inserted into the posterior part of the suprasylvian gyrus perpendicular to its surface, rotated 90° and advanced rostrally along the gyrus – parallel to its surface – for 13–15 mm, then moved back, rotated 90° and removed (from the same spot at which it entered the cortex). By transecting the white matter below the posterior part of the gyrus, partial cortical deafferentation is created by disruption of thalamocortical projections. Field potential (EEG) recordings were obtained with an array of 7–8 monopolar electrodes located 2.5 mm apart along the whole length of suprasylvian gyrus at a depth of 1.5 mm. Intracellular recordings were obtained from the partially deafferented and relatively intact areas of the suprasylvian gyrus. Importantly, all the recordings were performed under ketamine–xylazine anesthesia (10–15 and 2–3 mg kg^{-1} i.m., respectively), which is thought to best mimic the slow sleep oscillation during natural slow-wave sleep in experimental animals.

Findings

In the acute experimental period (immediately after – to 10 h post injury), the EEG activity in the partially deafferented cortex remained markedly decreased up to 1–2 h. Between 2 and 3 h after the undercut, in 60–75% of animals, EEG recovered to the pattern of 'normal' slow oscillation with absence of spindles at deafferented sites. The remaining 25–40% of animals demonstrated paroxysmal-like activity (high amplitude slow waves, spikes, sharp waves) or clear-cut electrographic seizures (spike-wave and polyspike-wave complexes at 2–3 Hz and fast runs at 10–15 Hz). Electrographic seizures were initiated in the intact postcruciate gyrus and $\sim$ 7 s later appeared in the undercut cortex. Seizures lasted 5–50 s (average 17.0 ± 3.3 s), and such paroxysmal activity could last from 1 to 5 h within an experiment. It was shown that increase in the synchrony toward the end of seizures induced larger neuronal depolarization, which activated Ca^{2+}-dependent K^+ currents and terminated seizures.

In the subchronic experimental period (week 1 to week 5 after the injury), Nita and colleagues showed that more than 90% of injured animals displayed 3–4 Hz spike-wave and polyspike-wave complexes intermingled with fast (10–20 Hz) runs, which were described as similar to the patterns found in patients with severe epileptic encephalopathies. Electrographic seizures re-appeared at the border of intact and undercut cortex; with time, paroxysmal activity involved neighbouring cortical regions, until

finally (at about 4–6 weeks after the undercut) epileptiform discharges were recorded in all electrodes. These investigators concluded that following the undercut, the neocortex displays progressively increased signs of paroxysmal activity, expressed by gradually enhanced amplitudes and synchrony of spike-wave complexes. Interestingly, with barbiturate anesthesia, seizures did not develop – neither in the acute nor in the chronic phase – but enhanced activity (isolated spikes and epileptiform discharges merged with spindles) was observed in areas surrounding the undercut cortex.

Recently, the relation between paroxysmal activity in the deafferented cortex and natural state of vigilance in chronically implanted cats was investigated by the same group. Out of six animals studied, three displayed small localized muscular jerks and abnormal tail wagging during the waking state, and 1 cat had a generalized tonic–clonic seizure. EEG recordings were performed from day 5 to day 120 after the injury. Ictal events on EEG were observed in all recorded animals and consisted of 3–4 Hz spike-wave complexes of 5 s duration (**Figure 1(c)**), occurring intermittently in waking state when they were associated with eye movements. Initially, paroxysmal events were exclusively present over areas around the undercut cortex. During later stages (30–40 days), there was a tendency toward generalization over the whole cortical surface. Ictal events in the waking state were quantified based on 3-min recordings (day 4–day 120). The number of ictal events increased during the first 2 weeks, followed by a 30% decrease between days 20–40, and then a secondary increase that reached a plateau (75% of the initial number) after day 40. The intermittent ictal events observed during the waking state became almost continuous during slow wave sleep, and were completely absent during the REM sleep. These investigators suggested that an increase in intrinsic and synaptic excitability of intact neurons in areas adjacent to the undercut cortex is sufficient for the generation of electrographic paroxysmal activity.

Caveats

A possible major role of ketamine–xylazine anesthesia (which is known to favor the development of seizures) must be considered. Further, slow wave sleep is considered a potent activator of epileptiform discharges, as seen also in humans. Long lasting (days) EEG recordings in control animals maintained under ketamine–xylazine would help to rule out the possibility of spontaneous occurrence of spike-wave/polyspike-wave complexes during slow wave sleep. It is of interest that the electrographic seizures were found only in animals that showed propagation of the slow sleep-like oscillation in control conditions. Latency to seizures (2–3 h following the injury) should also be taken into consideration, as it is much shorter than latency period observed in humans with PTE.

Both the cortical undercut model and partially isolated cortex model produce very focal, limited cortical lesion – as opposed to human penetrating head injury where the damage is usually more diffuse and multifactorial. Cortical stab wounds are relatively rare clinical precursors of PTE.

Fluid Percussion Brain Injury (FPI) (A Model of PTE Induced by Closed Head Injury in Humans)

Hypothesis of action

FPI is the most widely used and investigated model for human closed head injury. As shown in multiple studies, lateral FPI is capable of inducing cortical contusion as well as intracerebral and subdural hematomas in rat brain – the features that are associated with the increased risk of PTE. At the cellular level, lateral FPI causes hippocampal sclerosis and reactive gliosis, and alters neurotrophin and ROS levels in the hippocampus – that is, all neurobiologic processes which are thought to be associated with secondary injury, circuitry reorganization and possibly epileptogenesis. These data suggest that the model of lateral fluid percussion brain injury offers the neuropathophysiological features that reproduce the complex mechanisms of human PTE.

In 1992, Lowenstein and colleagues showed that selective loss of hilar interneurons in the dentate gyrus is associated with abnormal hyperexcitability of the dentate granule cells, as measured by extracellular field potentials in response to paired-pulse stimulation of the perforant path. Later, Coulter and colleagues conducted in vitro electrophysiological studies with combined hippocampal-entorhinal cortical slices from rats with FPI. They demonstrated that slices from injured rats showed greater disinhibition in the dentate gyrus and CA1 subfield of the hippocampus (compared with slices from control rats) and that 86% of the slices generated self-sustaining epileptiform activity after stimulation of Schaffer collaterals (the main afferent input from CA3 to CA1 pyramidal neurons). Toth and colleagues next showed that the number of parvalbumin- and cholecystokinin-positive dentate interneurons, supplying inhibitory innervation of granule cells, was decreased months after FPI. Subsequently, Santhakumar and colleagues showed that hilar interneuron loss was not preferential compared to non-GABAergic hilar cells (i.e., mossy cells) and suggested that the surviving glutamatergic mossy cells play a critical role in the hyperexcitability of the dentate gyrus after TBI. Later, the same investigators discovered a long-term persistent hyperexcitability of CA1 pyramidal cells in response to Schaffer stimulation. However, critical evidence that the model actually can cause development of chronic spontaneous seizures was missing until recently.

Lateral fluid percussion brain injury (Originally designed by McIntosh and colleagues)

In our laboratory, preparation of the animal involves a 5 mm diameter craniectomy over the left parietal cortex with preservation of dura mater. The center of the craniectomy is located half way between bregma and lambda, with the lateral edge of the craniectomy adjacent to the left lateral ridge. A rigid plastic cap, cut from a female Luer–Loc needle hub, is carefully inserted into the hole over the intact dura and sealed to the skull with the Vetbond glue. The 'injury cap' is filled with 0.9% saline to check the quality of the seal and to prevent drying of dura. The injury cap is then cemented onto the skull with dental acrylic. The animal is then ready for the FPI insult. There is a 90 min interval between the anesthesia preceding surgical manipulations and the injury, to allow for the anesthetic (a single i.p. injection of a mixture containing sodium pentobarbital and chloral hydrate) to dissipate. The fluid percussion injury device consists of a plexiglass cylindrical reservoir filled with isotonic saline and bound at one end by a cork-covered plexiglass piston. The other end of reservoir is fitted with a metal housing which terminates with a male Luer–Loc fitting. The animal is connected to this fitting via the injury cap mounted on its head. A metal pendulum is released from a predetermined height and strikes the cork piston. As a result, the fluid pressure pulse propagates through the cylinder and impacts on the intact dura (without penetration), which is considered to recapitulate the effects of closed head injury in humans. The impact has brief (21–23 msec) duration, and the force is adjusted to 2.9–3.2 atm to induce severe lateral fluid percussion brain injury (which is assumed to maximize the likelihood of posttraumatic epilepsy). Pressure pulses are measured extracranially by a transducer mounted on the metal housing. One week after the injury, animals are implanted with a depth hippocampal electrode ipsilateral to injury, and two cortical screw electrodes bilaterally over the parietal cortex.

Findings

Chronic (11 months) continuous video-EEG monitoring demonstrated the occurrence of posttraumatic epilepsy in 43–50% of injured animals during the course of follow-up; this percentage is similar to that in humans with severe TBI. In addition, the absence of PTE in about half the impacted animals allows the investigators to compare animals (epileptic rats with those which fail to develop epilepsy after the traumatic insult) for mechanisms of posttraumatic epileptogenesis. In the PTE animals, the latency period varied from 4 weeks to 11 months, and the number of epileptic animals increased gradually over time. Behavioral seizures also worsened with time in individual animals, from partial to secondarily generalized (mean score 3.3 ± 1.4 according to the Racine scale). The electrographic seizure activity was detected first by the hippocampal electrode located ipsilateral to the injury site, and then spread rapidly to the contralateral cortex. Electrographic seizures were represented by high amplitude rhythmic discharges containing spikes, polyspikes, slow waves and spike-wave complexes, which had a clear onset, temporal evolution in amplitude, and frequency, and were followed by postictal depression (**Figure 1(d)**). Mean seizure duration was 113 ± 46 s, and mean seizure frequency, 0.3 ± 0.2 seizures per day. There was a tendency towards increased seizure frequency in individual animals during the course of follow-up observations. Interictal activity was presented with isolated spikes or spike-wave discharges in the hippocampus ipsilateral to injury.

Animals had bilateral hippocampal damage, but neuronal loss was more substantial ipsilateral to injury, and included hilus, CA1, and CA3. The extent of TBI-induced neuronal loss in the hilus of the dentate gyrus did not correlate with the occurrence of posttraumatic epilepsy. Both injured hippocampus and thalamus showed substantial atrophy and shrinkage compared to the contralateral side. Subicular complex and enthorhinal cortex also underwent neurodegenerative changes. Further, while all experimental animals demonstrated mossy fiber sprouting in the injured (ipsilateral) dentate gyrus, the extent of synaptic reorganization in rats with epilepsy was more pronounced than in those animals with TBI but no epilepsy. In contrast to nonepileptic rats, mossy fiber sprouting in epileptic animals was bilateral. These results suggest that TBI-induced hippocampal injury and synaptic reorganization do not necessarily lead to epilepsy. Long-term MRI studies (T2, volumetry) performed in this model confirmed a progressive cortical and hippocampal pathology lasting over 8 month period, as well as atrophy of thalamus. Diffusion weighted imaging revealed an early (3 h post-injury) diffusion drop in the hippocampus ipsilateral to injury, similar to that previously described in human TBI. This early drop in diffusion, and subsequent increase at 23 days to 3 months post injury, showed significant correlation with increased susceptibility to seizures 1 year after the injury – suggesting that diffusion weighted imaging at early and subchronic time-points after traumatic brain injury can provide a potential surrogate marker for subsequent development of posttraumatic epilepsy.

Caveats

Despite close approximation to the clinical syndrome of human PTE, several methodological features – e.g., lack of uniformity in the precipitating insult, high levels of acute mortality after the injury, slow progression of disease, and low seizure frequency – represent a challenge in seizure monitoring and use of this protocol for the evaluation of efficacy of therapeutic intervention.

PTE in juvenile rats

D'Ambrosio and colleagues recently induced rostral lateral FPI in juvenile male Sprague–Dawley rats at

postnatal day 32–35. The center of the 3-mm diameter burr hole was located 2 mm posterior to bregma and 3 mm lateral to midline. Thus, the lesion was more rostral and medial than the lesion produced by lateral FPI in adult rats. A fluid pressure pulse of 3.75–4 atm was applied.

Findings

Intermittent extended video-electrocorticography (EcoG) with epidural electrodes demonstrated partial seizures originating in the neocortex proximal to the lesion site, with or without secondary generalization. The majority (60%) of animals displayed electrographic epileptiform activity within the first 2 weeks post injury. Nine weeks post injury, epileptiform activity was present in 100% of animals. Ictal EcoG events were categorized into three different grades: grade 1 activity originated from the cortical lesion focus and was limited to it; grade 2 activity originated from the focus, with subsequent spread; grade 3 activity (generalized) started simultaneously in several recording channels. Ictal episodes consisted of 7–10 Hz rhythmical spike-wave discharges of up to 10 s duration, with abrupt onset and end. During ictal discharges, injured animals typically demonstrated behavioral arrest, sometimes followed by facial automatismsms, myoclonus or 'ictal atonia.' Tonic–clonic behavioral seizures or full-blown electrographic seizures have not been reported. In vitro local field potential recordings demonstrated persistent hyperexcitablitiy of the neocortex at the site of and around the lesion, associated with increased glial reactivity. In a 7 months follow-up study, ictal-like episodes originating in the hippocampus were reported, which gradually increased in frequency, suggesting that the ictal focus had moved from the fronto-parietal cortex (at or near the site of injury) to the temporal lobe. Interestingly, at 7–8 months of age, idiopathic seizures were observed in 33% of sham-injured controls that had identical anesthetic and surgical manipulations without the FPI. Cresyl violet and glial fibrillary acidic protein-staining indicated that early pathologic changes were confined to the lesioned cortex and thalamus. Changes included neuronal loss, areas of calcification, and reactive gliosis. In a chronic follow-up study, atrophy of CA1 and CA3 subfields of the hippocampus ipsilateral to injury was also reported.

Caveats

It is unclear whether the etiological factor has led to distinct clinical outcome or not. Further behavioral and electrophysiological studies are required to prove that this model indeed imitates the features of human PTE.

The differences in outcome between the adult and juvenile rats that were exposed to FPI include: (i) latency period (longer in adult animals), (ii) frequency of occurrence of epilepsy (50% in adult rats versus 92% in juvenile rats), (iii) seizure frequency, (iv) appearance of electrographic seizures (wave morphology and duration), and (v) behavioral correlate of electrographic ictal events (generalized seizures versus freezing and facial automatisms). Several methodological factors might explain these differences, including the age of the animals, anesthesia, size of the craniectomy and location of lesion, construction of fluid-percussion injury device, and severity of the applied pressure pulse.

Future Challenges

Despite a considerable amount of research in this area, our understanding of the pathophysiology of trauma-induced seizures and epilepsy is still in its infancy. Continued evaluation is needed to explore and explain subtle and gross differences in epilepsy phenotype across the PTE models, and assess their validity in relation to the human condition.

Animal models are ultimately designed not only to study the mechanisms of a given epileptic syndrome, but also to assist the drug discovery process by testing whether each novel molecule is biologically active and does not have a negative impact on recovery from injury. It is possible that the models best suited to study basic mechanisms of seizures and epilepsy may be different from those models for therapy discovery.

As it was pointed out at the White House-initiated 'Curing Epilepsy: Focus on the future' conference held in 2000, the important characteristics of a desirable model are reproducibility, cost and suitability for the high-throughput screening of large numbers of drugs. Moreover, models should be age-appropriate (for the developing, mature, and aged brain) and offer the possibility to study the contribution of genetic background to epilepsy. Meeting these important requirements remains a major challenge in modeling posttraumatic epilepsy.

See also: **Epileptogenesis**: Surrogate Markers for Epileptogenesis; **Ion Channels**: Acquired Ion Channel Dysfunction in Epilepsy; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury; Posttraumatic Epilepsy Caused by Fluid Percussion Injury; Traumatic Hemorrhage and Iron-Induced Epileptogenesis; **Seizures**: Seizure-Induced Gene Expression.

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Traumatic Hemorrhage and Iron-Induced Epileptogenesis

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Introduction

Traumatic brain injury causes a complex sequence of events, which result in chronic seizures in some patients. This clinical observation has triggered a number of studies in our laboratory focusing on the sequence of reactions and factors that are associated with the development of chronic seizures (i.e., epileptogenesis).

Biochemical injury to neurons may cause a sequence of changes ranging from cellular loss with replacement gliosis, to subtle alterations in neuronal plasma membrane. Membrane changes initiated by biochemical effects of injury may alter densities and distribution of ion channels on neuronal membrane. Alteration of membrane ionotophores could affect Na^+ and Ca^{2+} currents, alter thresholds, and lead to progressive depolarization. Intrinsic cellular bursting also may develop with an increase in extracellular K^+ or reduction of extracellular Ca^{2+}. The mechanism or critical physiological changes causing posttraumatic epileptogenesis remains unknown. However, several processes may provide useful candidate areas for investigation. For example, trauma may cause mechanical shearing of fiber tracts with loss of inhibitory interneurons following anterograde transsynaptic neuronal degeneration. Trauma-induced release of aspartate or glutamate, with attendant activation of NMDA receptors, elaboration of nerve growth factor or enhancement of reactive gliosis, may also occur. In addition, trauma and associated hemorrhage may alter endogenous antiepileptic systems.

Our laboratory approach to this issue of posttraumatic epilepsy developed indirectly from experiments designed to understand the basis for seizure generation seen in animal models in which metallic cobalt was embedded into isocortex. Applying cobalt in aqueous solution to cortex caused epileptiform discharges, and we reasoned that cations of cobalt (in solution in the neuropil) caused physiological changes that altered neuronal calcium homeostasis. Since cobalt is in the transition region of the periodic table, we began to evaluate other elements in that group, including aqueous iron. We found that ferrous and ferric cations in aqueous solution also caused acute and focal epileptiform discharges. This finding was particularly intriguing given the fact that iron is liberated into the neuropil following an intracerbral hemorrhage when red blood cells hemolize. Intracerebral hemorrhage is a common feature of traumatic brain injury – and such injuries lead to epileptogenesis.

During transport in plasma, iron is sequestered in carrier proteins, thus preventing the generation of free radicals. Neural tissue does not possess a mechanism for sequestration of iron. As hemoglobin breaks down, heme compounds and blood products admix with brain lipids and neural tissue. Red blood cell contents pose hazards to neural tissue because autoxidation of iron in biological

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POST-TRAUMATIC EPILEPSY

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The Causes of Epilepsy after Traumatic Brain Injury

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Introduction

The Center for Disease Control (CDC) has estimated that each year 1.5 million people suffer a traumatic brain injury (TBI) in the United States, resulting in almost 100 000 patients per year with long-term disabilities and annual costs to the economy of over $50 billion. TBI and its sequelae have been referred to as an 'invisible epidemic' in which patients suffer devastating life-long cognitive, sensory, emotional, and motor deficits.

Head trauma and brain injury are important factors in epilepsy etiology, contributing about 4% of newly diagnosed cases in the United States. However, the underlying mechanisms remain unknown. The percentage of all victims of a serious head injury that go on to develop posttraumatic epilepsy (PTE) has been estimated at 10–34%, depending on the severity of the injury. One of the strongest predictors of the likelihood of PTE is a penetration of the dura. A unique characteristic of PTE is the variable delay, or latent period, between the trauma itself and the development of seizures – a delay which can last from weeks to years. This latent period is of considerable biological interest. What slow processes are triggered during injury that do not cause seizures until much later? The latent period provides a unique therapeutic opportunity. If we knew what was happening during the latent period, then therapies might be designed that stop this process and prevent a patient at risk from ever becoming epileptic.

The consequences of TBI are as diverse as its causes. Acute aspects of TBI include loss of blood, hemorrhaging, thrombosis, infection, inflammation, intracranial hypertension, and edema. Although these acute complications can often be managed with timely clinical care, long-term consequences, such as PTE, frequently leave TBI victims disabled. To devise effective therapeutic strategies for minimizing the long-term adverse consequences of TBI, we should understand their biological basis.

Background

Fundamental Elements of TBI

Which features of TBI need to be modeled to understand the genesis of PTE? Multiple processes undoubtedly act together to alter the normal balance of synaptic excitation and inhibition and render the brain incapable of functioning normally after injury. Given the overwhelming complexity of the injured brain, it is helpful to consider separately several of the fundamental consequences of lesions that are common to many forms of TBI and are likely to

strongly alter the affected cell populations' activity, such as axonal injury or neuronal deafferentation.

Cell death and glial scarring

TBI is initially characterized by diffuse or focal neuronal and glial cell death, owing to physical damage as a result of trauma and by release of excessive excitatory neurotransmitter such as glutamate. This physical insult is then followed by an intense inflammatory response, owing to breakdown of the blood brain barrier, microglial activation, and reactive astrogliosis. Secondary neuronal damage is often produced by delayed apoptotic cell death (>24 h post-injury). Finally, reactive astrocytes form a glial scar that may spatially restrict the injury, but may also impede healing.

There is a strong correlation between PTE and loss of brain volume after head injury in humans, notably bilaterally reduced hippocampal volume. Neuronal loss in the hilus of the dentate gyrus is the most consistent neuropathologic feature within the hippocampal formation, and its severity is correlated with the neuronal loss in CA1 and CA3 areas. Extensive cortical and subcortical neuronal loss occurs in several well-studied animal models as well, such as cortical weight-drop and fluid percussion injuries (see later text). The extent of the damage is proportional to the depth of the cortical deformation, and may include the hippocampus and thalamus in addition to the directly affected neocortical area. In the controlled cortical impact (CCI) model, diffuse damage in the ipsilateral hippocampus (including the loss of CA3 and CA1 pyramidal neurons) and dentate gyrus has been described, and is related to the depth of the impact. Degeneration in these models occurs between 6 and 72 h after injury.

Selective loss of neurons following several forms of hippocampal pathology is associated with the development of abnormalities in physiology. The loss of GABAergic interneurons and glutamatergic mossy cells in the hilus of the dentate gyrus is well known in injury models, and the vulnerability of specific subpopulations of hilar interneurons (including somatostatin- and parvalbumin-containing neurons) has been established. Little is known, however, about the loss of subpopulations of GABAergic neurons in areas CA3 and CA1 after TBI.

Mechanical and pathologic damage to the adult CNS induces an injury response known as 'reactive gliosis' (or glial scarring) at the site of the lesion. Formation of the glial scar is thought to be important for recovering the tensile strength of the tissue in addition to protecting the remaining parts of the brain. Glial scar tissue is composed primarily of hypertrophic astrocytes, microglia, and oligodendrocyte progenitor cells, and includes clotted blood and deposition of extracellular matrix molecules. Different cell types arrive and participate at different times following injury in the glial scar formation. Macrophages and microglia are the first cell types to be found at the site of injury. Oligodendrocyte precursors from surrounding tissue are then recruited, and finally, large numbers of astrocytes migrate into the site of injury and eventually fill in the vacant space. Dynamic changes in macrophages and microglia-mediating reactive astrogliosis occur 24–48 h after head injury in human TBI and in experimental TBI models.

Although many studies suggest that the glial scar inhibits axonal regeneration, recent data have shown that reactive astrocytes can also release molecules that promote axonal regeneration and that the microglia/macrophage response may support axonal sprouting by producing and releasing growth factors.

Axonal injury

Traumatic axonal injury is a common sequela of TBI. In many head injuries there is irreparable damage to axons, either through the direct physical trauma of a penetrating head wound or indirectly because of the shearing and stretching of axons that occurs when the brain experiences rapid acceleration and deceleration. These injuries may not be fatal to the cell whose axon is injured, but the axon itself may be irreversibly damaged and may ultimately degenerate.

Traumatic axonal injury involves a progressive and transient disruption of the axonal membrane allowing for unregulated calcium influx. Excess intracellular calcium can induce a metabolic cascade involving calpain activation, mitochondrial swelling with cytochrome *c* release, and caspase activation, leading to perturbed axonal transport and axonal disruption over time. In addition, axonal injury initiates a variety of changes in axotomized cells, including changes in the expression of ligand-gated or intrinsic voltage-gated ion channels.

Denervation

Head injuries can also result in a complete or partial loss of the normal inputs to large populations of neurons as the result of degeneration of severed and injured axons, combined with direct and secondarily triggered (excitotoxicity, apoptosis) death of populations of cells that are presynaptic to a given region. Decreased synaptic densities are commonly observed in many forms of brain injury, indicative of denervation.

There is abundant experimental evidence that these kinds of injury-induced changes in afferent activity may alter the excitability of the affected neuronal population. After deafferentation, many types of cells respond to the loss of normal excitatory drive by increasing their excitability by various means. In cell culture systems, cells respond to a global decrease in their average action potential discharge rate by compensating homeostatically in a manner that will restore their normal discharge rate. Turrigiano and colleagues have established that the expression of synaptic glutamate receptors in cortical cells is

up-regulated in response to lowered activity levels, unlike the denervation supersensitivity displayed by denervated muscle. The expression of intrinsic voltage-gated ion channels can also be affected by altered activity levels. There is evidence that changes in the level of neurotrophins may be the signal that triggers these homeostatic responses. Exogenously applied neurotrophins can compensate for decreased activity and prevent homeostatic responses under some conditions.

Models of PTE

Diffuse Brain Injury

Three models of TBI enjoy widespread use, although they have only found limited application to the study of PTE. It is also noteworthy that all three models induce severe changes in blood flow, breakdown of the blood brain barrier, edema, and traumatic axonal injury. While realistic models of human TBI, these factors complicate the study of the biological basis of PTE.

Fluid percussion injury (FPI) is the most commonly used model to study TBI. FPI is induced with a rapid injection of saline onto the intact skull. The pressure administered is equivalent to a sudden impact received by a human skull, resulting in similar physiological effects in animals and humans. FPI produces a very realistic and reproducible diffuse, closed skull injury and results in well established neurobehavioral and cognitive deficits.

Controlled cortical impact (CCI) involves a pneumatic piston that delivers a blow directly to the pial surface of the brain at a surgically placed opening in the skull. CCI results in injury to the cerebral cortex and underlying subcortical areas. This CCI device provides an accurate, reproducible injury, and reproduces many characteristics of human TBI, particularly focal cortical contusions.

Weight drop injury has some similarities to the CCI model. In this model a weight of several hundred grams falls freely for as far as one meter onto the closed skull to produce mild TBI. Alternatively, a weight of a few tens of grams falls a few tens of centimeters directly against the cortex (after craniotomy) to produce a more severe injury.

Acute seizures, occurring immediately during injury, have been reported in the CCI model. In addition, hippocampal hyperexcitability has been revealed after stimulation or proconvulsant drug exposure. It is important to note, however, that epilepsy – that is, spontaneous recurring seizures – has only been demonstrated after severe FPI and weight-drop injuries in rats. These injuries are associated with significant neural degeneration.

Undercut Cortex

One of the first experimental demonstrations of chronic epileptogenesis associated with brain injury was described 50 years ago, and involved aberrant excitability in regions of chronically isolated cat and monkey neocortex. The aberrant excitability was produced by undercutting the cortex so as to leave the pial blood vessels intact. Much like human PTE, hyperexcitability was only observed with a delay of weeks after the lesion. Once established, the epileptiform discharge in the isolated cortical areas remained for more than one year. More recently, this model has been thoroughly characterized by Prince and colleagues in rodents, particularly through detailed electrophysiological studies in ex vivo brain slices. Their finding that the hyperexcitability persists after removal of the tissue from the injured animals provides strong evidence for a persistent reorganization of excitatory and inhibitory neural circuits as a consequence of the injury. These studies have found evidence of alterations in intrinsic neuronal excitability, GABAergic inhibition, and glutamatergic excitation, as well as reactive axonal sprouting.

Schaffer Collateral Transection

We have modeled a penetrating TBI by transecting the Schaffer collateral pathway in hippocampal slice cultures. Lesions of Schaffer collateral axons of CA3 pyramidal cells were made using a razor blade shard after the cultures had been allowed to develop in vitro for >14 days (**Figure 1(a)**). Lesioned cultures are hyperexcitable (see later text). We are also developing a model of acute brain injury with dural penetration by transecting the Schaffer collateral pathway in rats in vivo. Preliminary results demonstrate a delayed increase in excitability, apparent in depth recordings and maintained in ex vivo brain slices, and the occurrence of behavioral seizures in these animals. Although the hippocampus is rarely involved in human PTE, this type of lesion is simpler to study than comparable injuries in the neocortex, (such as that produced by undercutting) because of the regular cellular organization of the hippocampus. Indeed, its organization allows the presynaptic (i.e., axonal injury) and postsynaptic (i.e., denervation) consequences of injury to be studied in spatially isolated cell populations (CA3 and CA1, respectively), without the complications present in other models of brain injury (e.g. cell death, interruption of blood supply, edema).

Methods

Slice Culture Preparation and Schaffer Collateral Transection

We have performed Schaffer collateral transections in hippocampal slice cultures from rat and mouse. Hippocampal slice cultures were prepared using the roller tube method. In brief, the hippocampi were dissected from neonatal pups (postnatal days 1–8), 400 μm-thick slices were cut on a tissue chopper, and the slices were attached

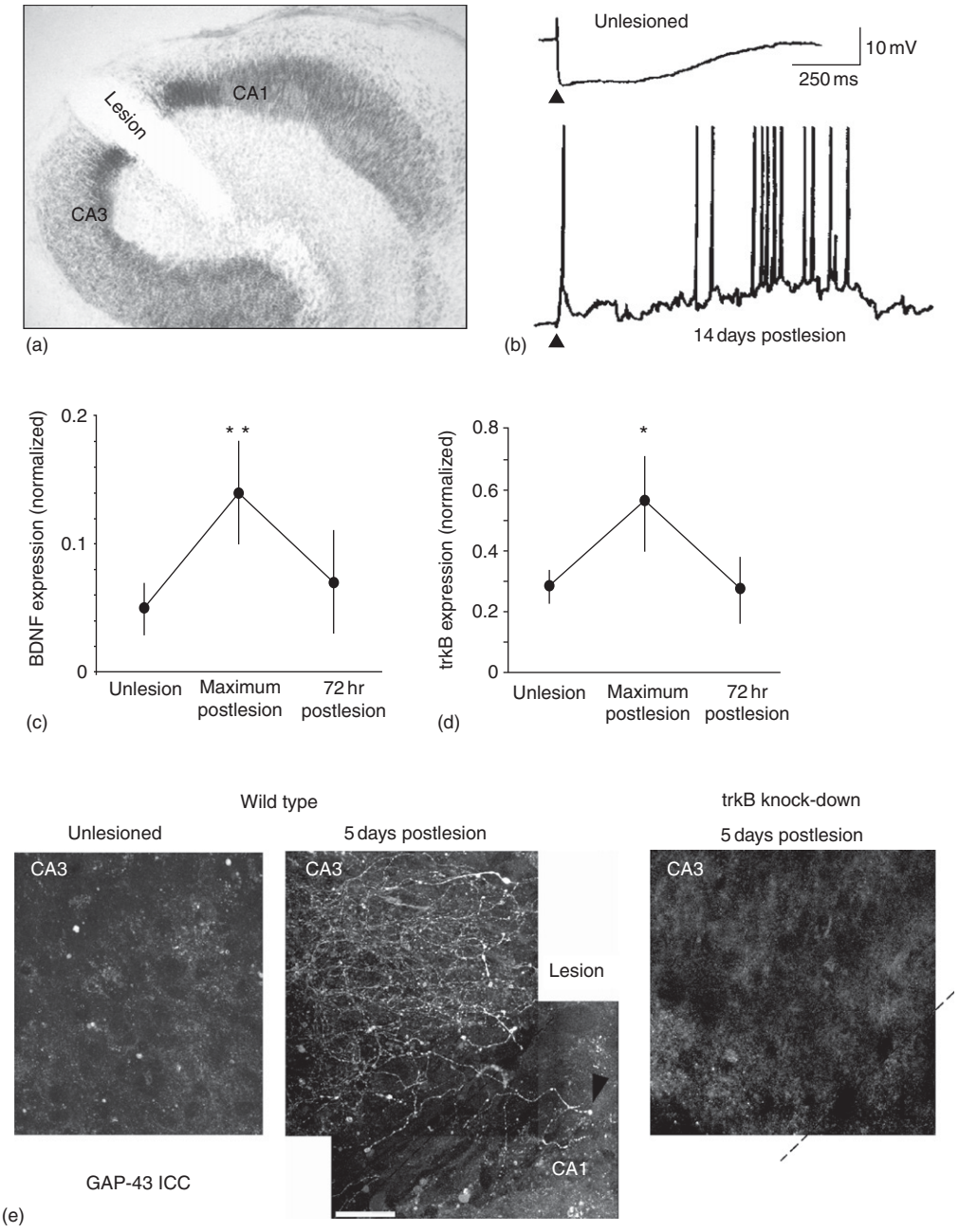


Figure 1 Reactive axonal sprouting and neurotrophin signaling after axonal injury. (a) Image of a Nissl stained hippocampal slice culture taken 14 days after making a transection of the Schaffer collateral pathway. Such lesions allow the consequences of axonal injury to be studied relatively selectively in area CA3 and the effects of chronic partial denervation to be studied in area CA1. (b) Membrane potential responses of CA3 pyramidal cells elicited after stimulation within area CA3 of an unlesioned control hippocampal slice culture (upper trace) and a culture in which the Schaffer collateral pathway had been transected 14 days earlier (lower trace). A normal EPSP–IPSP sequence was elicited in the control culture, whereas abnormal afterdischarge was elicited in the lesioned culture. (c) The expression of BDNF is increased transiently in area CA3 24–48 h after Schaffer collateral transection in hippocampal slice cultures, as determined with an Elisa assay. (d) The expression of the BDNF receptor trkB is also increased in area CA3 24–48 h after Schaffer collateral transection in hippocampal slice cultures, as determined with quantitative immunoblotting. (e) Single confocal images of rat hippocampal slice cultures stained with an antibody against the growth-associated protein, GAP-43 – a marker of growing axons. No immunoreactive fibers are seen in the unlesioned rat hippocampal slice culture (left), but many labeled fibers, some of which are tipped by growth cones (arrowhead), are seen crossing from area CA3 to area CA1 in the culture lesioned 5 days earlier (middle). Scale bar = 25 μm. In contrast, few or no immunoreactive fibers are seen when lesions are made in mouse hippocampal slice cultures in which the level of trkB expression has been genetically reduced (right). Adapted from McKinney RA, Debanne D, Gähwiler BH, and Thompson SM (1997) Lesion-induced axonal sprouting and hyperexcitability in the hippocampus in vitro: Implications for the genesis of posttraumatic epilepsy. *Nature Medicine* 3: 990–996, and Dinocourt C, Gallagher SE, and Thompson SM (2006) Injury-induced axonal sprouting in the hippocampus is initiated by activation of trkB receptors. *European Journal of Neuroscience* 24: 1857–1866.

to glass coverslips in a film of clotted chicken plasma-fibrin solution. The coverslips were then placed in sealed test tubes containing a semi-synthetic medium. The tubes were placed in a roller drum in an incubator. Cultures were allowed to develop for 14 days in vitro to allow for dendritic and synaptic maturation. At this age, cultures were removed and placed in a solution containing glutamate receptor antagonists under sterile conditions. A razor blade shard was drawn from the approximate position of the hippocampal fissure to the outer edge of the culture at the border between areas CA1 and CA3 in order to sever the projections from area CA3 to area CA1. Cultures are then returned to their test tubes and maintained for days or weeks after the lesions. This type of injury produces little neuronal degeneration, except within the immediate vicinity of the lesion, and thus represents a relatively selective form of axonal injury.

Immunocytochemistry

Tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline overnight, then rinsed. After overnight preincubation in a blocking solution containing 0.4% Triton X-100, bovine serum albumin, and goat serum, cultures were incubated for 24 h in a solution containing an anti-GAP-43 monoclonal antiserum (10E8/E7, diluted 1:10). Biotinylated secondary antibodies and fluorescently labeled avidin were used for visualization of GAP-43-immunoreactive processes. TrkB expression levels were assessed with immunoblotting using a polyclonal anti-trkB antibody from Upstate Biotechnology (Lake Placid, NY). Brain derived neurotrophic factor (BDNF) levels were assessed with a Promega ELISA kit (Madison, WI).

Electrophysiology and Glutamate Photolysis

Conventional whole-cell voltage- and current-clamp techniques were used to record from CA1 and CA3 cells in the hippocampal cultures. In some experiments, we used photolysis of caged glutamate to study the responses of distal dendrites. In brief, UV light from an argon ion laser was directed into a quartz multimode fiber (10–25 μm in diameter). The proximal end of the fiber was then positioned in a focal plane conjugate with the target dendrite via a pair of relay lenses. The light is then directed into the back of the objective via a dichroic mirror that allows for simultaneous imaging with conventional wide field illumination, in order to visualize the target dendrite or monitor changed in intracellular calcium concentration microfluorometrically. Caged glutamate (*N*-NCM-glutamate, synthesized by our collaborator Dr. Joseph Kao) was applied at 500 μM and a brief pulse of the UV light (1–32 ms, spot size = 1–10 μm) was used for uncaging.

Recent Results – Mechanisms of PTE

Disinhibition

Epilepsy is widely recognized to result from an imbalance between excitatory and inhibitory synaptic processes. Certainly acute blockade of GABAergic inhibitory synapses is the most powerful and simple means of inducing epileptiform discharge in tissue from many brain regions. Nevertheless, the evidence of persistent disinhibition in various chronic epilepsy models, including those induced by TBI, is surprisingly weak. Some older work demonstrated loss of inhibitory synapses after chronic neocortical isolation or neonatal hypoxia. These studies gave rise to the notion that GABAergic interneurons might be selectively vulnerable to injury (compared to excitatory pyramidal cells), thus resulting in an imbalance between inhibition and excitation. More recent studies have failed to corroborate these findings, however. The evidence favors preservation of GABAergic interneuron number (as detected immunocytochemically) in the undercut cortex model, although there are modest reductions in the hippocampus after FPI in most (but not all) studies. The evidence is better for more subtle defects in GABAergic inhibition. Chronic decreases in the frequency of spontaneous inhibitory synaptic responses have been reported after TBI in various models, typically attributed to reductions in the number of inhibitory synapses, as well as impairment of Cl^- extrusion and Cl^- transporter expression. Such changes would weaken inhibition during periods of elevated activity. The difficulty in identifying alterations in GABAergic inhibition after TBI, especially compared to the more obvious alterations in intrinsic and synaptic excitation (described below), suggests that disinhibition may make only a minor contribution to the genesis of PTE.

Reactive Sprouting After Axonal Injury

An important clue as to the nature of lasting changes underlying the genesis of PTE was the discovery that axonal reorganization can occur in the adult brain in response to neuronal injury. This process has been studied almost exclusively for the mossy fiber axons of dentate granule cells because of the ease and specificity with which they are labeled using the Timm stain for heavy metals (zinc being found at high concentrations in mossy fibers). Dentate granule cells display abnormal mossy fiber collaterals within the dendritic regions of the dentate gyrus in hippocampi removed from epileptic patients, but not in matched non-epileptic patients. This mossy fiber termination pattern, called supragranular sprouting, led to the initial suggestion that axonal sprouting after injury might be an important contributing factor to the genesis of PTE. In experimental PTE produced by cortical weight-drop,

mossy fiber sprouting appears 1 or 2 weeks after the injury and persists for many months afterwards. Physiological studies indicate that newly sprouted mossy fiber collaterals form excitatory synapses with dentate granule cells, and that the net effect of these new synapses is to increase the excitability of the tissue. Thus, the slow time course of this mossy fiber regrowth may account for the delayed development of PTE.

It is now clear that axonal sprouting is not limited to granule cells. Axonal sprouting by pyramidal cells has also been observed after brain injuries and may be more widespread than is currently appreciated. Tissue from hyperexcitable areas of undercut neocortex (see previous text) display evidence of axonal sprouting by pyramidal cells near the lesion. There is also evidence for upregulation of GAP-43 and other growth-associated proteins in the cortex after some forms of TBI. Using biocytin filling of the axons of single pyramidal cells, GAP-43 immunocytochemistry, and mice in which some CA3 pyramidal cells express GFP constitutively, we have provided evidence that CA3 pyramidal cells also sprout new axon collaterals after Schaffer collateral transection in hippocampal slice cultures. After injury-induced axonal sprouting, the probability that any two CA3 pyramidal cells in the vicinity of the lesion are connected by an excitatory synapse is increased by about 50%, providing a lasting injury-induced morphological substrate for the spread of excitation through the population of CA3 cells. We also observed that lesioned cultures are hyperexcitable (**Figure 1(b)**) and have an increased sensitivity to proconvulsive conditions. The strong correlation between the number of sprouted axons and the degree of excess excitation supports the hypothesis that injury-induced axonal sprouting contributes to the generation of PTE. In order to test this hypothesis, however, it is necessary to stop the reactive axonal growth.

What Factors Trigger Axonal Sprouting After Injury?

Neurotrophic factors (NTs) were first discovered because they promote neurite outgrowth during development. They are now known to exert this action in the adult CNS as well. The hippocampus displays the highest level of neurotrophin mRNA in the brain, particularly for BDNF. In the hippocampal formation, BDNF mRNA is particularly prominent in CA1 and CA3 pyramidal cells. Responsiveness to NTs is conferred by expression of the high affinity neurotrophin receptor tyrosine kinases, trkA, trkB, and trkC. trkB receptors are highly expressed in hippocampal pyramidal cells, and bind BDNF and NT-4/5 with high affinity. Binding of NTs to their receptors causes them to homodimerize and autophosphorylate tyrosine residues in their cytoplasmic domain. In the hippocampus, resting levels of phosphorylation are very low, indicating low levels of constitutive activation of these receptors.

Expression of BDNF and other NTs is up-regulated in several models of experimental epilepsy, and increases during induction of kindled seizures and after kainic acid-induced seizures. These observations led to the hypothesis that enhanced NT expression contributes to the lasting changes underlying epilepsy. Indeed, axonal sprouting and increased excitability are induced by exogenous trkB ligands in hippocampal slice cultures. NTs are thus prime candidates to trigger axonal sprouting after neural injury.

We have performed a first test the hypothesis that TBI promotes axonal sprouting by CA3 pyramidal cells by causing upregulation of NT signaling via the trkB receptor. Consistent with the hypothesis, an ELISA assay detected a threefold increase in BDNF levels in area CA3 within 24–48 h after Schaffer collateral transection and recovery by 72 h postlesion (**Figure 1(c)**). Immunoblotting also revealed that trkB expression in area CA3 increases two to threefold after transection (**Figure 1(d)**). In slice cultures from transgenic mice ($trkB^{fBZ/fBZ}$) in which trkB expression is reduced to about 20% of control levels, the percentage of cultures displaying GAP-43-immunoreactive axons after Schaffer collateral transection was significantly reduced to only 23%, as compared to the >70% of lesioned cultures from wild-type mice that display sprouted axons (**Figure 1(e)**). Similarly, immunoblot analysis indicated that the lesion-induced upregulation of GAP-43 was decreased in the $trkB^{fBZ/fBZ}$ cultures to only 44% of the level seen in wild-type cultures. These are the first results demonstrating that injury-induced NT signaling promotes axonal sprouting in the CNS. We now have an experimental approach with which to test the hypothesis that injury-induced axonal sprouting contributes to the hyperexcitability seen after lesions. We are performing these experiments using newly created mice with pharmacologically-sensitive trkB receptors.

Homeostatic Plasticity After Denervation

The discovery of the 'paroxysmal depolarizing shift' as the intracellular correlate of interictal EEG spikes led to considerable debate between proponents of the 'giant synaptic potential' and 'epileptic neuron' hypotheses. The giant synaptic potential hypothesis holds that synchronous, all-or-none interictal events result from the summation of large numbers of synchronized excitatory glutamatergic synaptic inputs. The epileptic neuron hypothesis holds that depolarization shifts are generated by intrinsic depolarizing conductances in pyramidal cells that are then synchronized by modest synaptic excitation. The intrinsic propensity to produce long duration, large amplitude bursting responses may normally reside in a

subset of cells or may be displayed by a subset of pathologically 'epileptic' cells. In the case of disinhibited hippocampal slices, it is now clear that both hypotheses are partially correct: epileptiform discharge results from synchronous synaptic excitation and recruitment of several intrinsic depolarizing conductances, particularly dendritic voltage-dependent Ca^{2+} conductances. How well this conclusion applies to other forms of epilepsy remains unclear.

While much of the current focus is on changes in synaptic circuitry and function as the cause of epilepsy, changes in intrinsic postsynaptic excitability or receptor expression are now receiving increased attention. There are several examples of injury-induced hyperexcitability in which amplification of intrinsic excitability has been implicated. First, *denervation supersensitivity* to acetylcholine was first described for denervated muscle in 1949. Denervation supersensitivity has since been attributed to the increased expression of receptors, both at extrajunctional and junctional sites. A similar phenomenon also occurs in neurons. Decreasing postsynaptic activity in cultured cortical neurons (as produced with tetrodotoxin-TTX) results in increased postsynaptic glutamate receptor expression. Second, cells also increase their *intrinsic excitability* following manipulations that decrease action potential discharge. Inactivation of cortical cell cultures with TTX, for example, leads to a selective down-regulation of voltage-dependent K^+ currents. The effects of TTX on the expression of both glutamate receptors and intrinsic conductances have been attributed to decreases in the activation of postsynaptic trkB receptors by endogenously-released BDNF. Recent studies have suggested that dendritic currents, in particular A-type K^+ currents and hyperpolarization activated cation currents, may have persistently altered function in some chronic epilepsy models. Persistent increases in the propensity for repetitive firing and enhanced glutamatergic excitation are also reported in experimental TBI.

We have obtained considerable evidence that Schaffer collateral transection induces an increase in the excitability of CA1 cell dendrites as the result of the deafferentation and decrease in neuronal discharge. Using whole-cell current-clamp recording from CA1 cells in slice cultures 7–10 days after Schaffer collateral transection, we observed that a single stimulus delivered in *str. radiatum* within area CA1 elicited synaptic responses that were identical in lesioned and unlesioned slices. In contrast, synaptic responses were significantly prolonged in lesioned slices compared to controls when short strains of high frequency stimulation were delivered. A similar pattern emerged when distal apical dendrites were stimulated with photolysis of caged glutamate. Weak photostimulation elicited responses that were statistically indistinguishable in their amplitude and kinetics in lesioned and unlesioned cultures, whereas strong stimulation elicited so-called 'plateau potentials' in denervated cells that were significantly increased in duration compared to normally innervated cells (**Figure 2(a)** and **2(b)**). The lack of change in the amplitude and kinetics of the small responses suggested that a change in the expression of postsynaptic glutamate receptors or dendritic electrotonic properties were not causing the hyperexcitability. We concluded, instead, that an increase in intrinsic excitability was responsible. But what channels are affected?

Plateau potentials represent all-or-none responses of single dendritic branches and are mediated by the activation of voltage-dependent Ca^{2+} channels in the stimulated dendrite. Plateau potentials are most easily evoked with the prolonged depolarization produced by NMDA receptor activation. We have discovered that SK-type Ca^{2+}-activated K^+ channels are primarily responsible for the repolarization of the plateau potential. Several specific SK channel blockers (such as apamin, **Figure 2(c)**), high concentrations of tetraetylammonium (TEA), and intracellular dialysis of the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N, N,-N′,N′-tetracefic acid (BAPTA) all double the duration of the plateau potential.

Because acute block of SK-channels prolongs plateau potentials in control cells to a duration comparable to that occurring after chronic deafferentation, we examined SK channel function in denervated dendrites. We found that apamin, TEA, and intracellular BAPTA all failed to produce a significant prolongation of plateau potentials in denervated cells. In addition, the SK2 channel-mediated medium-duration afterhyperpolarization, recorded from the soma in response to trains of directly evoked action potentials, was significantly reduced in denervated CA1 cells. We concluded that plateau potentials in denervated dendrites are prolonged, compared to control cells, because the normal SK channel-mediated repolarization process is dysregulated. Finally, we observed no changes in SK2 mRNA in denervated CA1 cells (using quantitative RT-PCR) or in SK protein levels (using Western blotting). We thus concluded that the SK-channels are present, but not functioning normally, after chronic denervation.

Why does plateau potential prolongation promote hyperexcitability within the affected cell population? We found that plateau potentials in distal dendrites were almost always ineffective in triggering action potentials. In contrast, acute prolongation of plateau potentials in control cells with apamin (**Figure 2(e)**) or chronic prolongation of plateau potentials by deafferentation (**Figure 2(f)**) substantially increased the probability that a plateau potential would trigger fast action potential discharge. We thus demonstrated that an increase in intrinsic excitability, in particular a change in the function of dendritic Ca^{2+}-activated K^+ channels, is a primary cause of hyperexcitability in denervated CA1 cells in hippocampal slice cultures. In preliminary experiments,

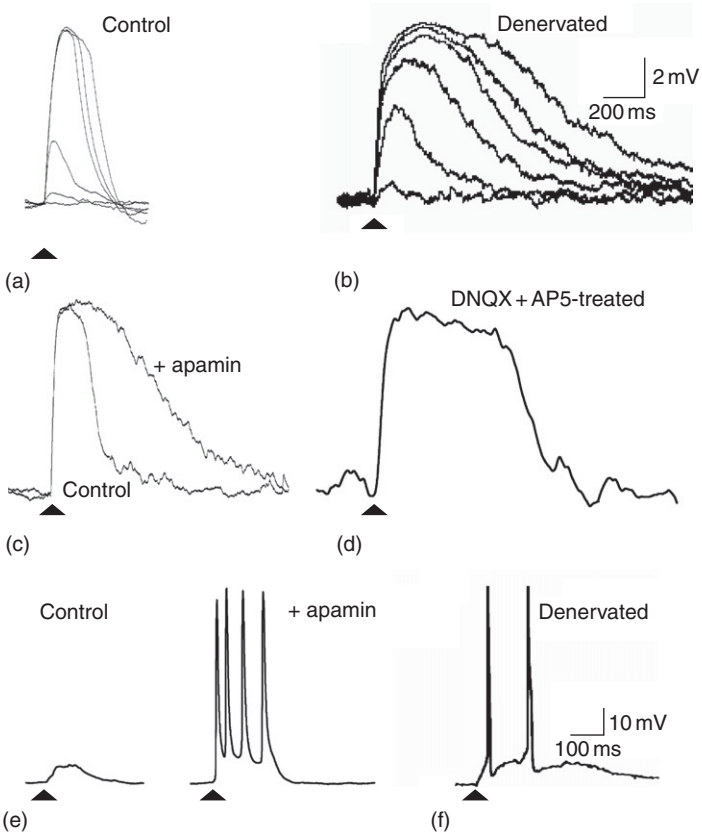


Figure 2 Changes in intrinsic dendritic excitability after chronic partial denervation. Traces represent membrane potential responses of CA1 pyramidal cells to photolysis of caged glutamate at a single distal apical dendrite at the time indicated by the solid triangle. All responses in a–d were recorded in the presence of TTX to block fast action potentials. (a) A series of photostimuli of increasing intensity elicited either graded, small EPSP-like responses or all-or-none plateau potentials. On average, plateau potentials are 10 mV in amplitude, as recorded with whole-cell pipettes from the soma, and have a duration of 100–200 ms. (b) Identical photostimulation in a CA1 cell recorded 7 days after partial denervation produced by transecting the Schaffer collateral inputs to the cell. Note that the amplitude is not different from the amplitude in control cells, but that repolarization is slowed and the duration of the plateau potential is threefold longer. Note also that the amplitude and time course of the small events are not different after denervation. Scale bar applies for panels a–d. (c) Block of SK-type Ca^{2+}-activated K^+ channels with apamin slows repolarization of plateau potentials and increases their duration in normally enervated cells. (d) The effects of partial denervation on plateau potential duration are mimicked by treatment of cultures for 7 days with the glutamate receptor antagonists, DNQX and AP5. (e) In the absence of TTX, plateau potentials elicited with dendritic photostimulation are not usually accompanied by fast action potentials. Application of apamin, however, greatly facilitates action potential discharge in response to dendritic stimulation. (f) In contrast to normally enervated cells, photostimulation of distal dendrites in denervated cells almost always elicits fast action potentials. Scale bar applies for panels e–f. Adapted from Cai X, Liang CW, Muralidharan S, Kao JPY, Tang CM, and Thompson S-M (2004) Unique roles of SK and Kv4.2 potassium channels in dendritic integration. *Neuron* 44: 351–364, and Cai X, Wei D-S, Gallagher SE, *et al*. (2007) Hyperexcitability of distal dendrites in hippocampal pyramidal cells following chronic partial deafferentation. *Journal of Neuroscience* 27: 59–68.

we have seen evidence that a similar process occurs in area CA1 of ex vivo hippocampal slices taken from animals with Schaffer collateral transections made in vivo.

The increase in intrinsic excitability after partial deafferentation represents a response of the cells to a decrease in postsynaptic activity levels. We found that treatment of slice cultures with a combination of the AMPA and NMDA receptor antagonists, DNQX and AP5, for 7 days caused an increase in the duration of plateau potentials elicited with glutamate photolysis that was even larger than that resulting from Schaffer collateral transection (**Figure 2(d)**). Because excitatory afferents were intact in these cultures, we could record miniature excitatory postsynaptic currents (mEPSCs) from CA1 cells after washout of the chronically applied DNQX + AP5, to assay the expression of postsynaptic glutamate receptors. We found that there were no statistically significant differences in mEPSC amplitude in cultures exposed to DNQX + AP5 for 7 days compared to untreated control cultures. In addition, there was no statistically significant change in the amplitude or kinetics of small glutamate responses, subthreshold for activation of plateau potentials, as in the denervated cells. We thus concluded that

an increase in glutamate sensitivity is not required for the prolongation of plateau potentials following deafferentation.

In conclusion, a decrease in neuronal discharge in cells that have been chronically denervated will react homeostatically to increase their excitability. We suggest that this increase in intrinsic excitability and reactive axonal sprouting act synergistically to cause hyperexcitability and PTE after TBI.

Challenges for the Future – Implications for Treatment and Therapy

PTE represents one of the few forms of epilepsy in which a true 'cure' can be imagined. Because of the long latent period between the time of the injury and the onset of clinical seizures, there is a relatively wide window of opportunity to interrupt the injury-induced processes underlying the generation of the epilepsy. In theory, a well-directed therapeutic approach could prevent patients at risk after TBI from ever developing PTE. This goal continues to drive research into the causes of PTE. Many important questions with regard to the genesis of epilepsy after TBI remain to be answered. Does axonal sprouting cause injury-induced hyperexcitability? Is the excitability of the postsynaptic targets of these newly sprouted axons increased after injury? Can evidence of these processes be seen in tissue from injured human brains?

The changes in neural function that occur after TBI, such as axonal sprouting, disinhibition, or altered intrinsic excitability, are likely to represent the brain's natural homeostatic mechanisms for healing itself. They also reflect the biological principle that recovery from injury often means reverting to a more immature state, perhaps produced by the re-expression of a constellation of genes normally active early in development. Both axonal growth and homeostatic regulation of synaptic and intrinsic excitability are prominent features of the young, growing brain. We suspect that these normally 'sensible' homeostatic responses to injury result in unfortunate, 'maladaptive' functional consequences that cause several of the neurological complications of TBI.

No question is more important than identifying better therapeutic approaches for the treatment of PTE. At least 30–40% of all patients with epilepsy are partially or totally resistant to currently available anticonvulsant medications, and an even greater percentage suffers from the negative side-effects of the drugs. Treatment of PTE is particularly problematic, perhaps because of the hard-wiring of the rearrangements that occur after the injury during the latent period. A major challenge for epilepsy researchers is to use our increasing knowledge of the cellular biology underlying epileptogenesis to offer better anticonvulsant therapies. It is to be hoped that the data gathered from the study of in vitro models of PTE will lead to the development of novel, well targeted therapeutic interventions that significantly improve on today's medications.

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; **Interneurons**: Functional Diversity and Selective Vulnerability of Hippocampal Interneuron Types: Implications for Epileptogenesis; **Models**: Seizure Models in Acute and Organotypic Slices; **Neurotrophic Factors**: Role of BDNF in Animal Models of Epilepsy; **Plasticity**: Circuitry Reorganization, Regeneration, and Sprouting; **Post-Traumatic Epilepsy**: Posttraumatic Epilepsy Caused by Fluid Percussion Injury; Posttraumatic Models; Traumatic Hemorrhage and Iron-Induced Epileptogenesis; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis.

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Traumatic Hemorrhage and Iron-Induced Epileptogenesis

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Introduction

Traumatic brain injury causes a complex sequence of events, which result in chronic seizures in some patients. This clinical observation has triggered a number of studies in our laboratory focusing on the sequence of reactions and factors that are associated with the development of chronic seizures (i.e., epileptogenesis).

Biochemical injury to neurons may cause a sequence of changes ranging from cellular loss with replacement gliosis, to subtle alterations in neuronal plasma membrane. Membrane changes initiated by biochemical effects of injury may alter densities and distribution of ion channels on neuronal membrane. Alteration of membrane ionotophores could affect Na^+ and Ca^{2+} currents, alter thresholds, and lead to progressive depolarization. Intrinsic cellular bursting also may develop with an increase in extracellular K^+ or reduction of extracellular Ca^{2+}. The mechanism or critical physiological changes causing posttraumatic epileptogenesis remains unknown. However, several processes may provide useful candidate areas for investigation. For example, trauma may cause mechanical shearing of fiber tracts with loss of inhibitory interneurons following anterograde transsynaptic neuronal degeneration. Trauma-induced release of aspartate or glutamate, with attendant activation of NMDA receptors, elaboration of nerve growth factor or enhancement of reactive gliosis, may also occur. In addition, trauma and associated hemorrhage may alter endogenous antiepileptic systems.

Our laboratory approach to this issue of posttraumatic epilepsy developed indirectly from experiments designed to understand the basis for seizure generation seen in animal models in which metallic cobalt was embedded into isocortex. Applying cobalt in aqueous solution to cortex caused epileptiform discharges, and we reasoned that cations of cobalt (in solution in the neuropil) caused physiological changes that altered neuronal calcium homeostasis. Since cobalt is in the transition region of the periodic table, we began to evaluate other elements in that group, including aqueous iron. We found that ferrous and ferric cations in aqueous solution also caused acute and focal epileptiform discharges. This finding was particularly intriguing given the fact that iron is liberated into the neuropil following an intracerbral hemorrhage when red blood cells hemolize. Intracerebral hemorrhage is a common feature of traumatic brain injury – and such injuries lead to epileptogenesis.

During transport in plasma, iron is sequestered in carrier proteins, thus preventing the generation of free radicals. Neural tissue does not possess a mechanism for sequestration of iron. As hemoglobin breaks down, heme compounds and blood products admix with brain lipids and neural tissue. Red blood cell contents pose hazards to neural tissue because autoxidation of iron in biological

fluid initiates a series of one-electron transfer reactions that yield free radical intermediates, with formation of superoxide radicals and production of lipid peroxidation. Indeed, evaluating data from patients subjected to traumatic brain injury, several investigators have noted that risk factors for epileptogenesis are determined not only on trauma 'dose' (i.e., severity), but also the presence of cortical contusion and intracerebral hematoma. Although shear injury and injury associated with patterns of pressure waves and cavitation were obviously important, we wondered particularly about how hemorrhage might be associated with epileptogenesis.

Background

Effects of Iron on Brain Tissue

Addition of iron salts or heme compounds to solutions containing polyunsaturated fatty acids (PUFA) or to suspensions of subcellular organelles results in the formation of highly reactive free radical oxidants, including perferryl ions, superoxide radicals singlet oxygen, and hydroxyl radicals. Although free radical species may form by iron-catalyzed Haber–Weiss reactions, these oxidants are also actively generated by iron in biologically chelated forms in heme or with ADP. Free radicals react with methylene groups adjacent to double bonds of PUFA and lipids within cellular membranes, causing hydrogen abstraction and subsequent propagation of peroxidation reactions. This nonenzymatic initiation and propagation of lipid peroxidation causes disruption of membranes of subcellular organelles, degrades deoxyribose and amino acids, and yields diene conjugates and fluorescent chromophores. Inorganic iron salts, hematin, and hemoproteins will stimulate peroxidation of lipids of microsomes and mitochondria, as well as change cellular thiodisulfide function. Alkyl hydroxyl and peroxyl species of fatty acids propagate until a termination reaction occurs with a membrane constituent capable of electron donation without formation of a free radical. Such constituents include tocopherol, cholesterol, proteins or the sulfhydryl group of glutathione. Histopathologic alterations following injection of aqueous iron into neural tissue can be prevented by pretreatment of animals with α-tocopherol and selenium, further supporting the contention that peroxidative reactions are of importance in trauma-induced brain injury responses.

Sustained membrane changes that are associated with generation of epileptiform discharges may result from free radical injury to neuronal nuclear or mitochondrial DNA. Cellular responses to generation of free radical oxidants following decompartmentalization of hemoglobin or iron containing heme compounds may depend upon the induction of protective mechanisms. Differentiation of susceptibility to develop epilepsy after a given trauma dose may be related to the ability of repair response induction following initiation of lipid peroxidation. For example, different strains of *Escherichia coli* may be differentiated based on their responses to peroxide, suggesting critical genetic factors that determine protection. Induction of enzymes to repair DNA damage induced by Fenton-derived free radicals appears to be critical for cellular survival.

The specific genetic factors that protect against, or cause vulnerability to, the development of posttraumatic epilepsy remain unknown. However, a possible genetic predisposition has been observed with the detection of decreased levels of serum haptoglobin in familial epilepsy. Haptoglobins are acute phase glycoproteins in the α-1-globulin fraction of serum; they form stable complexes with hemoglobin. Since antioxidants such as superoxide dismutase and peroxidases are not found in high concentration in extracellular fluid, control of oxidation in neural tissue must depend upon binding of reactive metals to carrier proteins, including transferrin, lactoferrin, ceruloplasmin, and haptoglobins. Since one mechanism of protection against induction of oxidant stress is sequestration of free hemoglobin with haptoglobins, an understanding of impairments in the synthesis of these glycoproteins may provide insight into an inherent susceptibility to development of epilepsy after head trauma.

Initial Studies on Iron-Induced Epileptogenesis

Our original methods to produce chronic seizures in rats at first involved injection of 100 mmol $FeCl_2$ (a concentration that is equiosmotic with CSF) into isocortex. Over the following 30 days, animals develop focal seizures and epileptiform discharges. In a colony of injected rats observed for over 1 year, 34% showed persistence of seizure. Subsequently, we have focused our studies on hippocampus in response to amygdala injections, taking advantage of the large literature on hippocampus within the context of amygdalar kindling. Targeting the amygdalar nuclear complex allowed observation of clear endpoints from resultant rodent behavioral changes, as predicted from kindling experiments.

Using this model, we began a series of experiments to elucidate the mechanisms of iron- and hemoglobin-induced epileptigenesis. Triggs *et al.* developed a microfluorescence method for detection and measurement of lipid peroxidation that results when iron cations are mixed with lipid. Although initial experiments used iron in aqueous solution, we demonstrated that not only iron in aqueous solution, but also blood products containing the iron moiety, would initiate lipid peroxidation. Subsequently, we found that pretreatment with α-tocopherol and selenium not only prevented epileptogenesis but completely inhibited the formation of a region of encephalomalacia with gliosis at the iron injection site. That striking observation was replicated by another laboratory,

using cat spinal cord injections. The tocopherol acetate pretreatment experiments led to an evaluation of the alcohol form of tocopherol as a treatment strategy.

Methods

Sprague-Dawley rats, weighing 200–280 g, were used for our studies (with approval from the appropriate animal use committees). Surgical procedures are performed with the animals anesthetized with pentobarbital sodium (37.5 mg kg^{-1}, i.p.). Animals were prepared for behavior and/or electrophysiological assessment. Control animals were injected with saline (0.9%), with pH adjusted to equal the acidity of the iron salts solution (pH of either ferrous or ferric chloride solutions were not adjusted, since pH manipulations will cause complex formation and cations to precipitate from solution).

Animal implantation involves stereotaxic placement of a 22-gauge guide cannula (lumen occluded with a stylet) just above the left amygdala. A bare-tipped insulated wire is affixed to the lip of the guide cannula orifice. The cannula is cemented to the skull with dental acrylic. After positioning the guide cannula, we remove the stylet and replace it with a fused silica tube that is attached to a microinjection pump (CMA Type 100); the tube is positioned in the left amygdala. Animals are then injected with 1.5 µl of 100 mmol $FeCl_3$ over 1.5 min, or with an equal volume of 0.9% NaCl (pH adjusted to 2.2, as for the ferric chloride solution). The cannula is removed at the end of the injection and the animals are allowed to recover. During injection, and for the following 30 min, all of our animals undergo continuous EEG recording (Grass Model 79). In addition, we used video recording (2 h, in a dimly lit room, on selected days between 8 and 10 a.m.) to monitor seizure behavior. Seizures are scored by the criteria of Racine; experimenters are blind to the nature of the injectate. Chronic recording from freely moving animals are carried out using teflon-coated stainless steel electrodes (<0.25 mm bare tips), positioned into the basolateral nucleus of the right amygdaloid body and into both dorsal hippocampi. Stereotaxic coordinates for amygdala are: 3.3 mm posterior from bregma, 5.0 mm lateral, and 8.5 mm below the surface of the skull; coordinates for the dorsal hippocampus are: 3.3 mm posterior from bregma, 1.5 mm lateral, and 3.4 mm below the surface of the skull. Stainless steel screws are placed in the occipital bone to provide reference and ground contacts. All electrodes are attached to wires with pin adapters and then affixed to the skull with dental acrylic. After 5 days of recovery from the surgery, the stylet is removed from the guide cannula and is replaced with an injection cannula consisting of a 24-gauge guide wire, the tip of which is a 0.5 mm length of fused silica (0.075 mm ID, 0.15 mm OD). These freely moving animals are injected with an aqueous solution (1.5 µl) of 100 mmol ferric chloride ($FeCl_3$, pH 2.2) at a rate of 1 µl min^{-1} using the microinfusion pump. Following injection, EEG recording and behavioral observations can be obtained daily for the duration of the experiment.

Animals prepared for neurochemical assessment or for molecular biological experiments are injected with $FeCl_3$, as described earlier, but chronic recording electrodes are not placed within the depths of the brain. Seizure behaviors are recorded for each animal during scheduled observations.

Results

Seizure Behavior and EEG

Acute injection of $FeCl_3$ into rat amygdala causes a gradual increase in spike discharges over 20 min, followed by quiescence of EEG patterns by the end of 30 min of recording. Animals injected with acidified saline have occasional isolated spike discharges. Of animals injected with $FeCl_3$ into the left amygdaloid body, up to 92% develop spontaneous seizures (stage 4 or 5) by the fifth day after injection. None of the acidified saline injected control rats have either sustained EEG discharges or behavioral seizures.

We have prepared parallel groups of animals with either EEG recording capabilities or with no intracranial electrodes – to allow for neurochemical experiments using microdialysis or molecular biological experiments. Among those animals prepared for chronic recording, all of those injected with $FeCl_3$ in our initial experiment developed spontaneous behavioral seizures and epileptiform discharges recorded from the depth electrodes. Epileptiform patterns are recorded from the ipsilateral amygdaloid body within 5–10 min after injection of $FeCl_3$. Over the following days, mainly epileptiform discharges are recorded from the ipsilateral $FeCl_3$-injected amygdala. During the following 3 days, focal spike discharges are much more frequent in the ipsilateral amygdala. However, by 5–7 days post-injection, isolated spikes can be recorded from both hippocampi and the contralateral amygdala. At 7 days after injection, we begin to record spontaneous rearing and limb clonus seizure (Racine stage 4–5) with rhythmic epileptiform discharges. Both the electrographic patterns and the behavioral seizures are observed until we terminate our experiments at 30 days post-injection. Saline-injected animals remain without spontaneous seizures or epileptiform abnormalities on the EEG recordings.

Histopathology

Timm staining of the hippocampus, as seen at 30 days following ipsilateral amygdala injection of $FeCl_3$, shows mossy fiber sprouting. In addition, with Berlin blue combined with H&E staining reveals cell loss in the ipsilateral and contralateral hippocampi (CA1 and 4 regions and the dentate hilus).

Genetic and Molecular Changes

Given that repetitive microinjection of glutamate into the amygdala causes seizure behaviors, we examined the regulation of neurotransmitter transporters by measuring mRNA and proteins responsible for glutamate and GABA removal. We extracted crude synaptic membrane fractions from hippocampal tissue and used immunoblotting and quantitative densitometry to measure the regulation of the high-affinity glutamate transporters GLAST, GLT-1, and EAAC-1. By 30 days following iron injection into the amygdala, the production of glial glutamate transporter GLAST is downregulated in both hippocampi. We hypothesize that the lost ability of brain to remove synaptic glutamate is fundamental to the process of posttraumatic epilepsy as modeled in these animals. Of interest, GABA transporters, particularly GAT3, are upregulated in a pattern that suggests compensatory responses within the inhibitory system.

Challenges for the Future

Within the context of this model, it is important to investigate the molecular basis of epileptogenesis, and the effects of drugs that might interfere with this process. Further, we continue to evaluate the impact of antiepileptic drugs on our model. Indeed, while epileptogenesis is correlated with the downregulation of GLAST and upregulation of EAAC-1, administration of valproate caused an increase in the quantity of GLAST protein measured in the hippocampus. Further, valproate treatment decreased GLT-1 in both control and experimental animals in both hippocampi, suggesting a unique mechanism of action. Specifically, valproate appears to affect the removal of glutamate by upregulating GLAST and decreasing GABA transport (which could result in increased tissue concentrations of GABA). Zonisamide administration increased the quantity of EAAC-1 protein in hippocampus and cortex, and downregulated the GABA transporters GAT-1, thus also increasing tissue and synaptic concentrations of GABA.

Fundamentals of neurochemical injury may offer clues for intervention in posttraumatic epileptogenesis. Hydroxyl radicals, superoxide radicals, and peroxides generated in biological systems – by oxidative chemistry or by actions of heme-containing compounds liberated within lipid systems – are quenched by the actions of enzymes such as catalase, peroxidase, and superoxide dismutase. Glutathione peroxidase, using glutathione as a cosubstrate and selenium as a metallic cofactor, reduces intracellular formation of hydrogen peroxide and free radicals. Oxidative stress increases activity of glutathione reductase, glucose-6-phosphate dehydrogenase, and glutathione peroxidase. Selenium, a metallic cofactor of glutathione peroxidase, also seems to act synergistically with α-tocopherol in preventing peroxidation of structural membrane components. These neurochemical sequences suggest that treatments designed to block free radical generation or quench the cascade of lipid peroxidation might lead to ways to prevent the development of epilepsy following trauma. α-Tocopherol prevents peroxidative injury of sulfhydryl groups of glycolipids and glycoproteins, apparently augmenting the antioxidant effects of enzyme systems such as glutathione peroxidase. Tocopherol also prevents peroxidation of unsaturated fatty acids and lipids by reaction of phenolic hydroxyl groups with propagating lipid radicals that were initiated by oxidative carbonyl hydrogen abstraction. Further, the phytyl side chain of tocopherol may intercalate within the acyl chains of polyunsaturated phospholipids, causing lipid membrane stabilization and reduction in membrane permeability. Tocopherol may also act as a free radical scavenger and singlet oxygen quenching agent. A novel nonglucocorticoid 21-aminosteroid with properties of inhibiting iron-dependent lipid peroxidation has been shown to have a protective effect on concussive injury to mice. Superoxide radicals induce cellular and vasogenic edema. Initiation of focal edema by cold-induced injury to the cerebral cortex of rodents causes increased levels of superoxide radicals. Administration of liposome-entrapped copper–zinc superoxide dismutase interferes with the development of cold-induced edema, suggesting that superoxide dismutase interruption of oxygen–free radical induced fatty acid injury may have potential for interruption of trauma-induced brain injury.

We continue to use amygdala injection of minute quantities of aqueous iron to initiate epileptogenesis, and then challenge that process with drugs and compounds. We believe this model provides a neurochemical and molecular framework for the study of epileptogenesis.

See also: **Epileptogenesis**: Role of Transporter Proteins and Lipid Peroxidation in Epileptogenesis; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury; Posttraumatic Epilepsy Caused by Fluid Percussion Injury; Posttraumatic Models.

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PSYCHIATRIC SYMPTOMS

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Norephinephrine: A Molecular Link Between Depression and Epilepsy?

Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures

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Introduction

Temporal lobe epilepsy (TLE) patients have a high incidence of schizophrenia-like symptoms. These symptoms may be present at all times between seizures (interictally) or only after seizure (postictal). I3n some patients, schizophrenic symptoms disappear following convulsive seizures. However, in other TLE patients, a single seizure, or more commonly, a cluster of seizures may result in psychiatric symptoms and occasionally in postictal psychosis that lasts for days.

The mechanisms of how seizures induce or suppress psychiatric symptoms are not well understood. A temporal lobe seizure may have time-dependent effects, on both local and remote structures in the brain. Seizures are known to alter synaptic transmission, either transiently or persistently. Excitatory synaptic transmission may be increased by frequency potentiation or other short-term potentation mechanisms, during or immediately after the seizure. Postictal potentiation, similar to long-term potentiation, may be long lasting. Inhibitory GABAergic transmission may be temporarily lost during and after a seizure, and permanently altered in epileptic patients. Seizures may also alter signaling pathways and gene function, and thus alter behaviors through gene expression.

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illness characterized by impoverished affect, nonsensical association, autistic behavior, ambivalence, delusions, and hallucinations. Psychotic symptoms can be relieved by a dopamine receptor antagonist. Some drugs that enhance dopamine function, e.g., amphetamine, induce schizophrenia-like symptoms in normal humans. Schizophrenia is also increasingly regarded as a disorder of an integrated system. Alteration of structure and function of the temporal lobe, particularly of the entorhinal cortex and hippocampus, may contribute to schizophrenia.

Many temporal lobe structures, including the hippocampus (CA1 and subiculum), entorhinal cortex, and amygdala, project excitatory glutamatergic afferents to the NAC, also known as the ventral striatum (Figure 1(c)). The NAC also receives glutamatergic afferents from the medial prefrontal cortex and dopaminergic afferents from the ventral tegmental area. The NAC has been suggested to be an interface between the limbic system and the motor system, and plays a critical role in reward and addictive behaviors.

Enhanced or disrupted synchrony of gamma waves of 30–100 Hz has been reported in the neocortical electrical activity of schizophrenic patients. In animals, increased gamma waves were reported in the hippocampus or NAC during schizophrenia-like behaviors induced by psychotomimetic drugs such as ketamine or phencyclidine. Electrophysiological studies of hippocampal gamma waves indicate that they are generated by purely GABAergic

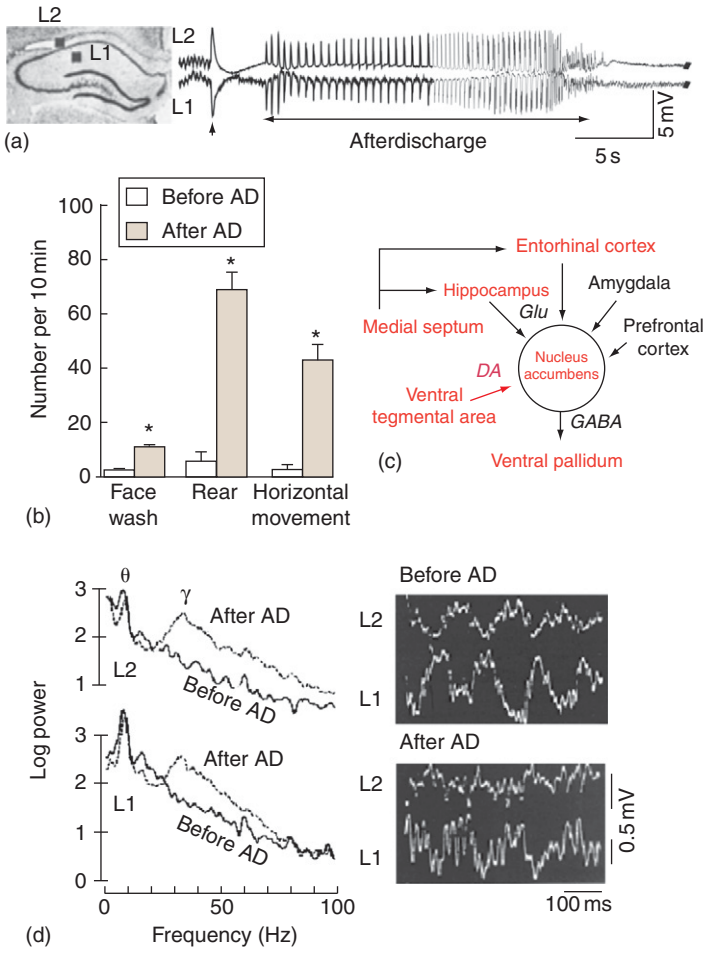


Figure 1 (a) A hippocampal afterdischarge (AD) of 21-s duration was recorded at electrodes L2 and L1 across the CA1 cell layer, evoked by a brief high-frequency stimulation (arrow) of the contralateral CA1 stratum radiatum. (b) Number of occurrence of face washes and vertical (rearings) and horizontal locomotion significantly increased in the 10 min after the AD, as compared with the 10 min before the AD. Adapted from Ma J, Brudzynski SM, and Leung LWS (1996) Involvement of the nucleus accumbens-ventral pallidal pathway in postictal behavior induced by a hippocampal after discharge in rats. *Brain Research* 739: 26–35. (c) Proposed neural circuit that mediates postictal behavioral hyperactivity, via dopamine release in the nucleus accumbens. Amygdala or prefrontal cortical AD did not induce postictal behavioral hyperactivity independent of the hippocampus. (d) Logarithmic power spectra of hippocampal EEG show enhanced gamma (γ, 30–100 Hz) but not theta (θ, 6–8-Hz peak) rhythm at the electrodes L2 and L1 (at stratum oriens and stratum radiatum of CA1, respectively) during the time of behavioral hyperactivity, 7 min after the AD. Reprinted from Leung LWS (1987) Hippocampal electrical activity following local tetanization. Afterdischarges. *Brain Research* 419: 173–187, with permission of Elsevier.

interactions among inhibitory interneurons, or by recurrent inhibitory interactions between excitatory pyramidal cells and inhibitory interneurons.

Background

Kindling of temporal lobe structures, notably the amygdala and the hippocampus, is a model of TLE. Kindling is the process of repeated and spaced stimulation of a brain structure, with the intention of evoking an afterdischarge (AD) with each stimulus. In classic studies, kindling of the amygdala increases the duration of the evoked AD and the severity of the accompanying behavioral convulsions. Full kindling is defined as attaining the stage of generalized convulsive seizures (stage 5 seizure of Racine). Chronic interictal behavioral changes are observed in the animals after amygdala kindling, in particular, after full kindling. Alteration of emotional behaviors, labeled as aggressive or defensive, has been reported in response to an animal intruder or to capture by a researcher. Kindled animals also show persistent changes in anxiety behaviors on an elevated maze, and deficient spatial behaviors in radial arm maze and water maze.

Dopamine hyperfunction after kindling has been indicated by several measures. Systemic injection of amphetamine (which releases dopamine) or a dopamine agonist may induce higher locomotor response in amygdala-kindled animals, as compared with non-kindled control animals. Changes in dopamine receptor sensitivity, dopamine release, and dopamine binding have also been found in chronic TLE models.

Postictal behavioral depression may also occur after a temporal lobe seizure, typically after a prolonged seizure accompanied by generalized convulsions. Behavioral depression after an amygdala AD (in a fully kindled animal) involves the release of endogenous opiates, which may decrease dopaminergic function in the NAC.

This article will focus on the postictal effects of a single AD in the hippocampus of rats, and on psychiatric symptoms in the partially hippocampally-kindled rats. Partial kindling refers to the repeated elicitation of ADs or electrographic seizures that are not accompanied by convulsive behaviors (i.e., Racine seizure stage 0 or 1).

Methods

We studied the changes in EEG and in synaptic transmission in the limbic system after hippocampal kindling. For studies in behaving rats, the rats were chronically implanted with indwelling electrodes for stimulation and recording. Guide cannulae were targeted at specific brain areas, including the hippocampus, medial septum, ventral pallidum, ventral subiculum, and NAC for infusion of drugs. Drugs included *N*-methyl-D-aspartate (NMDA) for excitation of glutamate receptors in the hippocampus, D_1 and D_2 receptor antagonists in the NAC, and $GABA_A$ receptor agonist muscimol for local inactivation of various structures. Electrically induced ADs were evoked by high-frequency stimulation (1-s train duration, 100–200-Hz frequency, stimulus intensity of 50–800 μA, 0.1–0.5-ms square pulse duration) in the dorsal and ventral hippocampus, ventral subiculum, amygdala, or medial prefrontal cortex of adult male Long Evans rats. For kindling, ADs were induced at intervals of not more than once every hour and 5 times a day. Partial hippocampal kindling reported here used 1, 10, or 21 ADs delivered to dorsal hippocampal CA1 area, without evoking behavioral convulsions.

Locomotor activity was quantified by analysis of the videotaped behaviors or by interruption of infrared beams in a $69 \times 69 \times 49\ cm^3$ high Plexiglas chamber. Locomotor activity was measured before and after injection of drugs. Sensorimotor gating deficits are found in schizophrenic patients, and sensorimotor gating is commonly tested by prepulse inhibition (PPI) of a startle reflex response. PPI was measured in rats by inhibition of an acoustic startle response, with a low-intensity noise serving as a prepulse (20-ms duration burst of noise of 73, 75, or 80 dB) presented 100 ms before a startle pulse of 120 dB. Percent inhibition of the startle response, averaged across the three prepulse intensities, provide a measure of PPI (see later). Muscimol (0.25 μg in 0.6 μl of saline) or saline (0.6 μl) was infused into the medial septum 15 min before evoking a hippocampal AD, and the rat was tested for PPI at 5–20 min after an AD.

Results

A Single Hippocampal AD Induces Behavioral Hyperactivity and PPI Deficit

In naive rats that were handled and implanted with chronically indwelling electrodes, but not otherwise experimentally manipulated, a long AD (>15-s duration) was evoked from the dorsal hippocampal CA1 area by a high-frequency stimulation (**Figure 1(a)**). The AD was followed by an increase in behavioral activity that included face washes, and most robustly, horizontal and vertical movements (**Figure 1(b)**). The behavioral hyperactivity lasted about 15 min after the AD.

PPI of the acoustic startle response showed a decrease when tested 5–20 min after a dorsal hippocampal AD, as compared with baseline in the same animal (pre-AD) or to control animals not given an AD (**Figure 2(b)**). There was no effect on the acoustic startle response itself (**Figure 2(a)**). Similarly, a PPI deficit was also found immediately after evoking an amygdala AD in a fully kindled rat. However, Howland *et al.* recently reported that PPI was not significantly altered immediately or two

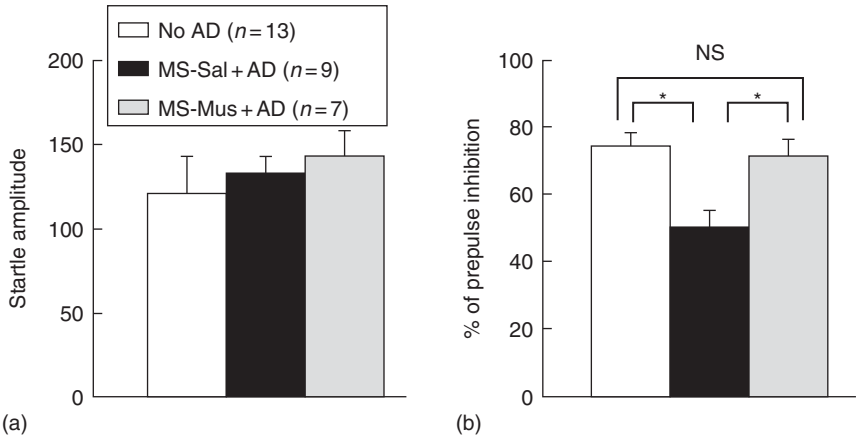


Figure 2 A hippocampal AD suppressed prepulse inhibition (PPI) of the acoustic startle (b) but not the startle itself (a). Muscimol infusion into the medial septum (MS-Mus) 15 min before the AD normalized the PPI, as compared with saline infusion into the medial septum (MS-Sal); * $p < 0.01$. Adapted from Ma J, Shen B, Rajakumar N, Leung LS (2004) The medial septum mediates impairment of prepulse inhibition of acoustic startle induced by a hippocampal seizure or phencyclidine. *Behavioral Brain Research* 155: 153–166.

days after a stage-5 AD evoked in the ventral hippocampus or the perirhinal cortex. For unknown reasons, it appears that a convulsive AD in the ventral hippocampus or perirhinal cortex, in contrast to an AD in the dorsal hippocampus or the fully kindled amygdala, may not induce significant functional disruption of structures involved in PPI, such as the NAC.

Structures and Pathways that Mediate Postictal Behavioral Alterations

Putative structures that are involved in postictal behaviors were identified by local infusion of drugs. Behavioral hyperactivity after a hippocampal AD was suppressed by infusion of a D_2 receptor antagonist sulpiride, or haloperidol, but not a D_1 receptor antagonist SCH 23390, into the NAC. This result suggests that hippocampal AD increased dopamine release and D_2 receptor activation. Inactivation of the ventral pallidum by local muscimol infusion also suppressed postictal hyperactivity (postictal PPI loss was not tested). Muscimol infusion into the medial septum suppressed both postictal hyperactivity and postictal PPI deficit. The medial septum projects cholinergic, GABAergic, and glutamatergic afferents to the hippocampus and modulates hippocampal excitability and EEG.

By recording electrical activity using chronically implanted electrodes, we showed that behavioral hyperactivity induced by infusion of NMDA into the ventral subiculum required the occurrence of a local AD. Little hyperactivity was induced by electrical or chemical stimulations in the ventral subiculum that did not evoke an AD. In addition, ADs evoked by electrical stimulation of the ventral subiculum had to last longer than 10 s in order to elicit behavioral hyperactivity. The number of horizontal movements was found to correlate with the duration of the AD evoked by ventral subiculum stimulation. Stimulation of other limbic structures gave different results: Stimulation of the ventral tegmental area typically induced stereotypic head movements, such as sniffing and forward locomotion, without evoking an AD. A nonconvulsive AD in the amygdala did not consistently change locomotor activity early in the kindling process, while a convulsive stage 5 amygdala AD in fully kindled animals was accompanied by a decrease in locomotor activity. Prefrontal cortical ADs only induced behavioral hyperactivity when the AD induced high-frequency gamma oscillations (of >70 Hz) in the NAC.

Partial seizures arising from the temporal or frontal lobe in patients are commonly associated with ictal or postictal automatic behaviors called automatisms. Postictal behavioral hyperactivity induced by a hippocampal AD in rats is a type of automatism, and it depends on dopaminergic D_2 receptor activation in the NAC. It is thus suggested that some postictal automatisms in TLE patients may be mediated by the NAC via a dopamine D_2 receptor mechanism.

Hippocampal Gamma Waves Are Associated with Postictal Behavioral Alterations

The power of gamma waves in the hippocampus was enhanced tenfold (one log unit) during the postictal behavioral hyperactivity induced by a hippocampal AD (**Figure 1(d)**). The period of enhanced hippocampal gamma waves lasted about 15 min after a hippocampal AD, generally similar to the duration of enhanced behavioral activity. However, the high gamma-wave activity did not abate during a brief period of behavioral immobility. Inactivation of the medial septum with muscimol (either before or immediately after a hippocampal AD) did not affect the AD duration, but it suppressed the AD-induced increase in both hippocampal gamma waves and

locomotor behaviors. The muscarinic cholinergic antagonist, scopolamine, partially suppressed the AD-induced increase in both gamma waves and locomotor behaviors. However, lesion of the cholinergic septohippocampal neurons in the medial septum by specific cholinotoxin 192IgG-conjugated saporin did not block the postictal increase in gamma waves or behavioral hyperactivity. Hippocampal disinhibition by both cholinergic and GABAergic septohippocampal afferents may be required for postictal gamma wave increase in the hippocampus.

Chronic Psychiatric Symptoms after Partial Hippocampal Kindling

Changes in behaviors were found after partial hippocampal kindling, as compared with nonkindled rats. Three behaviors – (1) increase in climbing episodes, (2) increase in locomotion after 1 mg kg^{-1} i.p. methamphetamine, and (3) decrease in PPI (**Figure 3**) – may be associated with an increase in dopamine functions in the NAC. Twenty-one hippocampal ADs, but not 10 hippocampal ADs, were needed to induce the latter behavioral changes. Similarly, kainic acid-treated rats (that experienced status epilepticus) showed increased behavioral hyperactivity with amphetamine challenge.

Kalynchuk *et al.* reported altered defensive and social behaviors in rats after 99 ADs in the amygdala, while Adamec showed that emotional behaviors in cats were induced by partial kindling (12 ADs) of the ventral hippocampus. A deficit of PPI was not found during the interictal state of amygdala-kindled rats, pentylenetetrazol-kindled rats, or in rats induced with kainic acid status epilepticus.

Future Goals

The ability of a single hippocampal AD to induce psychiatric symptoms such as locomotor hyperactivity and loss of PPI suggests that seizure activity in the limbic system alone can induce psychiatric symptoms. Locomotor hyperactivity is shown to be dependent on dopamine release in the NAC. Dopamine function appears to be sensitized and then suppressed during the process of kindling, but the mechanisms of sensitization and suppression are not fully studied.

The conditions (seizure origin, spread, and stage) whereby an AD can induce behavioral hyperactivity and PPI remain to be determined. Our data suggest that a bilaterally prolonged limbic AD has the ability to drive behavior by activation of the NAC. In the case of ADs evoked by stimulation of hippocampal CA3/CA1 or ventral subiculum on one side, the hippocampal AD spread bilaterally via the strong connection through the ventral hippocampal commissure. The AD was also prolonged, most likely because of the intrinsic physiology and/or the functional connections of the rat's hippocampus. Despite a direct connection to the NAC, the amygdala and the prefrontal cortex may not generate an AD of sufficient duration or sufficient bilateral synchrony to induce postictal psychiatric symptoms. Alternatively, a generalized AD in the amygdala or prefrontal cortex may mediate postictal behavioral depression by releasing opiates in the NAC. In humans, bilateral hippocampal ADs are relatively rare because of a small ventral hippocampal commissure.

Hippocampal gamma waves induced by a hippocampal AD do not have a moment-to-moment relation to the behavioral hyperactivity. However, periods of enhanced hippocampal gamma waves correlate with periods of behavioral hyperactivity, and medial septal inactivation reduces both gamma waves and behavioral hyperactivity. It is known that generation of hippocampal gamma waves depends on local inhibitory interneurons, but further studies are needed to address the alteration of hippocampal interneuronal interactions that correlate with schizophrenia-like behaviors. Procedures or drugs that modulate the inhibitory circuits in the hippocampus,

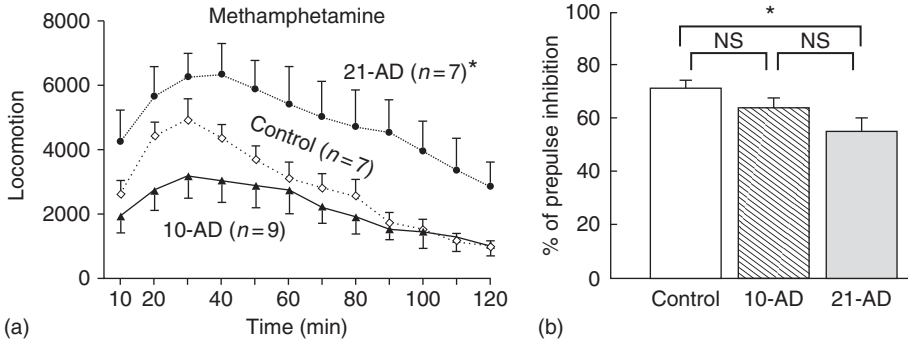


Figure 3 Methamphetamine-induced behavioral hyperactivity and decrease in PPI were found after partial hippocampal kindling with 21 ADs, but not with 10 ADs. Locomotion was measured by interruption of infrared beams counted in 10-min bins. Methamphetamine (1 mg/kg i.p.) induced increased locomotion in 21-AD group as compared with control or 10-AD group. A significant loss of PPI of the acoustic startle was found in the 21-AD group ($N = 6$), but not in the 10-AD group ($N = 9$), as compared with control group ($N = 11$) tested 3 days after the last AD. Adapted from Ma J and Leung LS (2004) Schizophrenia-like behavioral changes after partial hippocampal kindling. *Brain Research* 997: 111–118.

such as inactivation of the medial septum by a GABAergic agonist, may provide therapeutic treatment of psychiatric symptoms in TLE patients.

See also: **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures; **Psychiatric Symptoms**: Interictal Anxiety in Temporal Lobe Epilepsy; Interictal Behavioral Comorbidities in a Model of Epilepsy; Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Interictal Anxiety in Temporal Lobe Epilepsy

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Introduction

Epilepsy is a common and serious neurological disorder affecting almost 1% of the world's population. Most people with epilepsy lead normal lives; however, a significant proportion experience severe interictal (i.e., in between seizures) disturbances in cognition and behavior. Consistent with this observation, clinical studies have repeatedly shown that the rate of anxiety, depression, and psychosis is overrepresented in epileptic populations. These behavioral disturbances are especially high among patients with partial seizures that result from epileptiform discharges within mesial temporal limbic structures – a condition called temporal lobe or limbic epilepsy. Although seizures are the primary problem in epilepsy patients, the continuous and protracted presentation of these behavioral disturbances can profoundly affect their quality of life. However, these problems often go unrecognized and even when identified, they are frequently under-treated.

Considering the high prevalence of temporal lobe epilepsy (TLE) among adult seizure disorders, characterizing the nature and etiology of interictal anxiety is a worthwhile objective. However, studying interictal anxiety clinically has been difficult because of the heterogeneity in seizure-related factors among patient populations. The use of animal models of epilepsy has helped expand our understanding of how changes in cognitive and emotional behaviors relate to seizure-induced changes in neurobiology.

Background

Clinical Manifestations of Anxiety in TLE

Given the well-known role of the limbic system in regulating complex aspects of emotional behavior, it is not surprising that the major types of behavioral changes associated with TLE encompass areas of mood, personality,

such as inactivation of the medial septum by a GABAergic agonist, may provide therapeutic treatment of psychiatric symptoms in TLE patients.

See also: **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures; **Psychiatric Symptoms**: Interictal Anxiety in Temporal Lobe Epilepsy; Interictal Behavioral Comorbidities in a Model of Epilepsy; Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Background

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Given the well-known role of the limbic system in regulating complex aspects of emotional behavior, it is not surprising that the major types of behavioral changes associated with TLE encompass areas of mood, personality,

and thought. These emotional disturbances can range from mild fear to pathological levels of anxiety and depression, and may include episodes of psychosis. Although these symptoms may develop concomitant to seizure activity (in prodromal, ictal, or post-ictal periods), they are more commonly present during chronic interictal periods. Higher rates of emotional disturbances are observed in people with TLE than those afflicted with other types of seizure disorders. Estimates have suggested that the prevalence of emotional disturbances among TLE patients may be as high as 50% during the interictal phase.

Anxiety is one of the most common interictal behavioral disturbances reported in patients with TLE. Several studies have estimated that the prevalence of interictal anxiety in TLE patients is between 14% and 25%, and in some settings it is as high as 65%. However, relative to other psychiatric-epileptic dyads (such as depression and psychosis), the relationship between anxiety and epilepsy has been less thoroughly investigated. This is surprising considering the frequent comorbidity of anxiety with other psychiatric conditions (such as depression) and the considerable focus that current psychiatric research has placed on understanding the pathophysiology of anxiety. Clinical observations suggest that seizure-related factors such as age of onset, duration of epilepsy, antiepileptic treatment, seizure severity, and lateralization of seizure spread can be important predictors for the development of interictal anxiety in patients with TLE. In addition, several questionnaire studies have tried to examine the relative contribution of anxiety in reducing health-related quality of life measures in people with epilepsy. In one study, interictal anxiety was the most significant predictor for reduced quality of life in epileptic patients, explaining 27% of the variance in these measures compared with 12% for depression and 3% for seizure frequency.

Preclinical Research on Interictal Anxiety

The neurobiological study of interictal anxiety in human epileptic patients has been slowed by the diversity in seizure-related factors among patients and potential psychosocial influences that may affect their lives. Determining how seizures alter brain function interictally to produce lasting changes in emotionality has been difficult. The development of animal models of epilepsy has helped in this regard by removing many of the inherent problems associated with human clinical research and allowing for controlled investigations of the pathophysiology of interictal anxiety. The kindling model of TLE has been one of the most valuable methods for studying the neural mechanisms associated with interictal anxiety and emotionality. The remaining sections of this chapter will focus on the kindling model. We encourage the reader to consult the 'Further Reading' section for additional discussion on interictal behavioral disturbances found in other animal models of epilepsy.

Kindling as an experimental tool for measuring interictal anxiety

Kindling refers to the process whereby repeated application of initially subconvulsant focal electrical stimulations to subcortical or cortical regions results in long lasting, if not permanent, increases in seizure susceptibility. Although kindling has been commonly used to study epileptogenesis, several features of kindling have made it an attractive model for understanding the pathogenesis of interictal behavioral disturbances. For instance, because the site of stimulation is under the control of the experimenter, inducing epileptic seizures from different brain sites can allow for the investigation of neuroanatomical structures that might be important in mediating interictal anxiety. In addition, because the time-window between consecutive seizures can also be controlled and manipulated by the experimenter, the potential influence of ictal and post-ictal effects on the interpretation of behavioral data is minimized. Finally, because the behavioral and electrographic evolution of seizures during kindling is progressive, this model also offers the possibility of evaluating the behavioral consequences of seizures at different stages in the development of epilepsy.

Effects of kindling on interictal anxiety

Kindling can enhance fear and defensive responses in rodents and felines. From a clinical perspective, studying interictal changes in fear and defensive behavior is important because these animal behaviors model many aspects of human anxiety (**Table 1**).

Table 1 Common behavioral and clinical correlates of fear and defensiveness in kindled rats and in humans suffering from clinical anxiety disorders (generalized anxiety disorder)

Animal	*Fear/anxiety*	*Defensive*
Kindled rats	Increased startle response, increased freezing to novel situations, changes in heart rate, hypoalgesia, increased defection and urination in response to stress, autonomic dysregulation (such as sweating, piloerection, etc.), and hyperlocomotion	Fighting, biting, escape (flight or jumping) and avoidance (passive, active, and/or defensive burying)
Humans (w/GAD)	Exaggerated startle response, experiences of worry (and apprehension), inability to inhibit behavior, cardiovascular changes, autonomic dysregulation, diarrhea and increased urination	Irritability, outbursts of anger, and persistent avoidance of the aversive stimulus

Adamec was the first to systematically document the effect of kindling on interictal fear and anxiety-like behavior. In his seminal studies, partial kindling (i.e., kindling that produces afterdischarges but no generalized motor convulsions) of the amygdala or ventral hippocampus in cats resulted in long-lasting changes in emotional behavior that appeared to be independent of motor seizures or interictal spiking for their maintenance. He found that partially kindled cats displayed elevated defensive responses (such as defensive posturing, piloerection, ear retraction, and vocalizations) when exposed to rats, mice, or recorded vocal threats from conspecifics. These effects were lasting and could still be evoked 4 months after the last afterdischarge.

Many of these findings were soon replicated in the rat. Pinel and his colleagues demonstrated that increased interictal defensiveness (i.e., resistance to being captured, Table 2) followed protracted kindling of the amygdala but not the caudate nucleus. Similar changes in defensiveness in the rat were also reported by Post using a pharmacological kindling model of repeated lidocaine injections. Subsequent work by Caldecott-Hazard and her colleagues further showed increases in locomotor activity during exposure to a novel environment 21 days after bilateral amygdala kindling. Of particular interest was the fact that these investigators also reported a global reduction in deoxyglucose metabolism in the forebrain after kindling. This finding may be of clinical relevance because similar reductions in glucose metabolism are often seen in limbic regions of TLE patients who report high levels of anxiety and emotional disturbances.

It is well known that stress can precipitate the development of affective illnesses such as anxiety and depression. Several studies have revealed that kindling can exacerbate the effects of stress. Most notable are the studies of Henke and Sullivan, who reported that amygdala kindling potentiated the formation of stress-induced stomach ulcers. Furthermore, Weiss and her colleagues showed that administration of the stress hormone corticotrophin-releasing hormone (CRH) elicited defensive fighting in rats that had been previously kindled. These results suggest that repeatedly evoked seizures can lead to the subsequent dysregulation of stress circuitry and may contribute to the appearance of interictal behavioral disturbances.

Short-term kindling protocols – in which animals receive daily electrical stimulations until three to five consecutive generalized motor convulsions are elicited (i.e., 15 and 30 stimulations in the case of amygdaloid stimulations) – have been used to examine interictal changes in emotionality. Using this protocol, several researchers have shown that short-term kindling decreases open-arm exploration on an elevated plus maze, increases passive (e.g., freezing) and active defensive responses (e.g., flight and fighting) to both threatening and nonthreatening stimuli, increases withdrawal from social interactions, and increases fear-potentiated startle. The changes in interictal behavior associated with kindling appear to be highly specific and involve increases in fear and/or anxiety-like behavior. Despite the high concordance of depression in patients with TLE, we (and others) have found that kindled rats do not show differences – compared to non-kindled controls – on measures of depression such as sucrose preference (a measure of anhedonia) or forced swimming (a measure of helplessness).

Table 2 The 7-point scale for resistance to capture

0 = Easy to pick up
1 = Vocalizes or runs away from hand
2 = Shies away from hand and vocalizes
3 = Runs away from hand
4 = Runs away from hand and vocalizes
5 = Bites or attempts to bite
6 = Launches a defensive jump attack

Methods

We have developed an animal model of interictal emotionality in which rats receive 99 electrical stimulations of the amygdala. It was our contention that if interictal emotionality involves the development of hyperexcitable fear circuits, then this process would be far from complete after just a few consecutive generalized convulsions. Long-term amygdala kindling would produce changes in interictal emotionality that may not be apparent with partial kindling, and these changes would be more robust than the ones produced by short-term kindling. As a result, long-term kindling offered a unique advantage in that it would provide a robust and reliable method for studying changes in interictal behavior after seizures. An additional point of importance was that delivering 99 electrical stimulations ensured that our animals would be well kindled, but not so well kindled that they would begin exhibiting spontaneous convulsions, which would seriously confound the interpretability of behavioral testing. Early work by Pinel and colleagues showed that spontaneous seizures do not occur unless rats receive at least 200 amygdala stimulations. Indeed, we have never observed a spontaneous seizure in a rat subjected to 99 kindling stimulations.

Our general methods are as follows. Rats are brought into our animal facility from a commercial supplier. They are given a few days to acclimatize to the facility, and then they are subjected to stereotaxic surgery. During the surgery, a bipolar stimulating electrode is implanted into the structure of interest in the left side. This is usually the basolateral amygdala, but we have also studied the effect of kindling the central nucleus of the amygdala, hippocampus, caudate nucleus, claustrum, perirhinal cortex, and bed nucleus of the stria terminalis. Rats are given several days to recover from the surgery prior to the onset of kindling. During kindling, rats receive three stimulations per day, 5 days per week, with a minimum

of 2 h between consecutive stimulations. Each stimulation consists of a 1 s, 60 Hz train of square-wave pulses, and each pulse has a biphasic amplitude that is well above threshold to elicit an afterdischarge (e.g., 800 μA peak-to-peak for amygdala stimulation). We always include a separate group of control rats that receive sham stimulations, in which the stimulating electrode is implanted and subsequently attached to the stimulation apparatus – but no current is passed. Our primary measure of seizure severity is the convulsion class elicited by each stimulation. We use the following extension of the Racine scale: class 0 convulsions comprise an arrest in behavioral mobility; class 1 convulsions comprise orofacial automatisms; class 2 convulsions comprise orofacial automatisms and repetitive head nodding; class 3 convulsions comprise unilateral forelimb clonus and mastication with salivation; class 4 convulsions comprise generalized convulsions with rearing and forelimb clonus; class 5 convulsions comprise rearing with rapid bilateral forelimb clonus followed by a loss of equilibrium; class 6 convulsions comprise bouts of multiple class 5 convulsions; class 7 convulsions comprise running fits and jumping; class 8 convulsions comprise any of the preceding symptoms with intermittent periods of tonus.

We generally conduct our behavioral testing beginning one day after the final kindling stimulation, but in some cases we have delayed the behavioral testing for one week or one month. We have used a number of different behavioral tests to assess changes in anxiety and depression-like behavior, including the open field, resistance to capture from the open field (see **Table 2**), elevated plus maze, social interaction, acoustic startle, forced swim, and sucrose preference tests. Rats receive no more than one behavioral test per day. After the completion of behavioral testing, rats are sacrificed and brains are processed to analyze changes in gene and protein expression using several different molecular techniques.

Recent Results

Long-Term Amygdala Kindling and Interictal Anxiety

We consistently see increased fear and anxiety-like behavior in rats subjected to long-term kindling. For example, when placed into an unfamiliar open field, long-term amygdala-kindled rats show a short initial period of immobility or freezing that is followed by a longer period of explosive ambulation and escape-related behaviors (**Figure 1**). At the end of testing, these rats display extreme resistance to being captured and engage in several active defensive responses such as fleeing, biting, and defensive jump attacks. These fear responses are exaggerations of normal species-specific displays of fear (e.g., freezing, fleeing, and fighting) to unfamiliar environments and potentially threatening stimuli. Such liability in emotional responses towards novel and mildly anxiety-provoking stimuli is consistent with the idea that repeated seizures lead to sensitization in neural pathways related to fear and escape-related behavior. Two additional lines of evidence also support this idea. First, we found a monotonic relationship between the number of kindled stimulations (i.e., 99 > 50 > 30 > 20 stimulations) and the magnitude of fear behavior produced by amygdala kindling. Second, we found that elevations in resistance to capture were dependent on the novelty and unfamiliarity of the testing environment; i.e., kindled rats

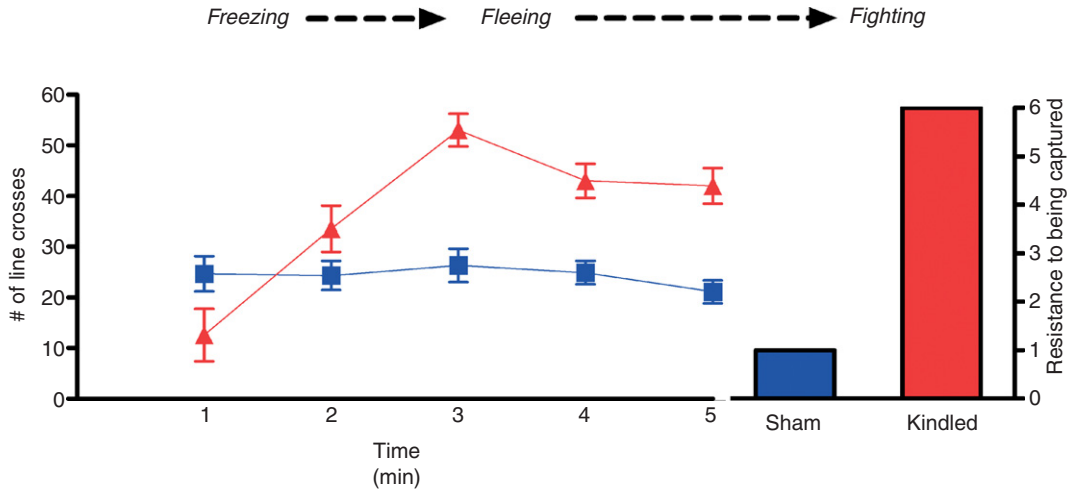


Figure 1 An example of the behavioral profile of kindled rats exposed to an unfamiliar environment. When placed in an unfamiliar open field, kindled rats first engage in a period of freezing, followed by enhanced exploration and darting from one end of the open field to another. After five minutes of this exploration, kindled rats display extreme resistance to capture from the open field, characterized by fleeing, vocalizations, attempts to bite, and defensive jump attacks (see text and **Table 2** for further discussion). These findings suggest that long-term kindling of brain structures related to emotionality, such as the amygdala, can lead to profound changes in interictal responses to mildly anxiogenic events.

displayed little resistance to capture from their home cages or from an already familiar open field.

The behavioral outcome of repeated seizures also appears to be highly dependent on the brain area that is stimulated. Our analyses revealed the following pattern of fear behavior after long-term kindling: basolateral amygdala = central amygdala > bed nucleus of the stria terminalis > perirhinal cortex = ventral hippocampus > claustrum = caudate = sham. These findings show that kindling of amygdaloid nuclei and related structures results in the greatest increases in fear behavior, whereas kindling of hippocampal and parahippocampal regions produces only intermediate increases in fear. Of importance is the fact that kindling non-limbic structures, such as the caudate and claustrum, does not increase interictal fear. Although these non-limbic structures are known to be involved in the spread of seizures, the fact that kindling them does not increase interictal fear suggests that it is not generalized seizure activity per se that is important for the development of interictal disturbances. Instead, it appears that the location of seizure initiating activity and/or potential seizure-inducing modifications is most critical.

An important question is how long the changes in interictal behavior last once the kindling stimulations have been terminated. The persistence of interictal emotional behavior is extremely important in forming hypotheses about the neural mechanisms that mediate sensitized fear and anxiety-like responses. For example, if interictal anxiety declines quickly once the seizures have stopped, then the anxiety might be simply an epiphenomenon (or by-product) of seizure activity. Alternatively, if interictal anxiety persists for long periods of time, then it is reasonable to speculate that potentially permanent modifications in emotional neural circuits may accompany chronic seizures. We have found that long-term amygdala kindling produces the greatest increases in fear behavior one day or one week after the final seizure, but kindled rats tested two months after the final seizure remain significantly more fearful than sham-stimulated controls. It should be noted that the biological outcome of a 30 to 60-day period in a rat is probably not functionally equivalent to a 30 to 60-day period in a human; a 30 to 60-day period in an adult rat would be functionally equivalent to a period of roughly 2–5 years in an adult human. Thus, the persistent elevations in interictal anxiety and emotionality for such a considerable period of time would certainly fit even the most conservative criteria of a clinical anxiety disorder.

Possible Mechanisms Underlying Interictal Anxiety

Electrophysiological studies

The lasting nature of interictal anxiety produced by kindling suggests that seizures modify the functional and perhaps physical relationships among sets of neurons. The neurocircuitry involved in fear and defensive behavior in the rat has been well characterized. The hippocampus and nuclei of the amygdala play important roles because of their direct and indirect innervation of the ventral medial hypothalamus (VMH) and midbrain periaqueductal grey (PAG) matter – two brain structures important in defensive behavior. Early studies by Adamec and his colleagues revealed that the threshold response to elicit defense reactions from VMH and/or PAG stimulation in the cat was significantly lower after partial amygdala kindling. Later, Adamec's group showed that partial kindling potentiated efferent transmission from the amygdala to the PAG. Indeed, amygdala-kindling-induced potentiation of neurons of the PAG and VMH could contribute to increased efficacy in transmission between these structures. Adamec's group went on to show that bilateral low frequency stimulation of the amygdala could selectively depotentiate the changes in efferent transmission to the PAG and VMH that occurred after partial kindling. Interestingly, potentiation of the right amygdala connection with the PAG caused defensive behaviors to return to pre-kindling levels. These findings may be of clinical relevance as epileptogenic foci lateralized to one particular side of the brain are associated with different psychiatric symptoms (e.g., depression is correlated with left hemispheric foci).

Receptor binding studies

In a series of receptor binding studies, we found that measures of interictal anxiety correlated with changes in a number of receptor subtypes localized primarily in the hippocampus. The pattern of results was clear: inhibitory receptor binding (e.g., for $GABA_A$, benzodiazepine, and $5\text{-}HT_{1A}$ receptors) was significantly increased within the dentate gyrus after kindling, whereas excitatory receptor binding (e.g., for NMDA and AMPA receptors) was significantly decreased in this region (**Figure 2**). Surprisingly, no significant changes in receptor binding were detected in the amygdala. We also found decreased *c-fos* immunoreactivity (a molecular marker of cellular activation) in the dentate gyrus and CA1 after kindled rats were exposed to fear-provoking stimuli. These findings led us to speculate that the hippocampus of kindled rats might be in a state of hyper-inhibition, which resulted in a type of functional lesion and ultimately, in dysfunction.

There is further support for the idea that hippocampal dysfunction might play a role in interictal anxiety. Several studies, most notably the work of Gallagher and colleagues, have shown that human TLE patients express increased levels of the stress hormone adrenocorticotrophic hormone (ACTH) between seizures. Surgical resection of the epileptogenic focus appears to normalize ACTH levels. In addition, several researchers have noted that limbic seizures increase the release of glucocorticoids (cortisol in man; corticosterone in rat). We have found that long-term amygdala kindling decreases glucocorticoid

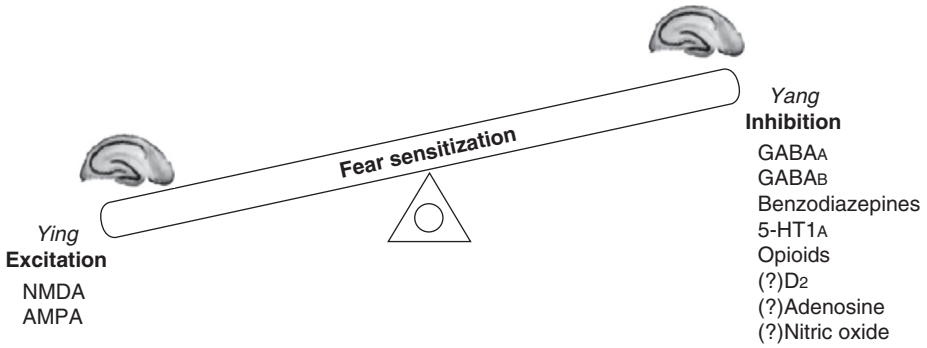


Figure 2 Hypothetical model for explaining increased fear sensitization and interictal behavioral pathologies after long-term kindling. (?) denotes possible changes in receptor subtype or neurotransmitters.

receptor (GR) mRNA expression in the dentate gyrus and hippocampal CA1 region. It is very likely that high levels of circulating glucocorticoids after kindled seizures lead to an eventual downregulation in GR expression in the hippocampus. These changes in GR expression may have important functional consequences for the development of interictal anxiety. First, alterations in the transcription of genes controlled by the GR promoter could lead to downstream effects on different genes and their protein products, some of which may be involved in anxiety. Second, because hippocampal GRs are instrumental in terminating the stress response, a downregulation of GR receptors could result in consistently elevated levels of circulating corticosterone or cortisol. Finally, high levels of glucocorticoids are known to induce dendritic remodeling of pyramidal neurons of amygdala and hippocampus, which may play a role in enhancing epileptiform activity.

Does increased inhibition play a role in maintaining the interictal state?

An important question is whether the increase in hippocampal inhibition after kindling plays a functional role in the pathogenesis of interictal anxiety. Considering the prominent role of the hippocampus in the processing of contextual information and in mediating some aspects of anxiety and fear behavior, increased inhibition in hippocampal circuits after kindling might explain the elevated anxiety and fear that occurs in response to novel environmental stimuli. The dentate gyrus is known to regulate hippocampal excitability and serves as a major gateway through which excitation from the entorhinal cortex enters the hippocampus. Some investigators have speculated that the development of inhibitory processes and other seizure suppressing mechanisms after epileptic seizures reflects a homeostatic response that serves to maintain the interictal state. Interestingly, kindling potentiates feedback and feedforward inhibition in the dentate gyrus, and there is some evidence that inhibitory mechanisms in CA1 might also be compromised in the chronically epileptic brain. Gutierrez and colleagues found that after seizures, dentate granule cells begin to synthesize and release the inhibitory neurotransmitter GABA from normally excitatory mossy fiber terminals. When taken together with the results from our receptor binding studies, these findings suggest that increased inhibition after kindling might reflect an endogenous compensatory response to reduce the likelihood of subsequent seizures. Viewed in this way, interictal anxiety could be a by-product of the brain's attempt to limit the future occurrence of seizures.

Neurogenesis and interictal anxiety

The discovery that seizures can increase neurogenesis in the adult hippocampus has become the focus of much experimental research. Although most of this work has focused on how aberrant neurogenesis might contribute to epileptogenesis, an interesting question is whether the birth of new neurons after seizures also plays a role in the emergence of interictal behavioral disturbances. Our preliminary work has revealed that hippocampal neurogenesis is substantially increased following short-term amygdala kindling to about 30 stimulations, but that it declines from there as more kindling stimulations are delivered. By the 99th kindling stimulation, the rate of cell proliferation is back to baseline levels (**Figure 3**). Given the observation that newly born neurons require 2 weeks to 1 month to fully integrate into preexisting adult hippocampal circuits, newly generated cells born after the 30th kindling stimulation would have time to differentiate, migrate, and functionally integrate themselves by the time the 99th kindling stimulation is delivered (~4 weeks after the 30th kindled stimulation). We believe that these newborn cells may play a role in the emergence of interictal anxiety after long-term kindling.

Some exciting recent findings from Katherine Jakubs of Olle Lindvall's laboratory in Sweden suggest that at least some of the new neurons born after epileptic seizures might serve to maintain network homeostasis after seizures. Jakubs and colleagues found that new neurons produced under seizure conditions received increased inhibitory and reduced excitatory synaptic input. The reduced excitability of these new neurons suggested to

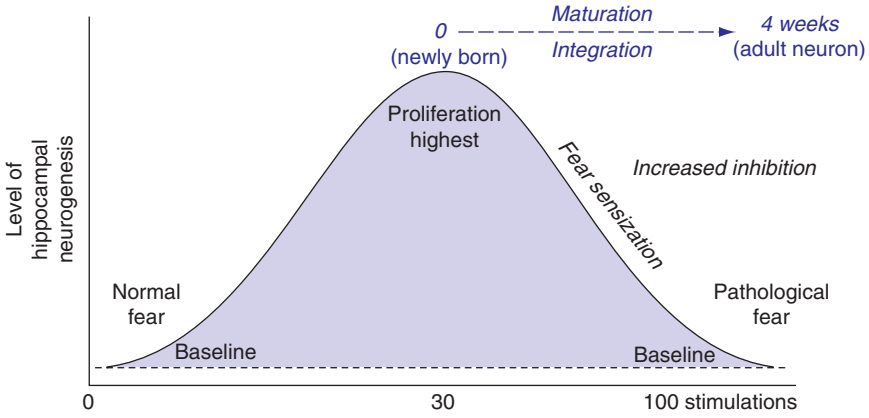


Figure 3 Time course of seizure-induced neurogenesis and interictal behavioral pathology.

them that at least some aspects of seizure-induced neurogenesis might serve to compensate for the increased excitatory activity associated with epilepsy. It is possible that increased inhibition within the dentate gyrus exacerbates kindling-induced emotional behavior during the interictal period. An intriguing idea is that the increased inhibitory drive on newly generated dentate neurons might play a role in this process. This hypothesis is consistent with the possibility suggested above, that some of the compensatory changes in inhibitory activity that serve to maintain a seizure-free state have the secondary consequence of increasing the development of behavioral disturbances.

Future Directions

Effect of Gene Expression on Interictal Anxiety

There has been much interest in examining how seizures lead to changes in gene expression. Despite the extensive use of microarray and proteomic technologies to examine changes in the expression of genes and gene products (proteins) associated with different types of epilepsy disorders, there has been no systematic study to date investigating which gene changes might relate to the development of affective psychopathology in epilepsy. Rats that have been selectively bred to be more or less susceptible to enhanced amygdala excitability and the effects of kindling (e.g., FAST and SLOW kindled rats) display altered levels of anxiety and fear in response to novel environments. These findings suggest that an important topic of future research should be directed at how specific genetic factors influence both seizure susceptibility and the development of behavioral disorders associated with epilepsy.

Effect of Increasing Hippocampal Activity on Interictal Anxiety?

If increased inhibition in the hippocampus leads to the development of interictal anxiety in individuals with TLE, then procedures that are known to increase hippocampal activity and adaptive plasticity might help reduce fearful behavior in kindled rats. We tested this hypothesis recently using procedures such as training on a Morris water maze spatial cognition task or housing in a complex (spatially enriched) environment. Importantly, these two procedures are known to activate and elicit synaptic changes within the hippocampal formation. In both cases, kindled rats exposed to these procedures were much less fearful than kindled rats that were not exposed to the procedures. Although the mechanism(s) responsible for the beneficial effects of hippocampal activation on interictal anxiety is unknown, one intriguing possibility is that these procedures may work by altering the course of hippocampal neurogenesis. We have hypothesized that new cells, born under conditions of continuing seizure activity, may be predisposed to take on abnormal inhibitory characteristics – partly in an attempt to reduce the probability of future seizure activity. However, new cells born under conditions of enrichment or learning may be predisposed to take on normal excitatory characteristics – which could re-set normal emotional responding to aversive stimuli. This idea is speculative, but it does provide a framework for future research aimed at understanding the particular features of adaptive plasticity that ameliorate seizure-induced changes in behavior.

Discovery of More Effective Anticonvulsant Treatments

Most anticonvulsant interventions are administered with the primary aim of preventing the complications of unprovoked seizures by reducing their frequency and/or their severity. However, several clinical studies have revealed that treatment with certain antiepileptic drugs can aggravate interictal anxiety in some patients. These disruptions in normal behavior can have profound effects on the quality of life for the patient, which can overshadow the beneficial effect of the drug on seizure occurrence.

If inhibitory systems are enhanced during chronic epilepsy, then it is possible that certain anticonvulsant drugs, whose mode of action is to potentiate inhibitory transmission and suppress ictogenic events, may exacerbate behavioral disturbances during interictal periods. There has been very limited study of this issue using experimental models of epilepsy. What is known is that administration of the anticonvulsants carbamazepine and valproate can exacerbate interictal defensiveness in spontaneously epileptic cats, whereas treatment with flumazenil (a benzodiazepine antagonist) can decrease interictal defensiveness in partially kindled rats. Further study in this area may help identify pharmacological as well as potential non-pharmacological interventions that simultaneously provide effective seizure relief and decrease the interictal behavioral comorbidities associated with epilepsy.

Conclusions

The study of epilepsy and its psychiatric comorbidites provides an excellent opportunity to explore the relationship between pathological brain activity and behavior. By determining how seizures impact the structural and function organization of the brain, we hope to gain a better understanding of the neural mechanisms that underlie the development of interictal anxiety. The information gained from these studies may help in the development of more effective treatments that can reduce the impact of these disturbances.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Effects of Seizures in Animal Models; **Models**: Kindling: A Phenomenon of Seizure-Induced Plasticity and a Model of Focal and Limbic Epilepsy; Seizure Predisposition and Expression in the Genetic Epilepsies as Exemplified by the Genetically Epilepsy-Prone Rats; **Neurogenesis**: Neurogenesis in the Dentate Gyrus and its Potential Role in Epileptogenesis; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures; Interictal Behavioral Comorbidities in a Model of Epilepsy; Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Interictal Behavioral Comorbidities in a Model of Epilepsy

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Introduction

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completely accepted and is even fervently dismissed by some. Recent studies have concluded that behavioral comorbidities with epilepsy are the rule rather than the exception. But are these behavioral disturbances due to the same altered neural functioning that gives rise to seizures? Are they a consequence of seizures themselves? Or are they due some other unrelated factors? With electrical kindling, we can assess the long-term behavioral consequences of seizures without confounding factors (e.g., heterogeneity of the population, seizure locus and frequency, undetected ictal events, anticonvulsant medications, social stigma) that make such assessments in patients so difficult. For instance, the focus and frequency of seizures, as well as the influence of anticonvulsant medications on behavior, can easily be controlled. Further, the influence of social stigma can be ruled out in kindling studies, which thus can generate neurobiological explanations for interictal behavioral disturbances. This article focuses on the major behavioral consequences of kindling and speaks to the behavioral disturbances in adults with focal (partial) epilepsy. We discuss issues related to the site and hemisphere of stimulation, the time course of changes, and whether kindling produces a functional lesion.

Background

Psychology and neuroscience have a long history of determining the contribution of a specific area of the brain to the generation of behavior. When a behavior is abolished, reduced, or even enhanced following creation of a lesion, this result is interpreted as evidence that the lesioned area was, at least in part, involved in the generation or regulation of that behavior. Although kindling is not associated with a gross lesion, we argue below that it does result in lasting changes in the behaviors or functions subserved by kindled circuits. Furthermore, behavioral capacity or functions can be increased or decreased, depending on whether the circuits are enhanced (perhaps due to potentiation of excitatory transmission) or suppressed (perhaps due to potentiation of inhibitory influences). In either case a functional lesion is created, meaning that normal behavior is disrupted by the seizures.

Emotional Disturbances

It appears that some people with temporal lobe epilepsy do differ from both healthy people and those with chronic non-neurological disorders in their behavioral traits. This difference is suggested by results from several early reports – e.g., by Waxman and Geschwind in 1975, who characterized an interictal behavioral syndrome that they believed was associated with temporal lobe seizures, and by the systematic 1977 Bear–Fedio Inventory – and by more recent studies using modern psychiatric diagnoses. The behavioral differences are primarily in emotional behaviors such as fear, anxiety, and depression, although there is no consensus on the exact nature of the emotional differences. The relation between seizures and depression is particularly intriguing, as depression can precede, and is a risk factor for, epilepsy. Interestingly, electroconvulsive therapy is an effective treatment for some people with chronic depression.

Using kindling in cats, Adamec first showed that kindling produces changes in predation and defensiveness, both measures of interictal emotionality. Domestic cats normally vary in their tendency to be either aggressive (killers) by displaying efficient mouse predation and little fear of humans, or to be defensive (nonkillers) by displaying stereotypic fear responses to rats, humans, and conspecific threat vocalizations. Partial kindling of the amygdala of killer cats converts them to nonkillers and increases their defensive behavior. After partial kindling of the ventral hippocampus, a region that facilitates attack behavior, speed of killing increases and defensiveness decreases in killer cats. These behavioral changes last for several weeks in some cats, and as long as the animals are maintained (4 months) in other cats.

There are a number of interesting correlates of the emotional behavior. Compared to defensive cats, killer cats have higher thresholds for after discharge (AD) in the basolateral amygdala, a region that tonically inhibits attack. The behavioral change after hippocampal kindling is associated with potentiation of the field excitatory postsynaptic potential (f EPSP) in the dentate gyrus. Furthermore, the potentials evoked in the medial hypothalamus by stimulation of the basomedial amygdala are greater in defensive cats than in aggressive cats, whereas potentials evoked in the ventral hippocampus are greater in aggressive cats than in defensive cats.

These findings suggest that baseline differences in behaviors, such as aggressiveness and defensiveness or fearfulness, are associated with preexisting differences in excitability of limbic structures. Furthermore, the changes in interictal behaviors after kindling are associated with lasting changes in the efficacy of synaptic transmission between the structures. Thus, endogenous differences in excitability interact with seizures to determine the behavioral outcome, with aggressive and defensive cats responding differently to partial hippocampal kindling. Adamec has also provided evidence that some of the interictal behavioral changes are related specifically to kindling-induced potentiation (KIP) of amygdaloid circuits in the right hemisphere.

Emotional behavior is also altered in a variety of tasks that measure anxiety and fear following amygdaloid kindling in rats. Kindling of the amygdala exaggerates conditioned fear-potentiated startle responses, increases immobility in a social interaction test, increases defensive fighting induced by corticotrophin releasing factor, and increases stress-induced stomach ulcers. Amygdaloid

kindling also decreases exploratory behavior and decreases the latency to killing mice in rats that are spontaneous mouse-killers. The alterations in interictal emotional behavior produced by amygdaloid kindling appear to be specifically related to changes in fear or anxiety-like behavior, rather than to a general change in motoric activity.

Anxiety-like behavior, measured in the elevated plus maze, is also affected by kindling in rats. In the elevated plus maze, anxiety in rats is characterized by a preference for the closed arms of the maze, avoidance of the open arms, and risk assessment (a behavior in which the rat stretches toward an open arm but keeps its hind quarters anchored in a closed arm) – all of which can be increased after amygdaloid kindling. Amygdaloid-kindled rats will even leap from the plus maze, presumably as an escape response. However, the situation is complicated by the fact that some studies have reported decreased anxiety, or anxiolytic effects, after kindling. Adamec relates this discrepancy to the hemisphere stimulated and the exact placement of the electrode. That is, he finds that anxiolytic effects are generally observed with kindling of the left amygdala, whereas anxiogenic effects are associated with kindling of specific nuclei in the right amygdala, including the ventral and anterior basolateral nuclei. In contrast, Hannesson and Corcoran have found that anxiogenic effects are produced by amygdaloid kindling, regardless of hemisphere stimulated or exact placement of the electrode. Controversies aside, there is general agreement that increased anxiety does not occur with kindling of all sites. For example, kindling of hippocampal field CA1 fails to increase anxiety-like behavior.

Learning and Memory

Over the last several decades, a number of studies have demonstrated specific declines in memory function in people with unilateral temporal lobe epilepsy, although there is some inconsistency in the literature surrounding the deficits associated with nondominant temporal lobe dysfunction. Generally, left temporal lobe epilepsy is associated with deficits in verbal learning and recall, and right temporal lobe epilepsy is associated with spatial and configural deficits. One problem with interpretation is the relatively unknown contribution of the damage or sclerosis seen in these populations versus the neural network reorganization due to the seizures themselves. Kindling provides insight into these issues.

We now have extensive knowledge about the effects of kindling on learning and memory. When considering the results, it is useful to distinguish between paradigms involving emotional conditioning, such as avoidance conditioning or conditioned emotional response, and more cognitively demanding paradigms, such as spatial learning or object recognition memory, as well as the retrograde and anterograde effects of kindling in the various paradigms. Other important variables are the site and extent of kindling, and the interval between kindling and behavioral testing.

Effects on Emotional Conditioning

Retrograde amnesia is the loss of information that had already been learned. Kindled seizures produce retrograde amnesia for aversively motivated tasks learned immediately before the seizure. As McIntyre showed in the earliest work on behavioral consequences of kindling, acquisition of a conditioned emotional response, which involves association of a neutral tone with the aversive consequences of shock, is disrupted when a generalized amygdaloid seizure (but not a focal seizure kindled from anterior neocortex) is triggered 6 s after each conditioning trial. Partial anterior neocortical seizures do not produce retrograde amnesia for passive avoidance, but neocortical seizures that include a generalized limbic-type component do. The amnestic effects of amygdaloid seizures involve the adrenal glands (the effects are abolished by adrenalectomy), as are the state-dependent (dissociative) effects of amygdaloid seizures. Thus, the amnestic effects of kindled seizures may in part be due to the seizures' ability to produce dissociation of memory. The dependence of both the amnestic and the dissociative effects of kindled seizures on the adrenal glands suggests that seizures trigger the release of adrenal hormones (adrenaline, adrenal steroids), which are responsible for both dissociation and the consequent amnesia.

In contrast to retrograde amnesia, anterograde amnesia is a deficit in the ability to form new memories. Significant and long-lasting anterograde effects of amygdaloid kindling are also evident in a variety of paradigms, including conditioned emotional response, passive avoidance, and conditioned taste aversion, in which rats readily learn to avoid a flavor paired with an internal state of malaise. In some of these tasks, disruptive effects have been documented as long as 55 days after kindling. Lesions of the amygdala also produce anterograde effects in the same behavioral tasks, suggesting that kindling the amygdala produces a functional lesion. The kindled amygdala may generate aberrant activity that interferes with subsequent learning; alternatively, kindling modifies plastic synapses in the amygdala and thereby 'uses up' the amygdala's capacity to be further involved in learning. In theory, kindling-induced changes in emotional conditioning could be secondary to nonspecific sensorimotor deficits. However, this possibility is likely not the case, given the selectivity of the effects on specific tasks. For example, amygdaloid kindling disrupts retention of brightness discrimination, but fails to affect acquisition of brightness discrimination or active avoidance.

Effects on Cognitive Learning

Kindling of partial or generalized seizures with stimulation of sites in the hippocampal formation disrupts

retention of spatial learning in the water maze, a form of retrograde amnesia. Deficits are produced by kindling of other sites such as the olfactory bulb or amygdala only when a large number of seizures (e.g., >100) are triggered, which we define as extended kindling. The retrograde effects of hippocampal kindling could be due either to disruption of consolidation or to a failure of retrieval, perhaps related to the state-dependent effects of kindling. They probably are not due to gross sensorimotor deficits, however, because kindled rats can readily escape to a visible platform that protrudes above the water, demonstrating that sensorimotor capacity and motivation to escape remain intact.

Skilled Performance

The ability of people with epilepsy to learn and perform a new motor task has received very limited experimental assessment, and the use of anticonvulsant medications has potentially confounded any interpretation. In a study from Lassonde's laboratory, a specific neuropsychological profile was obtained for children with frontal lobe epilepsy that distinguished them from children with temporal lobe epilepsy and generalized absence epilepsy on several measures that were purported to assess frontal lobe functions. It was shown that children with frontal lobe epilepsy exhibit many characteristics that are commonly associated with frontal lobe dysfunction in adult patients. More specifically, they show impairments in motor coordination, response generation, impulse control, and planning ability. Motor deficits are more marked for tasks involving bimanual coordination and asymmetrical movements, thus confirming the crucial role of the frontal lobes in interhemispheric integration of complex motor programs. In a conceptually related work, the hypothesis that kindling in the sensorimotor region results in a functional lesion in adult rats acquiring a new motor task has been experimentally tested.

Methods

Electrical Kindling

The electrical kindling technique involves the implantation of chronic electrodes targeted at specific brain regions, and the regular (usually daily) elicitation of self-sustaining afterdischarges and seizures by the application of 1 or 2 s of electrical stimulation. Interictal behavioral changes have been well modeled using the electrical kindling technique, with kindled animals showing persistently altered anxiety-like and aggression-related behaviors, changes in spatial memory abilities, and alterations of skilled motor performance – depending on the kindling location and number of seizures. Kindling has an advantage over models that result in spontaneous seizures, because unanticipated seizures can potentially interfere with interictal behavioral assessments.

Behavioral Testing

Spatial learning

The primary cognitive task studied with kindling is spatial learning, in either the water maze or the radial arm maze. The standard water maze involves placing a rat in a large circular swimming pool with milky water hiding a submerged platform. Rats are highly aquatic animals and very good swimmers, but they do try to escape from the cold water and prefer standing on the submerged platform to swimming. The inner walls of the pool are undifferentiated by intra-maze cues, and in order to escape from the water the rat must therefore learn the location of the platform based on extra-maze cues. In the radial arm maze, each arm of the maze is baited with food; the rats must visit each arm once, retrieve the food, and then go to the unvisited arms until all the food has been retrieved (and, ideally, no arms have been revisited). Arms are also distinguished by their position relative to extra-maze cues.

Resistance to capture

The rat's resistance to being picked up was scored on a 7-point scale, with lower values indicating no or low emotional response and higher values indicating more emotionality, such as the rat launching a jump attack at the experimenter's hand. After 100 kindling or sham stimulations of the amygdala, the experimenters measured the rats' resistance to capture, as follows: One day after the final stimulation, each rat was placed in an unfamiliar open field for a few minutes and then forcefully picked up from above by an experimenter who was wearing a large leather glove.

Nictitating membrane conditioning

This paradigm involves establishing conditioned closing of the nictitating membrane (a translucent secondary lid that covers the rabbit's eye) in response to a previously neutral tone, and then establishing a discrimination between a relevant stimulus (CS+) and an irrelevant control stimulus (CS−). After the response has been conditioned to the CS+, a reversal contingency is imposed, in which the rabbit must learn to respond to the CS− and withhold responding to the CS+.

Skilled forelimb behavior

The assessment of skilled forelimb behavior involves training a rat to reach through a narrow slit, and grasp and consume a small food pellet. The rat is normally trained on this task for 10 minutes a day, and it requires about 2 weeks of training before the rat reaches asymptotic levels of performance. The task generates not only an

end point of success (# of successful reaches/# of attempts × 100) but also allows for the determination of *how* the rat reaches with kinematic analysis. Kinematic analysis involves a frame-by-frame analysis of the high-speed recording of the behavior. The reaching behavior is broken down into 10 discrete subcomponents. If each movement appears normal, it is given a score of 0; if it is ambiguous, it is given a score of 0.5; if it is impaired but recognizable, it is given a score of 1; if it is absent or completely unrecognizable, it is given a score of 2.

Recent Results

Emotional Disturbances

To further explore kindling-induced alterations in emotionality, Kalynchuk and colleagues developed a new test of emotionality in rats called 'resistance-to-capture' (see Methods section). As predicted, the kindled rats had substantially higher resistance to capture scores than did the sham-stimulated rats. But the question of whether kindling increases defensiveness or aggressiveness in rats had yet to be addressed. This question was tested in a resident-intruder paradigm. The resident-intruder paradigm was originally developed so that sequences of aggressive and defensive behaviors that commonly occur in the interaction between resident and intruder rats can be differentiated. Kindled or sham-stimulated rat were tested as an intruder with naive resident rats. The kindled rats displayed more active defensive behaviors, such as defensive upright postures and defensive attacks, and fewer aggressive behaviors, such as lateral displays and biting, than did the sham-stimulated rats. Thus the results of this experiment suggest that kindling-induced emotional behavior is primarily defensive in nature. Kalynchuk has shown that increased defensiveness occurs only when the rats are tested in a novel environment (not in a familiar environment), an effect that may be related to the conditioned anticipatory responses to kindling that are environment-specific. Furthermore, she showed that increased defensiveness is specific to kindling of limbic sites. It is most dramatic after amygdaloid kindling, significantly less robust after hippocampal kindling, and marginal after caudate kindling. The increased defensiveness is correlated with increased binding at the benzodiazepine receptor and at a receptor for serotonin in the dentate gyrus, pointing to GABA and serotonin as possible molecular participants in the effect.

The fact that kindling-induced emotionality is defensive rather than aggressive in nature has important implications for people with temporal lobe epilepsy. For decades, it has been assumed, despite the lack of documentation in well-controlled studies, that people with epilepsy are prone to outbursts of aggression. The few appropriate studies that have attempted to document a relation between temporal lobe epilepsy and aggression have been largely unsuccessful. The finding that enhanced emotionality is fundamentally defensive may therefore help to change the common assumption that people with temporal lobe epilepsy are particularly aggressive. Furthermore, the finding that enhanced emotionality is revealed in unfamiliar situations may be useful to people with temporal lobe epilepsy.

Learning and Memory

A hint of the complexity of the neural changes underlying the effects of kindling on even simple learning is found in a research by Robinson and colleagues, indicating that perforant path kindling has a bidirectional effect on conditioning of the nictitating membrane response in rabbits. Kindling facilitates acquisition of the nictitating membrane response but impairs reversal learning. Because the enhanced acquisition resembles the effects of induction of LTP in the perforant path, and the impaired reversal resembles the effects of hippocampal lesions, perforant path kindling produces both enhanced hippocampal processing, and at the same time a functional lesion of the hippocampal formation. Enhancement of conditioned responding in fear-potentiated startle also occurs after partial kindling with amygdaloid stimulation, indicating that enhancement of processing can occur with kindling of several different sites.

By manipulating the conditions of the spatial task, it is possible to distinguish between working memory, a short-term memory store containing information that changes between trials, and reference memory, a long-term memory store containing information that is constant within and between trials. Hippocampal kindling can be shown to disrupt both working and reference memory in the radial arm maze, although the impairment in reference memory appears to be greater. In the water maze, on the other hand, both working and reference memory are impaired by hippocampal kindling, but the impairment in working memory appears to be greater. Hannesson has argued that the critical variable to be considered in explaining these discrepant results may be the overall memory load, rather than the dimension of working or reference memory. In other words, greater demands may be placed on memory in the 'working memory' version of the water task – for example, because it involves retention of multiple platform locations across days. This hypothesis has not yet been tested experimentally.

Kindling of generalized seizures with hippocampal stimulation also impairs acquisition of spatial information, evidence of an anterograde amnesic effect. Prior hippocampal kindling disrupts spatial learning or short-term spatial memory, but not long-term spatial memory, as evidenced by a disruption in a 1-day acquisition paradigm in the water maze but unimpaired retention (after the

kindled rats learn the maze) at 7 and 28 days. In contrast, Hannesson and Corcoran have shown that kindling of generalized seizures with stimulation of the perirhinal cortex fails to affect spatial cognition, but it does (as expected) impair acquisition of an object discrimination task in a modified water maze. Thus, there is considerable anatomical and task-related specificity of the effects of kindling on behavior.

Skilled Performance

Henry and Teskey asked the following questions: Does the kindling of seizures in adult rats impair their ability to perform a new task? When the rats do learn the task, do they perform it in the same manner as nonkindled control rats? The rat sensorimotor neocortex is a ripe target for examining both questions. Within the sensorimotor neocortex there are movement representations, better known as motor maps, that when stimulated result in the elicitation of movements in discrete muscle groups. When these areas are ablated, rats show a reduction in their ability to perform with the affected body musculature. For instance, when a region of sensorimotor neocortex known as the caudal forelimb area is removed, rats show both a lower success level on skilled tasks with the impaired forelimb and an alteration in the expression of the reaching behaviors. Thus, performing a skilled task using the forelimb is dependent on the integrity of the neocortical caudal forelimb area. Henry and Teskey kindled with stimulation of the midline corpus callosum at the level of the caudal forelimb area, and then examined skilled reaching (using the single pellet task, in which rats are required to repeatedly reach through an open vertical slit in a Plexiglas chamber for a single food pellet). Nonkindled rats performed the task as expected. Kindled rats made just as many attempts, but their success at retrieving the food pellets attained asymptotic levels significantly below the success level achieved by nonkindled rats. A detailed kinematic analysis of the reaching behavior made from the videotaped record showed that kindled rats employ an altered strategy for reaching. Normally, rats bring their elbow to midline before extending the forelimb through the vertical slit. However, the kindled rats do not perform this subcomponent of the behavior, and the elbow remains well to the side of the open vertical slit. Thus the kindled rats make more of a 'swipe' at the side of the pellet, rather than trapping the pellet from above as do nonkindled rats. A frequent consequence of the swiping motion is that the rats miss the pellet altogether, resulting in lower success at obtaining the pellet. The kindled rats that do successfully grasp the pellet often drop it as they bring it to their mouths. Instead of placing the palm of its hand so that it faces its mouth, the kindled rat only turn the wrist so that the palm is facing upward at approximately a 135° angle (as opposed to the 180° angle seen in the nonkindled rats). The food pellet thus slips off of the hand as the rat raises it to its mouth. To determine whether the altered motor performance and reduced reaching success in kindled rats generalized to other tasks, Henry also required rats to walk across a horizontal ladder that was missing some randomly removed rungs. Kindled rats make more forelimb faults than do control rats on this task as well.

On the single pellet and ladder walking tasks, therefore, kindled rats behave as if they have a reorganized caudal forelimb area of their sensorimotor neocortex, and indeed we have observed increased numbers of highly efficacious (perforated) synapses in the same region following kindling. Whether the impairment is strictly sensory, motor, or both has not yet been determined. What is clear is that repeated seizures elicited from this region can result in a long-term alteration in motor performance. These intriguing results lead to a novel prediction: People with adult onset epilepsy with foci in their sensory and motor neocortices will show impaired and altered performance when learning new motor skills. Apart from anecdotal evidence of clumsiness, there are few systematic studies that have experimentally investigated the way in which people with epileptic foci in sensory and motor areas perform behaviors requiring skill.

Conclusions and Future Directions

Some of these behavioral effects suggest that function in the stimulated region is enhanced by kindling, whereas other effects on behavior resemble a lesion. Possibly various output pathways from or to target sites of the stimulated area are differentially susceptible to failure or enhancement after kindling. In a general sense, the data suggest that kindling can affect neural circuits or cell assemblies involved in normal behavioral and psychological processes. Furthermore, susceptibility of limbic sites to epileptiform activity is affected by the subject's general behavioral predispositions. This result is precisely what would be expected if kindling represents a pathological distortion of the mechanisms of neural plasticity that participate in the regulation of conventional behavior. In a real sense, epilepsy and susceptibility to kindling are the price the nervous system pays for learning and memory.

An additional and perhaps surprising implication of the findings is that the behavioral effects of kindling are specific to the area stimulated. That is, kindling of virtually identical generalized seizures from different sites – for example, the amygdala and field CA1 of hippocampus – has radically different effects on behavior: Amygdaloid kindling increases anxiety-like behavior but fails to affect spatial cognition, whereas hippocampal kindling disrupts spatial cognition but fails to affect anxiety-like behavior. Thus, the functional effects of kindling are due to local alterations in the area stimulated

rather than to the more widespread consequences of seizures per se. The regional specificity evident in kindling of partial seizures (partial kindling) or a few generalized seizures (full kindling) is lost, however, when a large number of generalized seizures are triggered from various sites – as seen in the extended kindling model. The behavioral consequences of extended kindling can therefore be attributed to the anatomically distributed effects of multiple seizures rather than to local alterations in the area stimulated. The mechanisms of local alterations, as well as the more diffuse changes following multiple generalized seizures remain largely unexplored.

Central neural networks are the most proximate generators of behavior, and therefore a comprehensive understanding of the functional neurobiology of neural networks and their regulation should lead to insights into the generation and execution of behavior. Future experiments exploring interictal behavioral alterations should be performed using a variety of animal epilepsy models. Furthermore, understanding mechanistic differences between fully generalized, secondarily generalized, and focal seizure expression on behavior will aid both our understanding of seizure-induced pathological changes and how to mitigate or reverse their effects. Moreover, how sensory systems change following seizures remains almost completely unexplored and should be investigated more thoroughly.

See also: **Models**: Characteristics and Mechanisms of Kindling; **Plasticity**: Seizure-Induced Alterations in Motor and Sensory Maps; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures; Interictal Anxiety in Temporal Lobe Epilepsy; Norephinephrine: A Molecular Link Between Depression and Epilepsy?.

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Norephinephrine: A Molecular Link Between Depression and Epilepsy?

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Introduction

Epilepsy and Depression Comorbidity

The link between epilepsy and depression was first described by Hippocrates, who noted that a relatively high frequency of epileptic patients experienced 'melancholia,' and that melancholics were prone to develop epilepsy. Since this noteworthy observation, a preponderance of the epilepsy and depression literature reveals that the comorbidity is bidirectional. Epileptic patients are at high risk for depression, and patients with major depression have a higher frequency of epilepsy than the general population. As evidenced by the abundance of published reviews over the past few years, the association between these two

rather than to the more widespread consequences of seizures per se. The regional specificity evident in kindling of partial seizures (partial kindling) or a few generalized seizures (full kindling) is lost, however, when a large number of generalized seizures are triggered from various sites – as seen in the extended kindling model. The behavioral consequences of extended kindling can therefore be attributed to the anatomically distributed effects of multiple seizures rather than to local alterations in the area stimulated. The mechanisms of local alterations, as well as the more diffuse changes following multiple generalized seizures remain largely unexplored.

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Norephinephrine: A Molecular Link Between Depression and Epilepsy?

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Introduction

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diseases, and the challenges encountered in attempting to treat them simultaneously, have recently become topics of great interest. There is some debate about the magnitude of the risk factors for epilepsy and depression comorbidity because of variance in study design and diagnostic criteria. However, most estimates place the risk for epileptic patients developing depression, and for depressed patients developing epilepsy, at four to fivefold higher than the general population. Nearly all reviews caution that depression is severely underdiagnosed and undertreated in the epileptic population. Raising awareness among clinicians of this link, and developing experimental models and therapies by researchers, are critical for the future of treating comorbid individuals.

Many theories have been put forth to explain the comorbidity between epilepsy and depression, including arguments based on neurobiology, genetics, social environment, and drug treatment side effects. It is likely that all these factors contribute to the connection to some extent. Why is the link between these two diseases underrecognized and undertreated clinically? One reason could be that despite the numerous hypotheses concerning the basis for the comorbidity, none have been empirically tested or convincingly demonstrated. Although epidemiological data support comorbidity between epilepsy and depression, other types of experimental data – and particularly the identification of a common underlying mechanism – would substantially reinforce general acceptance of this phenomenon. Because many studies that would be definitive are unethical to perform in humans, animal models appear to be the best experimental alternative to study the biological basis of epilepsy and depression comorbidity. To date, very few animal models designed to test these hypotheses have been described.

Background

Norepinephrine: A Molecular Link between Epilepsy and Depression?

Epilepsy and depression are very complicated neurological disorders, and it is generally accepted that no one single neurotransmitter is responsible for the manifestations of either epilepsy or depression. Of all the candidates that may represent a molecular link between epilepsy and depression, a particularly strong case can be made for norepinephrine (NE) because of the overwhelming evidence of its ability to modulate both seizure susceptibility and mood, as first proposed by Jobe and colleagues.

NE is one of the three most widely recognized neurotransmitters (serotonin [5-HT] and dopamine [DA] being the others) in the etiology and treatment of depression. The evidence for the involvement of NE in depression comes from both human and animal studies, and can be summarized as follows: (1) NE reuptake inhibitors and monoamine oxidase inhibitors (MAOIs) that increase NE levels are among the most widely used and efficacious antidepressant drugs available. (2) Depletion of NE via neurotoxins or dietary restriction leads to recurrence of symptoms of depression in patients in remission. (3) Postmortem studies reveal multiple alterations in the noradrenergic system of depressed individuals, including changes in NE biosynthetic enzymes, the NE transporter (NET), and NE receptors. (4) Reduced NE levels have been detected in the cerebrospinal fluid of depressed individuals. (5) NE dysfunction contributes to aberrant behaviors in animals that in some ways resemble depressive symptoms. (6) NE is critical for the efficacy of antidepressant drugs in some behavioral tests in rodents. (7) Chronic treatment of rats with antidepressant drugs changes the firing properties of locus coeruleus (LC) neurons, the major noradrenergic nucleus in the brain. Therefore, the etiology of depression involves a reduced function of the noradrenergic system, and treatment involves increasing extracellular NE levels. It should be noted, however, that there is also strong evidence for the roles of DA and 5-HT in depression and its treatment, indicating that depression likely involves changes in multiple neurotransmitter systems.

Despite the particular focus of modern epilepsy research on amino acid neurotransmitters (i.e., GABA, glutamate) and ion channels, NE is a very potent endogenous anticonvulsant neurotransmitter in the brain. It is important to note that the function of NE in epilepsy appears to be of a modulatory nature, since a loss of NE function does not result in spontaneous seizures. Therefore, the evidence demonstrating a relationship between NE and epilepsy is not as overwhelming as that between NE and depression. The strongest evidence supporting an involvement of NE in epilepsy is the following: (1) A reduction in central NE levels, induced either by neurotoxins or genetic manipulation, increases susceptibility of animals to a large variety of convulsant agents. (2) Stimulation of LC neurons, and the subsequent release of NE at their terminals, reduces susceptibility of animals to convulsant agents. (3) LC noradrenergic neurons are activated when a seizure occurs. (4) NE is required for the efficacy of some antiepileptic therapies. (5) Noradrenergic receptor agonists can suppress seizures. A common link between epilepsy and depression, therefore, may be reduced noradrenergic function.

Animal Models of Epilepsy and Depression Comorbidity

Perhaps the best-characterized model of reduced noradrenergic function contributing to the comorbidity of epilepsy and depression is the genetically epilepsy-prone rat (GEPR). These rats were selectively bred for susceptibility to audiogenic seizures, but they have also been

reported to display behavioral responses related to depression, such as increased immobility in the forced swim test (FST), and decreased saccharin consumption that is possibly indicative of anhedonia. Importantly for the subject of this article, noradrenergic dysfunction appears to be at least partially responsible for the seizure phenotype of these rats, and antidepressant drugs that block the NET and increase extracellular NE prevent seizures in GEPRs. Thus, a noradrenergic deficit in NE may underlie both the susceptibility to seizures and depression-like behaviors in this model. A critical test of this hypothesis would be to determine whether drugs that augment NE signaling could attenuate the depressive-like behaviors as well as the seizure susceptibility.

Methods

Rat Model of Depression-like Phenotypes

We have recently developed a new animal model of epilepsy and depression comorbidity. With the idea of expanding upon the available rodent models of depression, Weiss and colleagues selectively bred normal Sprague-Dawley rats for several phenotypes relevant to human depression. These lines include: Swim-Lo active (SwLo), with low motor activity in a Porsolt forced swim test (FST); Swim-Hi active (SwHi), with high motor activity in the FST; Swim-test susceptible (SUS), which are highly susceptible to having their swim-test behavior disrupted by mild stress; Swim-test resistant (RES), which are highly resistant to having their swim-test behavior disrupted by mild stress; Monitor Hyperactive (HYPER), which experience long-lasting hyperactivity following stress; and Monitor Resistant (MON RES), which are resistant to changes in activity following stress. Randomly bred (nonselected; NS) rats served as controls. To determine whether genetic susceptibility to depression-like phenotypes also influences limbic seizure susceptibility, adult rats of each of the six selectively bred lines, and NS controls, were tested for maximal seizure severity after administration of kainic acid ($10\,mg\,kg^{-1}$, then $5\,mg\,kg^{-1}\,h^{-1}$ until tonic–clonic seizure).

Dopamine β-hydroxylase Knockout (*Dbh*$^{-}$<?tfqs-$^{/-}$<?tfqs-) Mouse

The previously described models used genetically susceptible animals, and we wondered whether an environmentally-induced depressive-like state could also enhance seizure susceptibility. Specifically, we tested whether mice subjected to a chronic mild stress (CMS) paradigm would be more susceptible to kainic acid-induced seizures. The CMS paradigm uses a random series of multiple daily unpredictable mild stressors (e.g., forced swim, food or water deprivation, physical restraint, exposure to white noise, reversed light/dark cycle, etc) to elicit a depressive state. At the same time, we investigated further the possibility that a reduction in NE may be a potential link between epilepsy and depression by utilizing a mouse model of NE deficiency, the dopamine β-hydroxylase knockout ($Dbh^{-/-}$) mouse. DBH converts Dopamine to NE in noradrenergic cells; thus, $Dbh^{-/-}$ mice completely lack NE. We have previously shown that $Dbh^{-/-}$ mice are hypersensitive to many seizure-inducing agents. We predicted that $Dbh^{-/-}$ mice might be particularly prone to changes in seizure susceptibility following CMS exposure. Adult male $Dbh^{-/-}$ and control ($Dbh^{+/-}$) mice that have normal NE content were exposed to 5 weeks of CMS. Following 5 weeks of CMS, the mice were assessed for seizure severity following KA administration. Mice were injected with KA ($20\,mg\,kg^{-1}$, i.p.) and observed for one hour. During this hour, seizure behavior was scored using a modified version of the Racine scale: 1 = staring, 2 = heading nodding, 3 = forelimb clonus, 4 = rearing and falling, 5 = generalized clonic–tonic (CT) seizure, 6 = death.

Recent Results

Seizure-Related Mortality in Rats Bred for Depression-like Phenotypes

The strains selected for susceptibility to depression-like phenotypes (SwLo, SUS, and HYPER) had significantly higher mortality following kainic acid seizures than the strains selected for resistance to depression-like phenotypes (SwHi, RES, and MON RES) (**Figure 1(a)**). Non-selected control rats fell roughly in between the seizure susceptible and seizure resistant strains. The dose of kainic acid required to produce a CT seizure was equivalent between each pair of strains, except for the SUS rats, for which the dose was slightly but significantly increased (**Figure 1(b)**). However, the higher CT threshold dose probably cannot account for the increased mortality of the SUS rats, because dose and mortality were not significantly correlated across all lines. For example, some NS control rats that received a cumulative dose of $20\,mg\,kg^{-1}$ survived, while others that received $15\,mg\,kg^{-1}$ or even as little as $10\,mg\,kg^{-1}$, died; similar patterns were also observed among the other strains. The kainic acid seizure-induced mortality phenotype was evident across multiple generations of these rat lines, indicating that the depression and seizure mortality phenotypes are co-inherited and may be controlled by the same genes. In addition, the seizure mortality phenotype is unlikely to derive from a brain-wide change in excitability, as there were minimal strain differences in susceptibility to flurothyl, which induces generalized seizures that are likely cortical in origin (**Figure 2**). These results demonstrate that rats selectively bred for susceptibility or resistance

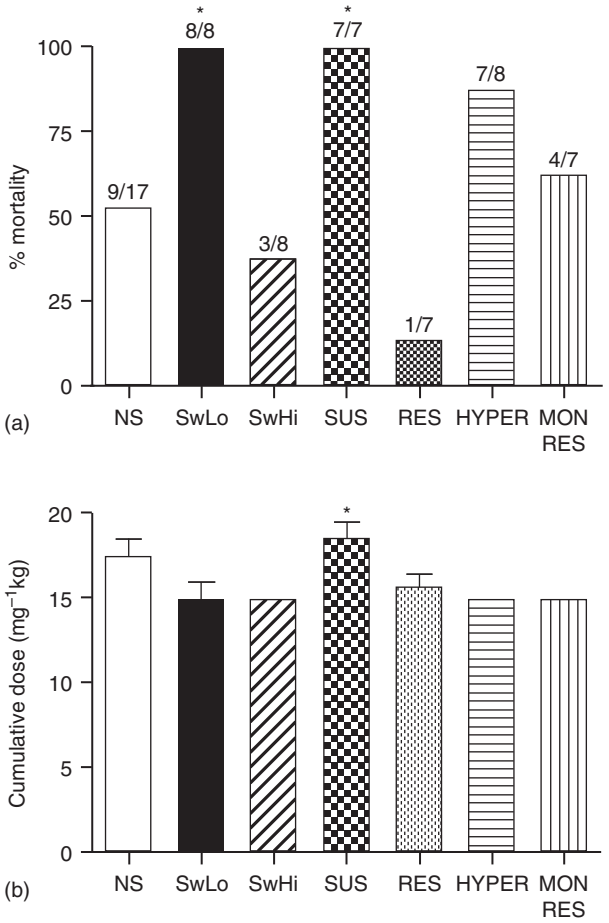


Figure 1 Kainic acid-induced seizure mortality. Kainic acid was administered to rats at a dose of 10 mg kg^{-1}, followed by 5 mg kg^{-1} booster injections until a clonic–tonic (CT) seizure was produced. Shown is (a) mortality, with the number of animals that died over the total tested above each bar, and (b) the cumulative kainic acid dose (mean ± SEM) required to produce a CT seizure. *$P < 0.05$ for the depression-susceptible line compared to its depression-resistant counterpart (SwLo vs. SwHi, SUS vs. RES, HYPER vs. MON RES, respectively). Reproduced from Tabb KD, Boss-Williams KA, Weiss JM, and Weinshenker D (2007) Rats bred for susceptibility to depression-like phenotypes have higher kainic acid-induced seizure mortality than their depression-resistant counterparts. *Epilepsy Research* 74: 140–146, with permission from Elsevier.

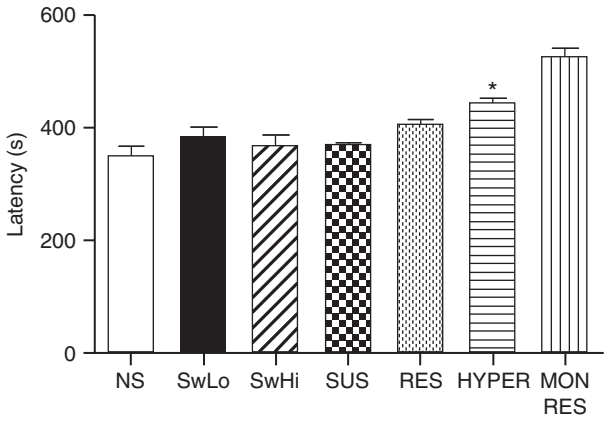


Figure 2 Flurothyl-induced seizure susceptibility. Flurothyl was administered to rats at a rate of 20 µl min^{-1}, and latency to CT seizure was observed. Shown is latency to CT seizure (mean ± SEM) for each line. $N = 6$–16 per group. *$P < 0.05$ for the depression-susceptible line compared to its depression-resistant counterpart (SwLo vs. SwHi, SUS vs. RES, HYPER vs. MON RES, respectively). Reproduced from Tabb KD, Boss-Williams KA, Weiss JM, and Weinshenker D (2007) Rats bred for susceptibility to depression-like phenotypes have higher kainic acid-induced seizure mortality than their depression-resistant counterparts. *Epilepsy Research* 74: 140–146, with permission from Elsevier.

to depression-like phenotypes are also susceptible or resistant, respectively, to kainic acid-induced seizure mortality. Consistent with this result, and perhaps more directly related to the issue of linking depression with seizure susceptibility, we recently completed a pilot study and found that SwLo rats have a shorter latency to CT seizure following administration of the muscarinic convulsant agent pilocarpine than SwHi rats (unpublished data).

Our model possesses two other salient features that are particularly relevant to the topic at hand. First, genetic load is a critical component of both epilepsy and depression, and a family history of depression is itself a risk factor for developing epilepsy. The selective breeding approach embodies a strong genetic component, because we used rats selectively bred for more than 30 generations for susceptibility or resistance to behaviors associated with human depression. Of particular note, all three lines bred for depression-relevant behavioral phenotypes were also susceptible to kainic acid-induced seizure mortality (compared to their counterparts that are resistant to these phenotypes), which suggests the lines may share genetic changes that contribute to the phenotypes. Furthermore, both the depression-like and seizure-related mortality phenotypes were stable over multiple generations, indicating co-heritability. Second, the incidence of major depression is particularly high in patients with temporal lobe epilepsy (TLE), ranging from ~20% to 70%. We found that our rat lines differed in response to kainic acid and pilocarpine, which are commonly used to model TLE, but not to any seizure phenotype related to flurothyl, which produces generalized seizures.

Strikingly similar to the GEPR strains, the SwLo and SUS rats have lower NE levels than their SwHi and RES counterparts, particularly in the hippocampus, and the depression-related phenotypes of both SwLo and SUS rats can be reversed by treatment with noradrenergic antidepressant drugs. The low NE levels in SwLo and SUS rats suggested to us that the reduction of NE in the depression-sensitive lines might underlie both the swim

test and kainic acid phenotypes, and provide a potential mechanistic link between epilepsy and depression. However, initial attempts to attenuate kainic acid-induced seizure mortality in the depression-sensitive lines with these antidepressant drugs have been unsuccessful (unpublished data). Further experiments will be required to fully test this hypothesis.

CMS in Dopamine β-hydroxylase Knockout Mice

As shown in Figure 3, two-way ANOVA revealed that KA-induced seizure severity was generally higher in $Dbh^{-/-}$ mice (main effect of genotype; F [1,41] = 6.7, $P = 0.01$), as we have previously shown. However, CMS did not increase seizure severity in mice of either genotype. Thus, the 'depression' induced by CMS in normal or NE-deficient mice does not appear to render them more seizure-sensitive, at least using the KA model. However, some caveats should be noted. While the mice were exposed to a CMS paradigm previously shown to elicit depressive-like behaviors, we did not conduct such measures in these experiments. Thus, it is possible that our CMS conditions were not severe enough to result in a depressive state and an increase in seizure susceptibility. In addition, KA-induced seizure severity is already quite high in unstressed $Dbh^{-/-}$ mice, so there may have been a 'ceiling effect.' Modified studies of this kind may provide further insight.

Treatments for Epilepsy and Depression Comorbidity

Many cases of depression are successfully treated with pharmacotherapy (antidepressants such as 5-HT and/or NE reuptake inhibitors). Similarly, many individuals with epilepsy are successfully treated with anticonvulsants (e.g., valproic acid, phenytoin, lamotrigine, or gabapentin). However, it is unclear how best to treat individuals suffering from both diseases simultaneously. This issue is particularly important and complex because some antidepressant drugs (e.g., bupropion) are reported to increase seizure risk, and some anticonvulsant drugs (e.g., phenobarbital) can precipitate depressive episodes.

Both NE and 5-HT have antidepressant as well as anticonvulsant properties, and it has been suggested that increasing the signaling within these neurotransmitter pathways could be useful for the treatment of comorbid individuals. We set out to test this idea by administering antidepressant drugs to mice, either acutely (single i.p. injection) or chronically (3 weeks via osmotic minipump) and assessing susceptibility to seizures induced by flurothyl. Importantly, our chronic administration paradigm was as therapeutically relevant as possible, taking into account both the delay between the start of administration and the antidepressant effect, and doses that produce serum drug levels comparable to those seen clinically. Surprisingly, we found that many of the antidepressant drugs tested had a bidirectional effect on flurothyl seizures; i.e., they were both proconvulsant (reduced the latency to seizure) and anticonvulsant (reduced seizure severity). This was particularly true of the selective NE reuptake inhibitors, and was confirmed using NET knockout mice. Interestingly, the dual 5-HT–NE reuptake inhibitor, venlafaxine, was the exception; it had no proconvulsant effect on seizure latency while retaining some of the anticonvulsant effect on seizure severity.

Our results suggest that, contrary to our hypothesis, pure NE reuptake inhibitor antidepressant drugs may not be the best choice for treating individuals with concurrent epilepsy and depression. One caveat to our experiments is that we tested these drugs only on flurothyl-induced seizures, which model generalized epilepsy. Given the prevalence of depression in TLE patients and the results with our selectively bred rats, it will be important to repeat these experiments using a limbic seizure model such as kainic acid.

What are some good candidates for epilepsy and depression pharmacotherapy? Our results in mice indicate that venlafaxine warrants further investigation. Other serotonergic drugs may also be of value. Although this article focuses on NE, there is growing evidence that changes in 5-HT could contribute to epilepsy and depression comorbidity. The involvement of 5-HT in depression is well established, and mice lacking the 5-HT2C receptor or the 5-HT7 receptor have increased seizure sensitivity. Furthermore, early life KA-induced seizures in rats lead to increased depressive-like behavior in adulthood, and evidence suggests an involvement of the 5-HT5B receptor in this process. Finally, it was recently shown that 5-HT1A receptor abundance in the brain is reduced

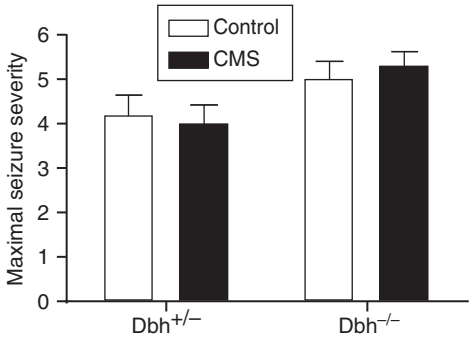


Figure 3 Effects of chronic mild stress on kainic acid-induced seizures in $Dbh^{+/-}$ and $Dbh^{-/-}$ mice. Following 5 weeks of CMS, mice were injected with kainic acid (20 mg kg^{-1}, i.p.). Shown is maximal seizure severity (1 = staring, 2 = heading nodding, 3 = forelimb clonus, 4 = rearing and falling, 5 = generalized CT seizure, 6 = death). $N = 10$–14 per group.

in TLE patients, and this reduction was significantly more pronounced in TLE patients with comorbid major depression.

The anticonvulsant drug lamotrigine has shown some promise in early trials to treat this comorbidity. In addition to its known therapeutic effect on seizures, it also appears to improve mood in epileptic individuals with depression. In initial studies, there was no correlation between improvement on mood and seizure frequency, suggesting that the effects of lamotrigine on depressive symptoms were not a secondary consequence of its anticonvulsant activity. Other intriguing possibilities include nondrug therapies such as the ketogenic diet and vagus nerve stimulation. Both of these treatments were originally developed for treating epilepsy, and later found to have a positive effect on mood. Interestingly, both the ketogenic diet and vagus nerve stimulation increase extracellular NE in the hippocampus and require NE for their anticonvulsant efficacy in animals, while lamotrigine inhibits monoamine reuptake in vitro. It remains to be seen whether the mood-enhancing properties of lamotrigine treatment also involves NE.

Conclusions and Directions for Future Research

Despite the prevalence of depression in epilepsy and the mounting interest in the subject, the neurobiological mechanisms underlying the comorbidity remain unknown. NE and other monoamines remain the best candidates for a common mechanism, although the relationship tying epilepsy and depression appears to be complicated. The role of NE and 5-HT in depression is very well documented, but detailed studies of potential changes in these transmitter systems in epileptic and comorbid individuals are needed. Further investigation of animal models, such as the GEPR rats and the Weiss selectively-bred lines, may yield clues that could evolve into new therapies for treating individuals suffering from both diseases. Treatments aimed at these neurotransmitters should be carefully considered.

Acknowledgements

We thank S. Dutton for assistance with the CMS experiments.

See also: **Cognitive Dysfunction and Other Comorbidities**: Behavioral and Cognitive Comorbidities; Cognitive Effects of Seizures in Animal Models; **Neurotransmitter Function**: Role of the Noradrenergic System in Seizure Regulation; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures; Interictal Anxiety in Temporal Lobe Epilepsy; Interictal Behavioral Comorbidities in a Model of Epilepsy.

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PYRAMIDAL CELLS

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Intrinsic Mechanisms Contributing to Hyperexcitability of Human Epileptogenic Cortex

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Introduction

The fruitful concept of a disturbed balance of synaptic excitation and inhibition underlying epileptiform activity, as opposed to alterations in intrinsic neuronal mechanisms, tacitly implies a view of synaptic processes and the voltage-dependent channels of a given neuron as distinct independent entities. Undoubtedly, either an increase of excitatory glutamatergic transmission or decrease of inhibitory GABAergic transmission has profound effects on the activity of arrays of interconnected neurons. Likewise, increases of neuronal conductances promoting depolarizations (Na^+, Ca^{2+}) or decreases of conductances opposing depolarizations (K^+) can decisively alter neuronal responses to a given input. The resulting increase of the neuron's output, in turn, increases excitatory activity of networks of neurons. Accordingly, the role of altered function of sodium or potassium channels in hyperexcitability – i.e., alterations of intrinsic neuronal mechanisms – has been discussed in several reviews.

The presence of inhibitory interneurons, however, complicates the simple extrapolation of a given alteration of a certain channel to neuronal activity in a network of neurons. A given change, for instance a functional decrease of a certain potassium channel, would have different effects on the activity at the network level depending on which type of neuron (excitatory pyramidal neuron or inhibitory interneuron) is affected. The chances that both types of neurons are affected by a given channel mutation are very small. Studies of the hippocampus, for example, have provided evidence indicating that subpopulations of neurons are endowed with molecularly distinct complements of channel subtypes (which in turn determine the discharge properties of different neuronal populations).

In the neocortex, three main types of neurons can be distinguished on the basis of firing behavior: regularly firing neurons, fast spiking neurons, and intrinsic bursting neurons. The different firing patterns may depend, to some extent, on the different morphologies of these neurons, but probably reflect different channel properties governed by the expression pattern of molecularly distinct channels. However, little is known about the molecular basis of these different firing patterns in the neocortex.

Regularly firing neurons typically generate, on injections of sufficiently large currents, a train of action potentials (APs). At low to intermediate current injections, the interspike intervals are typically about 200 ms and those intervals decrease progressively with further depolarization. These neurons have been morphologically characterized and mainly consist of pyramidal neurons. Synaptic stimulation elicits, in these neurons, a typical sequence of an excitatory postsynaptic potential (EPSP) followed by two inhibitory components. Typically, at high-stimulus intensities the EPSP reaches threshold for eliciting a single AP. Apparently regularly firing neurons often exhibit an initial deviation from regularity, at certain current injection amplitudes, the interval between the first two APs is much shorter than the intervals between subsequent APs, probably due to the presence of a transient low threshold Ca^{2+} current (T-current), which gives rise to an extra AP at short latency after the first one.

Fast spiking neurons have been shown to fire in high-frequency trains of APs, with regular interspike intervals on the order of 10 ms. This firing pattern is, for instance, obtained from aspiny stellate neurons – i.e., GABAergic interneurons. Understanding the basic function of GABAergic interneurons is complicated by the diversity

of morphologically distinct interneuron types, each of which has distinct targets on the soma and basal and apical dendrites of pyramidal neurons. In our experimental condition, such neurons are rarely encountered with sharp microelectrodes, thus precluding a detailed analysis.

Intrinsic bursting neurons generate, on moderate depolarizing current injections, a stereotype response consisting of a burst of APs superimposed on a slow depolarizing component followed usually by solitary APs. In these neurons, small excitatory synaptic responses can recruit a very similar burst response in a voltage-dependent manner. The bursting neurons are pyramidal-shaped, with a rich arborization of axon collaterals. This fact, together with the powerful amplification provided by the burst mechanism, is thought to facilitate synchronization of large arrays of follower cells. The currents underlying this intrinsic bursting behavior are in contention. Originally, the bursting of cortical neurons was attributed to the presence of a T-current. However, the persistence of the slow component with depolarization, which should inactivate T-currents, and the enhancement of bursting during reduction of extracellular Ca^{2+} concentration or antagonism of Ca^{2+} currents suggest the involvement of other mechanisms. A tetrodotoxin (TTX)-resistant sodium current had been proposed to generate the slow component of bursting neurons. Other studies have provided evidence indicating TTX-sensitive sodium currents to underlie bursting. Interestingly, the voltage- and time-dependent recovery from inactivation of bursts differs markedly from the T-current. The inactivation time constant is near 100 ms, which would therefore facilitate synchronization of large arrays of neurons up to about 10 Hz. Most investigators agree that these data suggest a key role of bursting neurons in synchronization of larger arrays of neurons, and therefore their critical role in epileptic discharge. The observation that extracellular Ca^{2+} concentration decreases before interictal-like activity emerges (in penicillin foci of rat cortex in vivo) would be consistent with the view that Ca^{2+}-dependent mechanisms limit the excitability of bursting neurons. Also, the reduction of the spectral power in the α-range by carbamazepine suggests that a sodium current (capable of synchronizing arrays of neurons) is attenuated by this drug.

It is important to point out, that the three categories of neurons mentioned earlier are an oversimplification. Nevertheless, intrinsic neuronal properties (governed by neuron-specific sets of voltage-dependent conductances) are modulated by various transmitters, and the effects of transmitters are in turn modulated by voltage-dependent conductances. These various mutual interactions between synaptic transmission and intrinsic properties are further complicated by the diversity of channels expressed in subtypes of neurons and by their subcellular distribution.

The classical interaction between synaptic potentials and intrinsic mechanisms is in the depolarization of the neuronal membrane by excitatory synaptic potentials – which eventually triggers the generation of APs. In the neocortex, synaptic potentials of regular firing neurons are considerably augmented in the subthreshold range (the time integral is about fourfold increased) by both a persistent sodium current and a T-current. Obviously, this modulation represents a voltage-dependent activation of intrinsic currents, but it also can be regarded as a modulation of the synaptic potential by intrinsic currents. The presence of hyperpolarization-activated cation current (I_H) and inwardly rectifying potassium currents (K_{ir}) attenuates synaptic potentials arriving in the soma. These modulations of synaptic potentials by intrinsic currents are complemented by a modulation of intrinsic currents by neurotransmitters. Depending on the channel being modulated and level considered, both decreases and increases of excitability occur. The modulation of Ca^{2+} currents by, e.g. GABA, would tend to decrease excitability in synaptic terminals, and the resulting depression of GABA release would increase excitability of the network. In contrast, activation of muscarinic receptors increases neuronal excitability, brought about by a reduction of the M-current (mediated by the family of KCNQ (K_V7) channels), which normally provides a conductance increase during depolarizations that attenuates the excitability. These few examples illustrate that intrinsic neuronal properties modulate synaptic input as well as being subject to a modulation by neurotransmitters.

To delineate the mechanisms contributing to excess activity in the human epileptogenic cortex, it is important to consider the various facets affecting the excitability of different types of neurons – and their interplay.

Background

Molecular Biology of Sodium Channels

Voltage-gated sodium channels are ubiquitously expressed in excitable cells such as neurons and muscle cells. Functional sodium channels of central neurons consist typically of an α subunit forming the pore and an auxiliary β subunit. A single α subunit contains four homologous domains, each consisting of six transmembranal helices. The sodium channels have various modulatory sites for phosphorylation by the protein kinases A and C (PKA, PKC) and binding sites for toxins (e.g., TTX). Sodium channels have been cloned, revealing a family of nine members, which have been termed $Na_V(1.1–1.9)$. The channels made of $Na_V1.1$, $Na_V1.2$, and $Na_V1.3$ subunits are widely expressed in the central nervous system. The possible combination of $Na_V1.1$ and $Na_V1.2$ with four types of β subunits, and $Na_V1.3$ combining only with $\beta1$ or $\beta3$, yields a rich diversity of functionally distinct sodium channels. Although present throughout the brain, the $Na_V1.1$ and $Na_V1.2$ subunit are not homogeneously distributed. Interestingly, the cardiac

sodium channel $Na_V1.5$ subunit has been detected in limbic areas of the rat brain. Similar to the $Na_V1.8$ and $Na_V1.9$ subunits, the $Na_V1.5$ subunit is fairly insensitive to TTX.

Sodium Currents and Epilepsy

About 10 mutations of sodium channels are known and some of them cause augmentation of the current, e.g., by a slowed inactivation. Mutation of $Na_v1.2$ for instance causes a slowed inactivation and an increased persistent current when expressed in *Xenopus* oocytes. This increase of persistent sodium current would augment the duration of depolarization produced by these currents, facilitating the generation of higher frequencies of APs via the fast transient sodium currents. Transgenic mice expressing this mutated channel exhibit focal seizure activity in the hippocampus, generalizing to the cortex. Recent evidence suggests that mutations in the β1 subunit of sodium channels may be crucial for the phenotype of generalized epilepsy with febrile seizures, and also play an important role in reduced channel responsiveness to the modulation by the anticonvulsant phenytoin.

As pointed out in a recent review, a mutation of a sodium channel subunit $Na_V1.1$ causes a decreased probability for channel activity in the inherited epilepsy, generalized epilepsy with febrile seizures plus (GEFS+). This effect is, at first glance, counterintuitive. It is due, however, to the dominance of this channel in interneurons; the reduced excitability of interneurons would tend to decrease the synaptic release of GABA. This effect illustrates how difficult it is to predict the functional consequences of a given channel mutation on the excitability of arrays of neurons, without detailed knowledge concerning the population of neurons affected.

Potassium Channels

Potassium channels are unquestionably the most numerous and diverse ion channels in mammals. To date, 70 different gene loci have been identified encoding potassium channels. The majority of potassium channels (40 genes) are members of the family of voltage-gated channels. The extraordinary complexity of potassium channels is obvious not only from the number of genes encoding them, but also from their structural diversity containing 2, 4, 6, or 7 transmembrane domains. Even the family of closely related K_V1–6 and K_V8,9 subunits exhibits an astounding variability. Each gene encodes a single subunit, and functional channels are homomeric or heterotetrameric assemblies of various subunits. Furthermore, functional channels are associated with modulatory subunits and accessory proteins. Finally, these channel subunit proteins are subject to posttranslational modifications. This potential for a huge stoichiometric variability of channels composed of K_v1–6 subunits presents an important safety factor for the control of neuronal excitability, despite possible adverse mutations. This speculation is based on the assumption that most moderately adverse mutations would impair a heteromeric assembly of channels only by a fraction compared with a monomeric assembly (if the function is some kind of 'average' of the subunits' function). With heteromeric assembly, a complete loss of a given subunit would perhaps be less devastating, since in a heteromeric channel, a missing subunit might be replaced by another subunit. Thus, the effect of the mutation would likely be much smaller than would be the case if the mutation resulted in a loss of a complete set of monomeric channels.

A detailed discussion of potassium channels is beyond the scope and space of this article. Therefore, I just briefly mention one example from another family of K_v channels, with a clear relevance to epilepsy. The mutations underlying benign familial neonatal convulsions (BFNCs) were mapped to chromosome 20 and 8, and demonstrated to encode the KCNQ2 and KCNQ3 ($K_V7.2$, $K_V7.3$) channels. Estimates suggest that the mutations associated with BFNCs reduce the total current through $K_V7.2/3$ channels by about 30%. The question of whether other types of epilepsy are also related to impaired M-currents remain unanswered.

A compelling line of evidence indicates that $K_V7.2/3$ channels are the substrate of the M-current, which tends to decrease excitability. Activation of muscarinic receptors reduces the M-current via G-proteins (Gq/11) thus sufficient activation of muscarinic receptors reveals the maximel excitability governed by the other conductances. Interestingly, the muscarinic receptors have been associated with cognitive functions, perhaps by a modulation of M-currents.

Among the voltage-gated channels, the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels mediating the I_H have recently attracted considerable interest. The HCN-channels belong to the superfamily of channels containing six transmembranal helices. Functional channels are homo- or heterotetramers of subunits encoded by four genes (HCN1–HCN4) and allow the flow of both K^+ as well as Na^+ ions to a different degree. The stoichiometric composition of the channels determines the activation kinetics, voltage dependence and their sensitivity to cyclic nucleotides (e.g., cAMP).

In cortical neurons, I_H has several functions, including a stabilization of membrane potential and modulation of temporal summation of synaptic input. The impact of this current on neuronal excitability has long been underestimated. Judging from the ~100% increase in resting membrane resistance in the presence of the established channel antagonist ZD7288, the conductance mediated by I_H channels represents about 50% of the resting conductance of cortical neurons. Interestingly, such a blockade also reveals intrinsic burst firing in previously apparently

regular firing pyramidal neurons. The role of I_H in epileptic disorders is currently the focus of a considerable debate. Some authors suggest that increased I_H contributes to hyperexcitability. Evidence from three laboratories, however, strongly suggests that I_H function is decreased in epileptic disorders. In the kainate model of epilepsy, a decrease of I_H was observed in entorhinal cortex neurons. In a genetically epilepsy-prone rat (WAG/Rij), I_H is also greatly reduced in the neocortex, associated with a decreased expression of HCN1 subunits. The augmented temporal summation, brought about by a reduced I_H, may account for the rapid spread of focal activity in the WAG/Rij rats. In addition, HCN2 subunit knock-out mice exhibit seizure activity.

Methods

The methods for tissue handling, slice preparation, and intracellular recordings with sharp microelectrodes have been described previously and are discussed elsewhere. In brief, human tissue was collected in the operating theatre and transferred to the laboratory. The tissue was cut into slices (nominal thickness, 400 μm), which were stored in beakers containing artificial cerebrospinal fluid (ACSF) equilibrated with carbogen. Individual slices were transferred to submerged-type recording chambers. Intracellular recordings were carried out with sharp microelectrodes (about 100 MΩ, containing 1 M K-acetate plus 1 mM KCl, pH 7.2) connected to a current-clamp amplifier (SEC10L, npi, Tamm, Germany). The signals were fed to a PC-based acquisition system (Digidata 1200, p-clamp 9, Axon Instruments).

Some slices were mounted in a submerged-type chamber attached to an upright microscope (Axioskop FS, Zeiss, Oberkochen, Germany) equipped with an IR camera (Hamamatsu, Herrsching, Germany). Patch-clamp recordings were carried out on visually identified pyramidal-shaped neurons using a conventional patch-clamp amplifier (EPC9, HEKA, Lambrecht, Germany). Electrodes suitable for patch clamping were made on a puller (DMZ Universal Puller, Zeitz, Munich, Germany). Neuron recordings were accepted if membrane potential exceeded −60 mV and AP amplitude 70 mV. For voltage-clamp recordings, series resistance was below 20 MΩ. Hyperpolarizing command steps (10 mV increments, down to −140 mV) were applied from a holding potential of −60 mV to elicit hyperpolarization-activated inward currents (I_H and K_{ir}). To quantify the magnitude and kinetics of I_H, we employed a modification of the established back-extrapolation method, whereby a triple exponential function is fitted to the current trace to yield estimates of the K_{ir} and the two I_H components. Direct measurement of the I_H amplitude is not possible because the onset of the I_H is contaminated by the concurrent instantaneous K_{ir} component and the capacitive transient. Standard leak subtraction protocols cannot be used either, because leak subtraction requires an ohmic behavior, which is not the case with K_{ir} and I_H present.

Recent Results

KCNQ Channels in Human Neocortical Neurons

As mentioned in the Introduction, pyramidal neurons of the cortex usually exhibit a regular firing behavior. The phasic firing pattern, typically associated with the presence of M-currents, is rare. Nevertheless, we evaluated a possible involvement of KCNQ channels in the altered excitability of the resected human epileptogenic tissues. To this end, we began characterizing the effects of the unselective muscarinic agonist, carbachol. Neuronal excitability was tested with a family of current injections, typically from −0.5 to 1 nA. The number of APs were counted and plotted versus the injected current magnitude. At current levels above threshold for initiating AP discharge, the number of APs increased fairly linearly with increases in current amplitude (equivalent to the steep part of *F–I* curves), and then deviated from linearity with a reduced increase of the number of APs with further increases of current. The initial part of this curve was approximated by a linear fit, yielding two parameters: the slope of the curve relating APs versus injected current (firing–current relation); and the extrapolated intercept of the curve – i.e., the current that is just below threshold (rheobase). Human cortical neurons had markedly lower rheobase values than did rat cortical neurons, despite comparable resting membrane potentials; that is, human neurons require less current injection to reach threshold. This approach revealed, moreover, a slightly steeper slope in human neurons (human, 36 APs per nA; rat, 26 APs per nA; 600 ms pulses); that is, the human neurons responded with more APs to a given current injections. This difference in slope between rat and human cortical neurons might be due to the attenuation of M-current. Application of carbachol (10 μM) increased the slope in both rat and human neurons. However, the slope of the firing–current relation increased in rat neurons by about 90% (to: 49 APs per nA), but in human neurons it increased by only about 10% (to 44 APs per nA). Thus, the average values obtained from human and rat cortical neurons in the presence of carbachol were indistinguishable. Although these data are from a small sample of neurons (human, $n = 10$; rat, $n = 5$), they indicate that the initial difference in slopes may relate to differences in the M-current. Co-application of pirenzepine (a presumed M_1-type receptor antagonist) prevented these changes in slope, indicating the involvement of M_1-type receptors. We therefore assume a deficit in M-current-mediated control of excitability in human neurons from epileptogenic cortex. Further data are

necessary, including data from nonepileptogenic tumor resections.

Considering possible links between cholinergic transmission (particular muscarinic receptors) and cognitive functions, the increased neuronal firing in response to the same input when carbachol is present may reflect an important feature of normal cortical neurons. The enhanced firing induced by carbachol represents an increased gain of neuronal coding. In addition, the higher frequency of APs would facilitate temporal summation of follower cells. The observed reduction in muscarinic modulation in human epileptogenic neocortex, therefore, may not only contribute to hyperexcitability but also to cognititve deficits in cases of severe epilepsy.

Hyperpolarization-Activated Inward Currents of Human Cortical Neurons

Our original observations, from a small number of tissue samples, indicated a relatively small component of I_H in human neurons from epileptogenic cortex. We further investigated the biophysical properties of I_H in human cortical neurons to resolve possible changes in epileptogenic tissues. Hyperpolarizations elicited three distinct current components: an almost instantaneous current and two slow components with different kinetics. Pharmacological manipulations revealed that the instantaneous current was greatly reduced by the application of the Ba^{2+} ions, known to antagonize K_{ir}-type currents. Application of ZD7288, an accepted antagonist of I_H, greatly reduced the two slower components. The amplitude of the fast I_H component was, on average, smaller than those seen in rat cortical neurons and the kinetics much slower (time constant (at -140mV) in human neurons: 43.6 ms, in rat neurons 17.0 ms). Comparison of the current amplitudes per se is questionable when the neurons are of different sizes. Therefore, the currents were normalized with the membrane capacitance as an index of the neuronal surface area. Even after such normalization, the resulting current densities of human cortical neurons were significantly smaller than those of rat neurons (human, $-2.6\,\mathrm{pA\,pF^{-1}}$; rat, $-6.9\,\mathrm{pA\,pF^{-1}}$). This slower kinetics and smaller I_H density in neurons from human epileptogenic tissue is similar to our observations on tissue from epilepsy-prone WAG/Rij animals.

To establish a possible relation between the smaller I_H and epilepsy is difficult, given the lack of adequate human control tissue and the arguable comparison with rat neurons. Nevertheless, we tried to correlate the magnitude of I_H with the duration and severity of epilepsy in the human patients from whom tissue had been excised. To this end, we averaged the I_H densities from each tissue sample (based on measures from 3 to 11 neurons per sample, with an average of about 5 neurons). The average current densities revealed no significant correlation with the duration of epilepsy. Interestingly, the majority of data points suggested a decrease of I_H with increasing duration of epilepsy, but two clusters of data (one at short duration and small currents, and one cluster at long duration with large currents) prevented a significant correlation. Regarding the severity of epilepsy, we first used as a simple index the number of grand mal (GM) seizures the patients suffered. This index revealed a clear dichotomy; about half of the patients had a relatively small number of GMs annually, the other half had numerous GMs monthly. Comparing the I_H densities of these two groups revealed a significant difference in the I_H densities. The average neuronal I_H density was smaller in tissues from patients suffering many GMs than in tissues from patients suffering few GMs.

Some of the tissues investigated were from patients receiving the antiepileptic drug (AED) lamotrigine. Since lamotrigine has been proposed to augment I_H, we evaluated the effects of lamotrigine on I_H of human neurons. In our experiments, lamotrigine had no consistent effect on I_H. We considered the possibility that the lack of effect of lamotrigine may relate to the pharmacoresistance of these patients. To investigate this point, we applied lamotrigine under identical conditions to rat neocortical neurons. Interestingly, lamotrigine failed to exert consistent effects on the I_H elicited in rat neocortical neurons.

Future Goals

The data discussed indicate an increased slope of the AP firing–current relation, as well as a lower rheobase value for APs of human neurons from epileptogenic tissue. Obviously, an increased firing of pyramidal neurons for a given input may contribute to cortical hyperexcitability. The depression of M-currents by carbachol results in an increased slope, consistent with current views about muscarinic modulation. Interestingly the effect of carbachol on the slope was much smaller in human neurons than in rat neurons; in the presence of carbachol, the *F–I* slopes are not significantly different. Considering the established view, a depression of M-currents by muscarinic receptors via G-proteins, the smaller response to carbachol of human neurons may be due to a decreased density of receptors or channels. In this context, we plan to further identify the receptor (presumably M_1) involved in this muscarinic action, as well as the associated channel. Evaluation of the effects of the AED retigabine (an activator of K_V7 channels) and linopirdine (a blocker of K_v7 channels) on the firing behavior is planned. Quantification of the M-current with patch-clamp recordings, and assessing the effects of retigabine and linopirdine under these recording conditions, might reveal whether the M-current is reduced. This approach would also allow to characterize

the muscarinic modulation of morphologically identified neurons. In the long run, these cellular data might be correlated with cognitive functions of the patients.

One of the physiological roles of I_H is the modulation of temporal summation of EPSPs. In parallel experiments, the temporal summation of synaptic responses should be further evaluated in the human cortex. The attenuated I_H should cause an enhanced temporal summation at a given synaptic frequency; further, because of the frequency dependence of temporal summation, reduced I_H should also increase the temporal summation at lower frequencies. Together with a voltage-dependent recruitment of, for example T-current, the altered temporal summation would facilitate cortical synchrony at lower frequencies and hence the probability for precipitation of seizures.

Our data indicate a surprising absence of lamotrigine effects on somatically recorded I_H, which may be due to altered stoichiometry of the heteromultimer channel (containing fewer HCN1 subunits) than in normal rat tissue. However, this absence of effect was also seen in rat cortical neurons, suggesting that the reported augmentation of I_H by altered voltage dependence may be a peculiarity of hippocampal neurons (the subject of earlier investigations) or is not accessible with somatic recordings. To examine these possibilities, we will first test whether the effects of lamotrigine obtained with dendritic recordings can also be obtained with somatic recordings in hippocampal neurons. If in these cells the somatic recordings fail to reveal effects of lamotrigine (seen at more distal dendritic sites), the more demanding dendritic recordings must be attempted in human cortical neurons. This task will be challenging, since the visualization of neurons – let alone dendrites – is difficult in human tissues. The clarification of this mechanism is important. If I_H is indeed small in epileptogenic human cortical tissue, then drugs augmenting this residual I_H would be very useful, considering the impact of I_H on the stability of membrane potential and temporal summation of synaptic potentials. Whether a pharmacological enhancement can compensate for the ~70% smaller current density existing in human cortical neurons remains to be seen.

The biophysical and pharmacological properties of transient and persistent sodium currents have not been investigated so far in human epileptic cortex. The patch-clamp technique with infrared videomicroscopy would also allow voltage-clamp experiments characterizing the properties of transient and persistent sodium currents of morphologically identified neurons and their modulation by relevant drugs. The inherent problems associated with voltage-clamp recordings from neurons in situ (e.g., space-clamp, access of drugs) could be alleviated by using acutely isolated neurons. Obviously, this approach could also yield data for other channels (e.g., Ca^{2+}) with intra- and extracellular symmetrical Cs^+ solution, plus a cocktail of appropriate drugs (TTX, TEA, 4-AP).

In the future, we would like to address the cause of different intrinsic neuronal properties (e.g., expression pattern of Na^+ channels in fast spiking interneurons versus regular firing pyramidal neurons with single-cell RT-PCR) and consequences (e.g., input–output relation of each type of neuron with paired recordings) complemented by immunohistochemistry of individually labelled neurons.

In conclusion, the data presented here demonstrate that various electrophysiologically accessible parameters can be quantitatively evaluated in human cortical neurons. The stable recordings (up to hours) from slices in our experimental setting allow for a detailed analysis of the effects of various drugs on membrane currents. Currently, we prefer to take a given neuron through a series of drugs in various combinations (to progressively eliminate distinct components) rather than to compare populations of neurons under various conditions. This approach allows us, with a certain degree of confidence, to extrapolate from a pharmacologically well-defined measurement to the role of a given parameter in the physiological situation – when the impact is obscured by interacting channels and transmitters. Although the slices are viable for up to 20 h and sometimes even longer, only a limited number of neurons are obtained (up to 12 with patch-clamp technique and up to 8 with sharp electrodes, partly due to the different duration of protocols used with the two recording techniques). Perhaps with acute isolation and short-term culture approaches, the yield from all measurements could be increased – so that for each key parameter (e.g., I_H density), a reliable number of neurons are assessed for statistical evaluation. If the number of recorded neurons from a given patient is sufficiently large, and the drug effects on pivotal parameters are determined, we could provide a rational basis for prescribing this patient with a distinct combination of drugs. This rationale approach to drug prescription might not only alleviate the sufferings of the individual patient (especially in the cases were surgical outcome is suboptimal), but might also help to predict the outcome of drug combinations in other patients.

Acknowledgements

I am indebted to R. Fleischmann and Dr. S. Gigout, who did some of the carbachol experiments, and to S. Wierschke, who allowed the inclusion of some of his data on I_H from his PhD work. I am most grateful to Dr. T.-N. Lehmann for providing the human tissues and to Prof. H.-J. Meencke and his team at the Epilepsiezentrum Berlin–Brandenburg for their care for the patients and their interest in my work.

See also: **GABA**: Alterations in GABAergic Transmission in Human Temporal Lobe Epilepsy; **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Ion Channels**: Ion Channel Mutations in Familial Eplepsy; Sodium Channel Mutations that Cause GEFS+; **Pharmacoresistance**: Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis.

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Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis

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Introduction

CA1 pyramidal cells (PCs) provide a major output of the hippocampus proper. They integrate information arriving directly from the entorhinal cortex via the temporoammonic pathway, and indirectly via the polysynaptic dentate gyrus–CA3–CA1 loop. Both pathways terminate in a spatially segregated manner onto CA1 PC apical dendrites. The temporoammonic pathway targets the distal dendrites in the stratum lacunosum moleculare, while CA3 PC axons terminate within the stratum radiatum. These inputs are integrated in the somatodendritic compartment and converted to an output consisting of action potentials propagating along the CA1 axons, a process controlled by the intrinsic membrane properties of CA1 neurons. We will first consider the mechanisms determining input–output properties of normal CA1 PCs. We will then address how the properties of CA1 PCs change in acute and chronic epilepsy models, and how they might contribute to increased excitability.

Background – Control of Intrinsic Excitability in Hippocampal Pyramidal Cells

In a physiological extracellular milieu, CA1 PCs in the normal rodent brain display characteristic, relatively

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Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis

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Introduction

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Background – Control of Intrinsic Excitability in Hippocampal Pyramidal Cells

In a physiological extracellular milieu, CA1 PCs in the normal rodent brain display characteristic, relatively

uniform intrinsic firing properties. As a threshold response to brief current injections, the vast majority of CA1 PCs generate a single action potential, or spike (see **Figure 1(a)**, panel b). When current injection is prolonged, CA1 PCs respond with a series of independent spikes (**Figure 1(a)**, panel a). This type of discharge behavior has been classified as regular firing and sets CA1 PCs apart from neurons that respond to a threshold depolarization with a high-frequency burst of spikes as a minimal response. The latter category of neurons has been termed 'intrinsically bursting.' Bursting neurons are rarely encountered in the normal CA1 region under physiological conditions, but appear under pathological circumstances (**Figure 1(b)**, panels a and b). In other brain regions, however, bursting neurons are prevalent under normal conditions (e.g., in the subiculum or certain thalamic nuclei).

What are the mechanisms that control whether a neuron discharges in a regular or a burst-firing mode? In many types of central and peripheral neurons, the rapid repolarization of the somatic spike is incomplete, and the spike is followed by a slow afterdepolarization (ADP) lasting tens or hundreds of milliseconds. The size of this graded afterpotential is a major factor in determining the firing mode of many neurons. If sufficiently large, the spike ADP redepolarizes the neuron to action potential threshold, thus generating one or more additional action potentials. Thus, the size of the spike ADP is a critical determinant of neuronal discharge behavior.

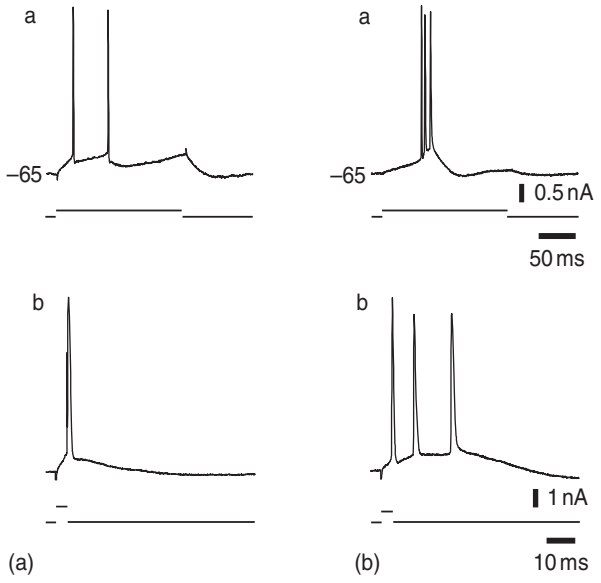


Figure 1 Regular firing and intrinsically bursting CA1 neurons. (a) Representative example of a regular firing CA1 neuron in a normal rat recorded with a sharp microelectrode. Upper traces represent voltage recordings; lower traces represent current injection steps. Responses of CA1 neurons to brief (4 ms, panel b) and long (180 ms, panel a) current injection steps are depicted. (b) Representative example of a bursting neuron in the CA1 region in a pilocarpine-treated rat. This neuron bursts to threshold brief (panel b) and long (panel a) stimulation. Bursting neurons are exceedingly rare in the normal hippocampal CA1 region.

Adult hippocampal CA1 PCs display a conspicuously active somatic spike ADP, but it does not reach spike threshold in most cells. Spike ADPs are composed of a passive and an active component. The passive component is brief, declines exponentially, and reflects passive recharging of the membrane capacitor. This passive ADP component may be augmented in size and duration by a slow inward current (active component) that activates during, or immediately after, the spike. The ion channels responsible for this active spike after-current vary across different types of neurons, and include voltage-gated Ca^{2+} and Na^{+} channels, as well as Ca^{2+}-activated nonspecific cationic channels. In adult CA1 pyramidal neurons, a major component driving spike ADPs is the persistent Na^{+} current (I_{NaP}), a low-threshold, noninactivating Na^{+} current. Patch-clamp studies in CA1 neurons have revealed that I_{NaP} is active within the voltage range of the spike ADP, and that this current gives rise to substantial sustained Na^{+} spike after-currents. Compounds that potently block I_{NaP} also block spike ADPs and associated bursting. In comparison, Ca^{2+} spike after-currents are much smaller in CA1 PCs, and suppressing them has no noticeable effect on spike ADPs.

The size of the spike ADP is not only determined by the magnitude of inward currents, but also depends on the density ratio of the inward spike after-current and opposing outward currents, such as the M-type K^{+} current (I_M). M-currents are noninactivating K^{+} currents that are active in the subthreshold voltage range. Among other cellular functions, they oppose the depolarizing drive furnished by I_{NaP} that causes spike ADPs in adult CA1 PCs. Recordings from CA1 neurons have revealed that I_M activates during the spike ADP. Selectively blocking I_M converts regular-firing CA1 PCs to a burst-firing mode. This result shows that I_{NaP} and I_M constitute two opposing low-threshold currents that in concert govern the magnitude of active spike ADPs.

Because somatic voltage-gated Ca^{2+} currents are mostly high voltage-activated subtypes that deactivate quickly after the spike, they produce only small spike after-currents in adult CA1 PCs. Unexpectedly, blocking them reduces the spike ADPs. The reason for this phenomenon is that Ca^{2+} influx during the spike causes intracellular inhibition of I_M, thereby shifting the balance between I_{NaP} and I_M in favor of the former current, leading to spike ADP facilitation.

Several lines of evidence suggest that both I_{NaP} and I_M originate in proximal axo-somatic compartments of CA1 PCs. First, channel subunits that give rise to I_M (K_v7 subunits), as well as subunits that contribute to I_{NaP} (i.e., $Na_v1.6$) are known to be aggregated at the axon initial segment of CA1 PCs. Second, focal applications of blockers of I_{NaP} or of I_M to the somatic region of CA1 PCs

suppress or augment the spike ADPs, respectively; whereas their focal application to the distal apical dendrites does not affect these afterpotentials. Finally, physically separating apical dendrites from the cell soma (with a fine cut) does not affect spike ADPs of CA1 neurons. Thus, the spike ADP in adult CA1 PCs, under normal conditions, is generated by the interplay of two low-threshold, persistent conductances at or near the soma of the neuron.

Available evidence suggests that under certain conditions dendritic ion channels also play a role in determining the firing mode of adult CA1 PCs. The apical dendrites of these neurons contain high densities of A-type K^+ currents (I_A) and T-type Ca^{2+} currents that are active within the action potential subthreshold range and are activated when action potentials back-propagate into the dendritic tree. Under normal conditions, dendritic excitability is limited by I_A. However, when dendritic I_A is blocked pharmacologically, the invading spike triggers a regenerative slow Ca^{2+} spike in the apical dendrites that spreads to the soma in much the same way as the dendritic excitatory postsynaptic potentials (EPSPs) excite the soma. This causes the somatic ADP to enlarge and escalate to a high-frequency spike burst. Thus, in normal conditions, apical dendrites of adult CA1 PCs harbor a Ca^{2+}-driven burst mechanism that is controlled by I_A (much the same way as the somatic I_{NaP}-driven mechanism is controlled by I_M). Intriguingly, the dendritic mechanism is unleashed in developing CA1 PCs at postnatal days 10–25, causing them to transitionally manifest a Ca^{2+}-driven bursting phenotype that is not ordinarily operative in adult neurons.

Methods

To address the physiological properties of CA1 neurons, electrophysiological techniques have been widely used. Such techniques include sharp microelectrode recordings (in which neurons are impaled) or patch-clamp recordings. Such recordings allow us to determine the resting electrophysiological properties of CA1 PCs (such as the membrane potential and passive electrical properties), and the properties of individual action potentials and their repetitive discharge behavior. In addition, these techniques also allow investigators to record the responses of CA1 neurons to the activation of excitatory and inhibitory synapses. These techniques have been used to glean information on the intrinsic and synaptic properties of CA1 neurons under control (i.e., 'normal') conditions and in chronically epileptic tissue. In CA1 pyramidal cells, patch-clamp recordings can be obtained from the main apical dendrite; such recordings have yielded useful information about dendritic processing and the underlying dendritic voltage gated channels, and their changes in chronic epilepsy.

Results

Plasticity of Intrinsic Excitability in Acute Models of Epilepsy

In vitro or in vivo, epileptiform activity can be induced by changing the ionic composition of the extracellular fluid in various ways. Such models have proved useful for studying the mechanisms underlying the development of synchronized population activity in normal brain. Epileptiform activity can be readily induced by lowering extracellular Ca^{2+} concentration ($[Ca^{2+}]_o$), raising extracellular K^+ concentration ($[K^+]_o$), increasing extracellular pH, or decreasing extracellular osmolarity. In addition, blocking K^+ currents with various blockers, including 4-aminopyridine (a blocker of A-type K^+ currents) causes epileptiform activity in vitro. Though all these manipulations differ in their effects on synaptic activity, they all induce intrinsic bursting in CA1 neurons. Examples for the effects of lowering $[Ca^{2+}]_o$ and raising $[K^+]_o$ are shown in **Figure 2(a)** and **2(b)**, respectively. Cumulatively, these data suggest that the epileptogenicity of hippocampal tissue correlates very well with the propensity of CA1 principal neurons to fire in burst mode.

How these diverse changes in the composition of the extracellular milieu cause the conversion of CA1 PCs to a bursting mode has been addressed in a few instances. Intrinsic bursting induced by lowered $[Ca^{2+}]_o$ is inhibited by blockers of I_{NaP}, indicating that this conductance supplies part of the depolarizing drive for the burst discharge, and suggesting that I_{NaP} may be augmented when $[Ca^{2+}]_o$ is decreased. Indeed, the activation curve of I_{NaP} was potently shifted in a hyperpolarizing direction by lowering $[Ca^{2+}]_o$ from 2 to 1.4 mM. Given such results, it is important to determine how the different voltage-dependent conductances that shape CA1 PC firing behavior are modified by altering the ionic composition of the extracellular milieu. Seizure activity results in large changes in extracellular K^+, Ca^{2+}, osmolarity, or pH, that may feed back onto the intrinsic properties of hippocampal neurons.

Plasticity of Intrinsic Excitability in Chronic Models of Epileptogenesis

In experimental rodent models of temporal lobe epilepsy (TLE), where seizures originate in the hippocampus or related temporal lobe structures, pronounced changes in intrinsic properties of CA1 PCs are also observed. These changes are, in most cases, caused by altered expression or modulation of ion channels that affects the magnitude of the corresponding currents and associated discharge behavior. In the widely used pilocarpine model of TLE, in which epilepsy develops following a single episode of status epilepticus (SE) induced by this convulsant, changes in intrinsic properties of CA1 neurons have been examined in detail. As explained earlier, most CA1

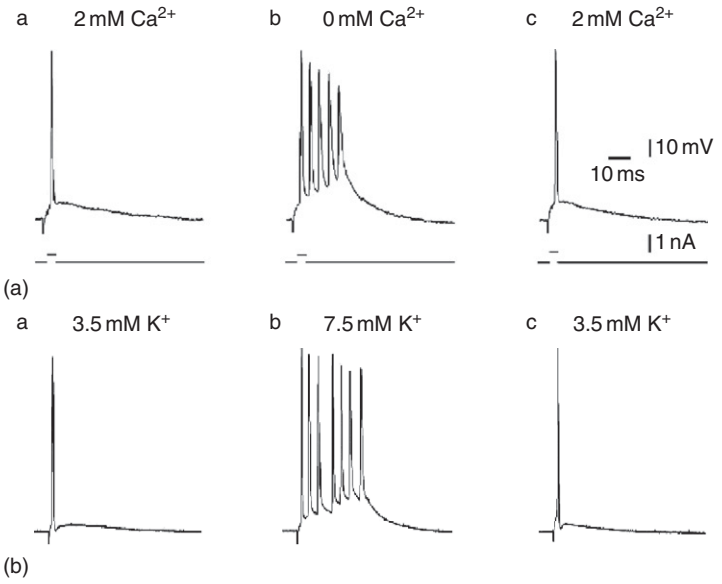


Figure 2 Changes in firing behavior in acute in vitro models of seizures. (a) Removing Ca^{2+} from the extracellular solution causes a conversion of a regular-firing CA1 neuron (panel a) to a bursting mode (panel b). This effect is reversible (panel c). (b) A similar phenomenon is observed following elevation of the extracellular K^+ concentration (panels a–c). Reproduced from Yaari Y and Beck H (2002) 'Epileptic neurons' in temporal lobe epilepsy. *Brain Pathology* 12: 234–239, with permission.

PCs in the normal adult brain are regular firing neurons. Following SE, however, about half of these neurons convert to a low-threshold bursting mode, in which their response to a threshold depolarizing stimulus is a burst of 3–8 spikes. A similar, but less pronounced change has been observed in the kindling model of epilepsy. Further analyses in the pilocarpine model have revealed that this novel bursting behavior is due to the up-regulation of a Ni^{2+}-sensitive T-type Ca^{2+} current. Interestingly, blocking T-type Ca^{2+} channels or Na^+ currents by focal drug application to the dendrites, blocks aberrant bursting, while applying blockers of T-type channels to the soma does not. This differential effect indicates that T-type Ca^{2+} currents in dendrites, presumably activated by back-propagation of action potentials, cause a dendritic Ca^{2+} spike that, in turn, propagates to the soma, eliciting further spikes (the same mechanism invoked following in normal CA1 PCs by blocking dendritic A-type currents; see the earlier sections). Interestingly, down-regulation of dendritic I_A has also been observed in CA1 PCs following SE and likely contributes to the de novo emergence of the dendritic Ca^{2+}-driven burst mechanism.

In addition to altered bursting behavior, a number of other intrinsic membrane properties are potentially altered in epilepsy. For instance, induction of early life seizures with flurothyl in rats markedly reduces spike frequency adaptation and the afterhyperpolarizing potential recorded in adult CA1 PCs. Thus, neonatal seizures also lead to persistent changes in intrinsic membrane properties of CA1 pyramidal neurons. Like the increase in intrinsic bursting behavior, these alterations reflect an increase in neuronal excitability.

How Changes in Intrinsic Excitability Contribute to Epileptogenesis

How relevant are changes in properties of CA1 neurons for the development of chronic epilepsy? Modeling studies from Traub and Wong were the first to suggest that neurons generating spontaneous bursts of action potentials may be critically important in entraining additional neurons into a synchronized population discharge. If this is true, then discharges originating in intrinsically bursting neurons would be expected to precede epileptiform activity. Indeed, in both acute and chronic models of TLE, spontaneously active intrinsic bursters were shown to be the forerunners in the generation of epileptic discharges (**Figure 3(b)**). In contrast, regular firing neurons always discharged in synchrony with the ensuing population discharge (**Figure 3(a)**). Likewise, in neocortical slices treated with blockers of inhibitory transmission, threshold stimulation of afferent fibers evoked long-latency epileptiform bursts, which originated in the cortical layers containing intrinsically bursting pyramidal cells. In the hippocampus, burst discharges of intrinsically bursting neurons are likely to recruit additional neurons in the CA1 region via recurrent excitatory connections. Such recurrent synapses between CA1 PCs are sparse in normal rats, but enhanced in chronically epileptic animals; local sprouting of axon collaterals leads to the formation of novel excitatory synapses between surviving CA1 pyramidal cells that significantly strengthen recurrent feedback excitation. The efficacy of local excitatory feedback is also controlled by GABAergic inhibition. In normal rats, even the sparse connectivity between CA1

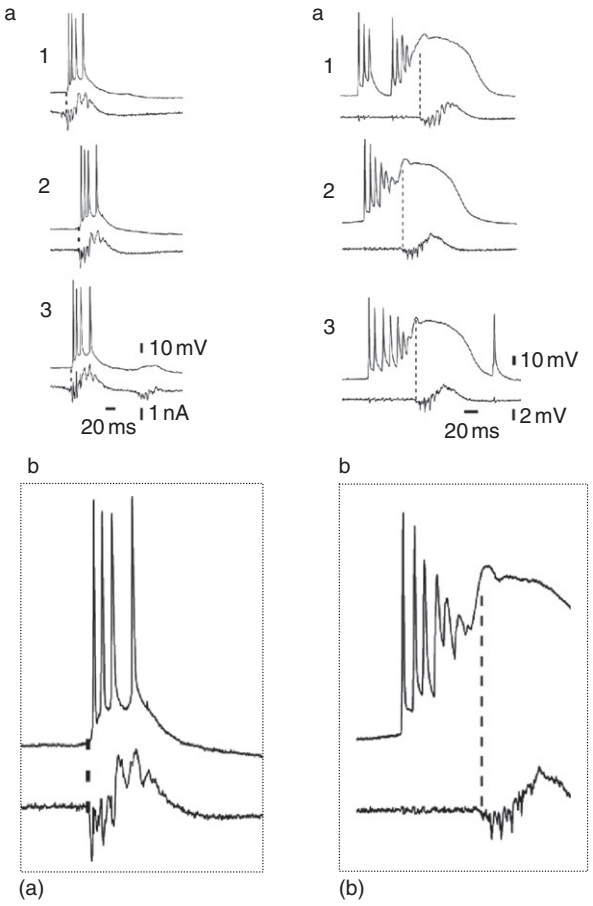


Figure 3 Intrinsically bursting neurons precede population discharges in epileptic tissue. (a) A regular firing neuron in the CA1 region recorded with a sharp microelectrode (upper traces in panel a) obtained simultaneously with a field recording of an epileptiform population discharge (lower traces in panel a). Three different instances of an epileptiform population discharge are shown (1–3, enlarged in panel b). The discharge of the nonbursting CA1 neuron always occurs together with the population discharge. (b) Analogous recording with a bursting neuron that was capable of spontaneous burst discharges. The bursting CA1 neuron invariably discharges previous to the population discharge (panel a, enlarged in panel b), suggesting that spontaneously bursting CA1 neurons may entrain other CA1 neurons into the population discharge. Reproduced from Sanabria ERG, Su H, and Yaari Y (2001) Initiation of network bursts by Ca^{2+}-dependent intrinsic bursting in the rat pilocarpine model of temporal lobe epilepsy. *Journal of Physiology* 205–216, with permission.

neurons can become functionally relevant if $GABA_A$ergic inhibition is blocked, leading to the spread of excitation within the CA1 region. In chronic epilepsy, there is a deficit in some forms of inhibition in CA1. Both enhancement of recurrent excitation and loss of inhibition are likely to provide pathways for the spread of intrinsic bursting within the neural aggregate, thus facilitating the generation of epileptiform events. Clearly, intrinsic and synaptic features in the chronically epileptic hippocampus conspire to produce epileptiform activity in the CA1 region.

Are there other consequences to the conversion of CA1 neurons to an intrinsically bursting mode? A number of studies suggest that the bursting mode affects plasticity at synaptic connections onto these neurons. For instance, synaptically evoked bursts of action potentials, but not solitary spikes, enable *N*-methyl-D-aspartate (NMDA) receptor-dependent long-term potentiation (LTP) of EPSPs in CA1 pyramidal cells. Presumably, the burst discharge is more effective than a single spike in removing the Mg^{2+} block from postsynaptic NMDA receptor-channels, which is a prerequisite for the induction of LTP. In addition to promoting LTP of excitatory synaptic transmission, it is tempting to speculate that intrinsically bursting neurons in epileptic tissue may be more liable to exhibit sprouting of axon collaterals, because this process also depends on NMDA receptor activation. Thus, intrinsic bursting may interact with various plasticity mechanisms, which conspire to cause the progression of the epileptic condition in TLE.

Apart from contributing to the increased propensity to generate burst discharges, changes in dendritic ion channels also affect processing of EPSPs generated at dendritic sites. Several types of dendritically enriched channels, such as A-type K^+ channels, T-type Ca^{2+} channels, and h-channels, are active in the subthreshold range and can therefore be activated by EPSPs that are subthreshold for spike initiation. The activation of all the three channel types can have substantial effects on the propagation of EPSPs from distal dendritic sites to the soma. In addition, dendritic channels affect the back-propagation of action potentials into the dendritic tree. I_A in particular causes attenuation of back-propagating action potentials. A reduction in I_A in chronic experimental epilepsy has been shown to cause a reduced attenuation of action potential amplitude at dendritic sites.

Future Directions

Intrinsic plasticity is a complex process that has often been cited to help explain phenomena observed in experimental epilepsies. Such plasticity can profoundly alter the firing behavior of CA1 PCs. The expression of intrinsic plasticity relies on an altered magnitude of dendritic and somatic voltage-gated ion channels. Even though some progress has been made, extensive further studies will require to dissect the underlying cellular and molecular mechanisms. Several intriguing questions remain to be addressed. First, changes of dendritic conductances dramatically affect synaptic integration. Due to the difficulty of recording from small dendrites, however, our knowledge of the active properties of dendrites is restricted to the main apical dendrite of CA1 neurons. Whether the properties of oblique or basal dendrites are altered in the epilepsies is unknown. Second, it would be important to learn more about the cellular cascades that are important in mediating intrinsic plasticity. A precise

identification of transcriptional and translational pathways underlying the regulation of specific ion channels by activity will be an intriguing area of research in the coming years. Finally, it will be important to use novel techniques that allow investigators to record activity from many neurons simultaneously, to determine the impact of a subpopulation of intrinsically hyperexcitable neurons on the greater CA1 network.

See also: **Granule Cells**: Granule Cell Properties in Seizure Discharge; **Ion Channels**: Post-Translational Modifications of Ion Channels in Epilepsy; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; **Non-Synaptic Mechanisms**: Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; **Post-Traumatic Epilepsy**: The Causes of Epilepsy after Traumatic Brain Injury; **Pyramidal Cells**: Intrinsic Mechanisms Contributing to Hyperexcitability of Human Epileptogenic Cortex; Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization.

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Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization

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Introduction

The CA3 region of the hippocampus is composed of pyramidal neurons and a myriad of interneurons that are inhibitory and synapse on pyramidal neurons (as well as on each other). The classic inhibitory neuron is the basket cell, which has numerous synaptic contacts on the cell bodies of CA3 pyramidal neurons and mediates somatic inhibition. Other interneurons synapse on dendrites of pyramidal neurons to produce dendritic inhibition. The CA3 pyramidal neurons receive excitatory synaptic input from dentate granule neurons via the mossy fiber synapse as well as perforant pathway input from the entorhinal cortex to the most apical dendrites. In addition, there are recurrent excitatory connections among the CA3 pyramidal neurons (autoassociational). The CA3 neurons project to the CA1 region via the Schaffer collateral pathways to complete the classic trisynaptic pathway of the hippocampal formation (perforant pathway to dentate granule neurons, dentate granule neurons to CA3, CA3 to CA1 neurons). Recent evidence also demonstrates some CA3 pyramidal neuron axons projecting back to dentate granule neurons. The CA3 pyramidal neurons send axons out of the ipsilateral hippocampus via the alveus and fimbria of the fornix; these axons project to the septal nuclei bilaterally and to the contralateral hippocampus. Inputs from the contralateral CA3 region

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Introduction

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and septal nuclei (primarily ipsilateral) also enter the hippocampus through the alveus and fimbria.

The hippocampus is important for memory, and the dentate gyrus/CA3 connections have been shown to produce pattern separation for similar stimuli or inputs, as opposed to pattern completion associated with the CA1 region. Thus dentate/CA3 activity will be selective for the exact orientation or features of a memory cue, whereas the CA1 neurons will not respond so selectively (and a similar pattern of activity will occur for recall of similar objects). The autoassociative (recurrent excitatory) connections among excitatory neurons are hypothesized to produce episodic and one-trial learning. These association pathways also provide a synaptic substrate for abnormal recurrent excitation that favors synchronization and seizures.

Background

CA3 pyramidal neurons were among the first mammalian CNS neurons from which intracellular recordings were made. Kandel and Spencer made recordings from CA3 neurons of the cat; neurons were impaled with intracellular electrodes that were introduced via an intraventricular approach, through the alveus. Neurons were identified as CA3 pyramidal neurons if they demonstrated an antidromic response to fornix stimulation (neurons that were not activated by antidromic stimulation were considered as unidentified). These early studies demonstrated that a burst of action potentials could be generated in identified CA3 neurons by either synaptic activation delivered by subiculum stimulation (presumed to be perforant pathway stimulation) or following depolarizing current injection. Furthermore, these investigators showed inhibitory postsynaptic potentials could be produced by weak fornix stimulation. The burst of action potentials and the spike afterdepolarizations that Kandel and Spencer described were hypothesized to be mechanisms of maintaining an excitatory state. Later work determined this pattern of burst firing and spike after depolarizations were generated (at least in part) by calcium currents.

The CA3 region of the hippocampus appears to be the pacemaker region for spontaneously occurring synchronous epileptiform activity in the hippocampal slice. When convulsants are added to the bathing media of hippocampal slices, the CA3 region is most likely to generate spontaneous epileptiform activity and to drive activity in the CA1 region via the Schaffer collateral pathway. The underlying basis for CA3's susceptibility to epileptiform activity appears to be related to the burst-firing properties of the CA3 pyramidal cells, and to the recurrent excitatory connections among these CA3 neurons. Subtle alterations that promote excitatory synaptic transmission, decrease inhibitory synaptic transmission, or alter the firing properties of CA3 neurons allow for the emergence of network-driven activity that underlies epileptiform discharges. Because the CA3 region displays spontaneously occurring synchronous in vitro activity that resembles interictal and ictal in vivo activity, experiments can be carried out to define variables that favor or suppress epileptiform discharges. Furthermore, the in vitro slice preparation allows investigators to obtain intra- and extracellular recordings, and to thus evaluate changes in membrane or synaptic properties that can be correlated with network-based epileptiform events (see **Figure 1**).

Methods

Most intracellular recordings in the hippocampus are now made from slice preparations (including organotypic hippocampal slice cultures). In general, hippocampal slices are prepared by sectioning the hippocampus using a tissue chopper or vibratome. The slices may be prepared in the transverse, longitudinal, or horizontal planes, depending on what circuitry the investigator wants to preserve and study. The hippocampus from newborn animals may be used to produce organotypic slice cultures that preserve many features of hippocampal anatomy, including synaptic connections from granule to CA3 neurons and CA3 to CA1 neurons.

Using such slice preparations, maintained in a recording chamber, both intracellular and extracellular

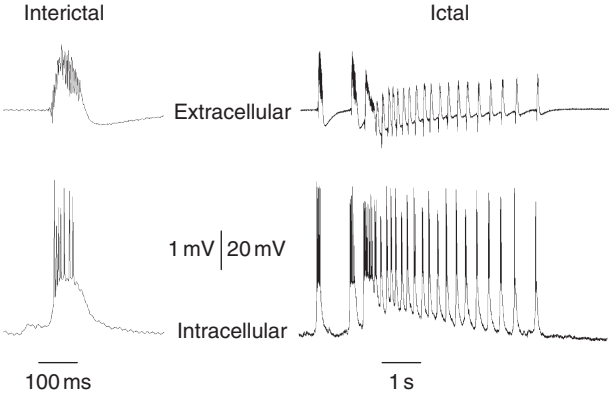


Figure 1 Intra- and extracellular recordings of spontaneously occurring epileptiform activity from the CA3 region of the hippocampal slice. This slice had been exposed to the group I metabotropic glutamate receptor agonist dihyroxyphenlyglycine (DHPG) and the activity continued after the agonist had been removed. The traces on the left show an interictal discharge that is analogous to the interictal spike that is recorded in the EEG. The interictal discharges are relatively brief compared to the discharge depicted in the right side of the figure that is more prolonged and resembles a seizure or ictal discharge. Intracellular recordings from a CA3 pyramidal neuron ($V_m = -64$ mV).

recordings can be obtained by introducing the appropriate type of electrode into the region (or cell type) of interest. Extracellular recordings are often obtained to determine if there is synchronous activity associated with an intracellular signal. Stimulation of various pathways within the slice preparation (often with bipolar electrodes) can be used to induce either antidromic or synaptically mediated activity in the cell of interest. Sharp electrodes were initially used to study intracellular activity in 'current clamp' mode. Whole-cell voltage-clamp recordings can also be made, using either blind techniques (i.e., not directly visualizing neurons but identifying the cell body layer), or a microscope with infrared diffusion interference contrast (IR-DIC) to visualize neurons directly. Data presented in article are drawn primarily from studies of acute slices from young rats (10–40 days old). The younger animals are particularly useful for IR-DIC guided recordings.

Results

Membrane Properties of CA3 Pyramidal Cells

Burst firing

First noted in in vivo studies, some CA3 pyramidal cells tend to fire in brief bursts of 2–3 action potentials when depolarized to threshold, either by current injection or synaptic input. This burst firing property defines pyramidal neuron activity in unit recordings and differentiates these neurons from interneurons, which fire at more sustained higher frequencies. Granule neurons do not burst under normal conditions and CA1 neurons are less likely to show intrinsic burst activity compared to CA3 neurons. Approximately 20% of CA3 pyramidal neurons demonstrate burst firing patterns when depolarized by current injection. Using intracellular recordings, investigators have attempted to elucidate the underlying bases of this burst pattern of discharge. This burst discharge property appears to be dependent on the activation of calcium channels, some of which are activated near rest (i.e., T-type calcium channels). The burst activity is also influenced by higher threshold channels (L, N, or R-type) that are also present in CA3 pyramidal neurons. The dendritic location of calcium channels allows them to amplify synaptic input, and to enhance synaptic activity as a consequence of back-firing action potentials from the soma. When action potentials are generated in the soma, they can back-propagate and activate high threshold calcium channels not originally activated by lower voltage synaptic depolarization. Such a back-propagation may be important for strengthening synapses active during a back propagating potential.

Some investigators have hypothesized that intrinsic bursting neurons are necessary for epileptiform activity. Intrinsic bursting will enhance the probability of synchronization by synaptic mechanisms in a network with recurrent excitatory synaptic connections. Burst firing with appropriate timing between spikes enhances temporal summation of EPSPs, and will increase the likelihood of action potential generation in the postsynaptic neuron. Furthermore, if the postsynaptic neuron itself has burst-generating properties, multiple action potentials may be generated that will lead to enhanced synaptic transmission onto other synaptically connected neurons. Interestingly, in epileptiform bursting induced by elevated extracellular potassium, intrinsic bursting may be less common and the network is driven more by an enhanced frequency or EPSPs.

Afterhyperpolarization

The burst firing of CA3 neurons is terminated and modulated by a number of potassium conductances activated by depolarization and the burst activity. These conductances include voltage dependent channels like the delayed rectifier and M-channel, and calcium-activated potassium channels. There are three calcium-activated potassium conductances that are activated by action potential firing that cause an afterhyperpolarization (AHP) following the burst. The first is the fast AHP (fAHP) which is activated by single action potential generation and controls the spike duration. This fAHP is mediated by channels that have a large conductance (so-called BK channels). These channels appear to be located near calcium channels that are involved in action potential generation. The second AHP, termed the medium AHP, is mediated by calcium activation of the small conductance (SK) class of channels that are sensitive to apamin, a toxin in bee venom. The longest duration AHP is the so-called slow AHP (sAHP) that is mediated by a calcium-dependent potassium conductance and lasts for seconds. The sAHP activates slowly, appears to be activated by higher levels of calcium influx, and is perhaps associated with calcium diffusion into the dendrite.

Some of these channels are negatively modulated by neurotransmitters that enhance epileptiform activity (e.g., muscarinic, beta noradrenergic, and metabotropic glutamate receptors, **Figure 2**, see below). Reduction of the medium and slow AHPs are associated with a faster spontaneous interictal discharge rate and favor more prolonged ictal discharges Finally, it should be noted that the M-current may also contribute to repolarization and an AHP that lasts for 2–300 ms. The M-current is activated by voltages just above the resting potential and controls spike rates when neurons are depolarized. The channels that are responsible for this current are closed when muscarinic receptors are activated and phospholipase C is produced.

Spike frequency modulation

The frequency of action potential generation is greatly influenced by a number of channels that are activated by

depolarization and calcium entry. As in many cortical pyramidal neurons, the frequency of spiking in CA3 pyramidal cells decreases during a prolonged depolarizing current pulse (adaptation) because of channels that repolarize the membrane potential. Under normal conditions, some neurons fire only a short burst of action potentials; the AHP repolarizes the membrane potential and prevents the cell from generating continuous spike. When neuromodulators exert their effects to reduce the various forms of the AHP (see below), the action potentials may be generated at a higher frequency for a more prolonged period. More sustained firing favors higher frequency of interictal discharges and longer lasting network activity, presumably by increasing the likelihood of excitatory recurrent synaptic transmission.

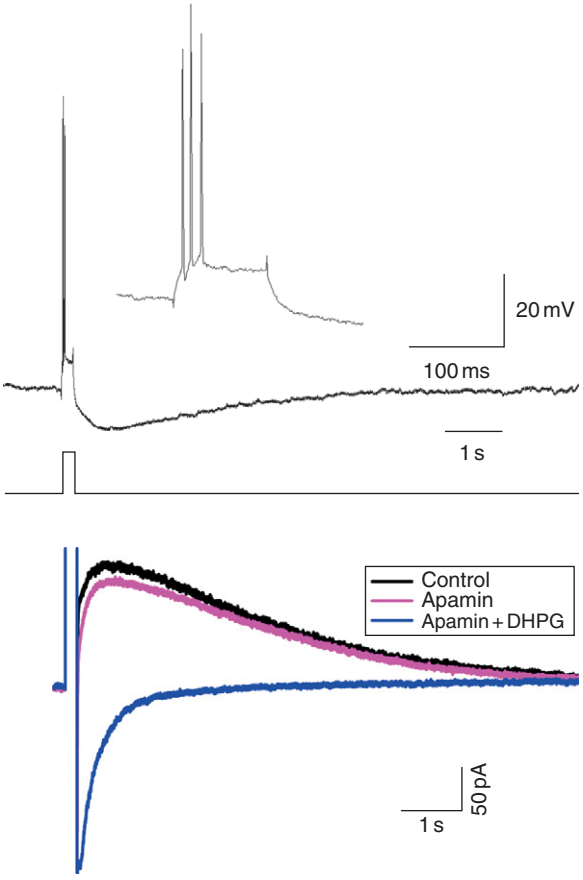


Figure 2 Sharp electrode recording from a CA3 pyramidal neuron ($V_m = -60$ mV) showing a burst of action potentials evoked by an intracellular depolarizing current pulse (top traces). Note that there is a burst of action potentials that accommodate and stop firing by the end of the current pulse that is followed by a long lasting after hyperpolarization (slow AHP). The bottom traces recorded using whole-cell voltage-clamo techniques show AHP currents evoked by a depolarizing voltage step from -50 to $+70$ mV recorded in the presence of TTX, TEA, and bicuculline. A long-lasting outward current develops after the pulse (I_{sahp}). Addition of -50 nM apamin to block the medium AHP produces a slight reduction in the amplitude of the earlier outward current. The group I mGluR agonist DHPG totally eliminates the slow AHP and also produces an inward tail current that probably results from a cationic current activated by the voltage step. These effects greatly enhance excitability and result in the development of spontaneously occurring activity depicted in Figure 1.

Synaptic Circuitry and Synchronization: Recurrent and Feedforward Circuitry

The hippocampus in general, and the CA3 region in particular, have extensive local synaptic circuitry that favors synchronization (Figure 3); this synchronization may be manifested in pathological activity – i.e., epileptiform activity. The basis for this synchrony is the rich recurrent circuitry that includes both recurrent (or feedback) inhibition and recurrent excitatory synaptic connections (autoassociational). Strong afferent excitatory input to CA3 pyramidal cells is mediated by the mossy fiber synapse, the specialized synaptic terminal of the dentate granule cell axons onto the CA3 dendrites. This complex synapse is close to the CA3 pyramidal neuron soma (on proximal dendrites) and provides a powerful drive that is subject to relatively little dendritic filtering or inhibition (compared to more remote associational/commissural synapses or perforant path input to the most distal apical dendrites). The mossy fibers also contact many inhibitory interneurons (many more inputs to

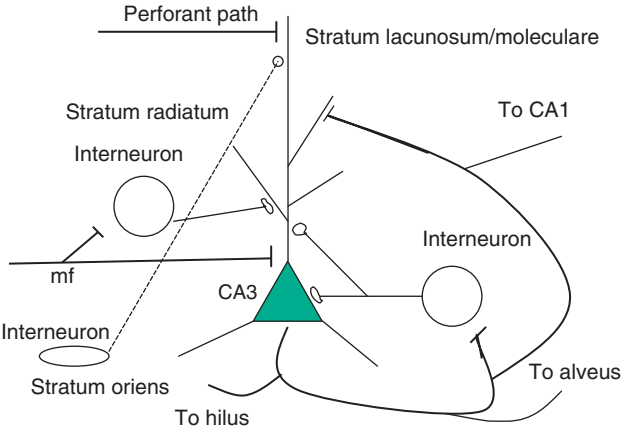


Figure 3 Schematic of the local circuitry of the CA3 region. Input from dentate granule neurons are carried by the mossy fibers (mf) which synapse on interneurons to activate feed forward inhibition and pyramidal neurons. The pyramidal neurons also receive input in the distal apical dendrites from the entorhinal cortex via the perforant pathway. In addition commissural and local associational inputs from other CA3 neurons activate interneurons as well as pyramidal neurons. The local excitatory synaptic connections promote interictal and ictal epileptiform activity. Other interneurons help regulate local circuits including the stratum alveus oriens interneuron that sends it axons to the distal apical dendrites (dotted line).

interneurons than to pyramidal neurons) and mediate feedforward inhibition (see Figure 3).

In addition, strong feedback inhibition onto CA3 pyramidal cells is mediated primarily by CA3 axon collaterals – which activate local interneurons, particularly basket cells. The basket cell provides a dense terminal plexus around the CA3 pyramidal cell soma, and produces a powerful inhibition through $GABA_A$ receptor activation. These somatic synapses are well positioned to prevent action potential generation. Although the somatic inhibition may not produce much change in membrane potential (the resting membrane potential of the CA3 pyramidal neurons is close to the reversal potential of the $GABA_A$-mediated IPSP), it does create a large shunt that will prevent the cell from being depolarized by other (e.g., EPSP) currents.

Blocking inhibition using a $GABA_A$ receptor antagonists (e.g., bicuculline, penicillin, and picrotoxin) produces spontaneously occurring epileptiform activity in the CA3 region that is mediated by a large excitatory postsynaptic potential generated by recurrent excitatory synaptic circuitry. Unlike the burst activity due to the activation of calcium and sodium channels (that can be turned off by hyperpolarizing the membrane potential), the epileptiform burst activity is not reduced by hyperpolarization and is actually increased in amplitude – consistent with an underlying excitatory synaptic potential. Furthermore, the epileptiform burst is accompanied by an extracellular potential that represents synchronous activity of many neurons. The recurrent excitatory synaptic potentials are more likely to synchronize when they are not inhibited by GABAergic synaptic input.

The excitatory recurrent circuitry is mediated by axon collaterals of CA3 pyramidal cells. The CA3 axon provides the major input to CA1 (the Schaffer collateral pathway), collaterals that ramify within the ipsilateral CA3, and collaterals directed to the contralateral hippocampus as well as to the septal nuclei blaterally. The transmitter that is released at the recurrent collateral synapses within CA3 is glutamate. Release of glutamate at these synapses activates AMPA/KA and NMDA receptors. Interestingly, NMDA-antagonists do not suppress epileptiform activity in the CA3 region, whereas low concentrations of AMPA receptor blockers do. In the slice preparation, one can easily see the consequences of the excitatory connections among CA3 neurons; cells are recruited by, and drive activity in other CA3 pyramidal cells when convulsant drugs (e.g., $GABA_A$ blockers, potassium channel blockers, increased extracellular potassium concentration) are added to the medium. The synchronous activity produced by these manipulations appears to be mediated by the AMPA/KA component of recurrent collateral synaptic transmission. The synchronous activity generated within the CA3 region drives synchronous discharges in the CA1 region via the Schaffer collateral pathway.

Modulation of Membrane Properties by Neurotransmitters

A number of neurotranmsitters activate or modulate ion channels through second messengers, and have a metabotropic rather than an ionotropic effect. Metabtropic receptors are not directly coupled to ion channels but are coupled to second messengers that then cause secondary effects (e.g., closing a potassium channel or activating calcium release from intracellular stores). The receptor for these modulating neurotransmitters is often coupled to G-proteins that then cause an effect mediated through a second messenger (either the G-protein itself or another second messenger, such as cAMP).

Cholinergic (muscarinic) modulation

Systemic injection of pilocarpine, a muscarinic agonist, produces status epilepticus, neuronal loss, and eventually spontaneously occurring limbic seizures. Pilocarpine-treated animals have been widely used as a model of temporal lobe epilepsy. The muscarinic receptors in the CA3 region are linked to second messenger systems that activate phospholipase C (PLC) and depolarize the membrane by decreasing the M-current (a potassium current activated near rest), decrease the slow AHP that controls neuronal firing, and causes calcium release from intracellular stores by inositol triphospate (IP3). IP3 is produced by PLC activation and binds to a receptor on the surface of intracellular calcium stores that cause release of calcium. When applied to hippocampal slices, pilocarpine produces synchronous CA3 firing that can last for seconds and mimics seizures in the intact animal.

Adrenergic (alpha and beta) modulation

Norepinephrine (NE) receptors in the hippocampus include the beta and alpha (1 and 2) receptors. NE action at the beta 1 receptor causes a decrease in the slow AHP by activation of cyclic AMP. Presumably, cAMP leads to phosphorylation of either the calcium-activated potassium channel or other effectors of this conductance, and thus reduces the activation of the conductance by calcium. This effect is associated with an increase in the rate of interictal discharges produced by disinhibition or elevations of potassium in the hippocampal slice. The beta receptor also enhances calcium currents carried by L-type channels. Overall, the actions of norepinephrine on noradrenergic beta receptors are proconvulsant.

The alpha-2 adrenergic receptor appears to activate anticonvulsant mechanisms that suppress epileptiform activity (as seen when $GABA_A$ blockade is induced in hippocampal slices). Alpha-1 adrenergic activation increases the rate of spontaneously occurring IPSPs in CA3 pyramidal neurons, an effect that appears to be due to a depolarization of interneurons. Alpha-1 activation also

appears to depress excitatory synaptic transmission by presynaptic mechanisms.

GABAergic ($GABA_B$) modulation

Besides activating $GABA_A$ receptors, GABA also binds to a G-protein coupled receptor termed $GABA_B$. This receptor activates a potassium channel via a G protein and produces a slow IPSP postsynaptically as well as inhibiting transmitter release (both glutamate and GABA) from presynaptic terminals by reducing calcium influx. Baclofen is an agonist at these $GABA_B$ receptors, and usually suppresses epileptiform activity in the CA3 region. In some cases, however, baclofen may lead to longer lasting discharges and can favor ictal-like activity. The reasons for this excitatory effect are not totally clear, but could relate to a reduction in GABA release from inhibitory interneurons. Baclofen may also reduce the amount of glutamate released with each CA3 pyramidal cell action potential, and may enhance prolonged synchronization by limiting transmitter release so that successive action potentials result in less transmitter release. One theory is that the epileptiform burst in CA3 neurons leads to depletion of releasable stores of transmitter, and the time needed to replenish these stores accounts for the period between interictal bursts. When glutamate release is not dampened by this $GABA_B$-mediated effect, only brief synchronous interictal activity occurs – perhaps because maximal glutamate release then leads rapidly to depletion of releasable vesicles in the terminal.

Glutmatergic (metabotropic) modulation

Under conditions of elevated glutamate release, as occurs during seizures, metabotropic glutamate receptors (mGluRs) are activated. This diverse group of receptors includes receptors subtypes located postsynaptically (group I mGluRs, mGluR1 and 5) in a perisynaptic location, as well as presynaptically (group II {mGluR2 and 3} and III mGluRs {mGluR 4, 5 6, 7, and 8}). Group I mGluRs activate second messenger cascades that include phospholipase C and, subsequently, diacylglycerol and inositol triphospate (IP3). IP3 activation results in calcium release from intracellular stores, and may contribute to excitotoxicity. Group I mGluRs also decrease a number of potassium conductances (thus increasing neuronal excitability) and also activate a cation channel that can mediate a slow EPSP. Group I mGluR activation results in seizures in animals, and in epileptiform activity in slices that persists following removal of the agonist.

Group II and III mGluRs appear to be anticonvulsant in action by inhibiting glutamate release presynaptically through calcium channel inhibition. mGluR2 receptors are located at mossy fiber presynaptic terminals, and agonists of mGluRs can be used to block mossy fiber EPSPs in CA3 pyramidal neurons.

Peptide Modulation of CA3 Excitability

A number of peptides play roles in controlling the excitability of the CA3 region and impact the tendency for CA3 pyramidal neurons to synchronize. Neuropeptide Y (NPY) is contained in interneurons, and these terminals increase in number in humans and animals with seizures. NPY has strong presynaptic actions that decrease excitatory transmitter release and suppresses interictal epileptiform activity in the CA3 region. Galanin is another neuropeptide that inhibits epileptiform activity in the hippocampus. Terminals from the medial septum contain galanin and activation of galanin receptors have anticonvulsant effects in vivo.

Dynorphin is a neuropeptide located in mossy fiber terminals, and release of dynorphin leads to activation of kappa opiod receptors. Dynorphin decreases excitatory glutamate synaptic transmission, enhances the M current, and has anticonvulsant properties. Activation of mu opiod receptors inhibits the release of GABA from interneurons resulting in disinhibition and epileptiform activity.

Somatostatin, another neuropeptide localized to interneuron axon buttons, appears to have antiepileptic effects. Somatostatin has been shown to enhance the M-current and reduce the frequency and duration of epileptiform discharges in the CA3 region of the hippocampal slice. Somatostatin also appears to inhibit recurrent excitatory connections.

Summary – Directions for Future Research

The network epileptiform activity that can be produced in the CA3 region of the hippocampus has proved to be a model of epileptiform activity in general. The organization of the recurrent excitatory synaptic connections of CA3 neurons and the firing properties of the CA3 neurons give rise to a system in which to study perturbations that favor epileptiform activity and seizure occurrence. Our understanding of pro- and antiepileptic properties of neurotransmitters and modulators has been improving through study of CA3 epileptiform activity and its modification.

Future studies will continue to define pharmacological manipulations that alter network synchronization in the CA3 region. Furthermore, other recording techniques – including multielectrode monitoring and optical imaging with voltage- or calcium-sensitive dyes –will provide insights into how a small network is capable of generating spontaneously occurring epileptiform activity. The CA3 region also should provide a useful model to study ways to modulate epileptiform activity and seizures by various electrical stimulation protocols. The ability to create prolonged epileptiform activity should help explain key mechanisms for the transition from interictal to ictal

patterns. These experimental approaches should lead to better therapies for temporal lobe epilepsy and epilepsy in general.

Acknowledgements

This work was supported by a Merit Award VA research grant to PAR.

See also: **Models**: Acutely Dissociated Neurons; **Networks**: Cellular Properties and Synaptic Connectivity of CA3 Pyramidal Cells: Mechanisms for Epileptic Synchronization and Epileptogenesis; Network Interactions in the Limbic System and Epileptiform Synchronization; Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Pyramidal Cells**: Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain.

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Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain

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Introduction

The hippocampus is a component of the temporal/limbic network, which is thought to play an important role in temporal lobe epilepsy (TLE). The evidence supporting this view includes imaging, surgical, and experimental data. For example, positron emission tomography (PET) studies have demonstrated that the degree of hypometabolism in the temporal lobe correlates with the severity of hippocampal pathology. Further, removal of the hippocampus (with some adjacent parts of the anterior medial temporal lobe) successfully controls seizures in patients with medically intractable TLE. Finally, a large series of studies on experimental models of epilepsy implicate the hippocampus as an epileptogenic zone.

The molecular anatomy of hippocampi removed from the brains of patients with medically intractable TLE has provided important insights. These studies reveal two broad types of organization in these TLE-associated hippocampi. A majority of these hippocampi involved in mesial temporal lobe epilepsy (MTLE) show a pattern of extensive (>50%) neuronal loss across all regions of the hippocampus, including area CA3, along with an increase in the population of astrocytes. These changes

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The molecular anatomy of hippocampi removed from the brains of patients with medically intractable TLE has provided important insights. These studies reveal two broad types of organization in these TLE-associated hippocampi. A majority of these hippocampi involved in mesial temporal lobe epilepsy (MTLE) show a pattern of extensive (>50%) neuronal loss across all regions of the hippocampus, including area CA3, along with an increase in the population of astrocytes. These changes

are accompanied by a distinctive reorganization of the neural circuits involving the surviving neurons. This pathological appearance is described as 'hippocampal sclerosis.' Several lines of evidence suggest that these sclerotic hippocampi are epileptogenic. Other surgically removed hippocampi do not show such extensive neuronal loss, gliosis, or reorganization, and are often described as nonsclerotic hippocampi. The latter closely resemble hippocampi from neurologically normal subjects, and are often considered to be nonepileptogenic. The cause of the epileptogenicity in patients with nonsclerotic hippocampi is sometimes a mass lesion (tumor or arteriovenous malformation) outside the hippocampus (mass associated temporal lobe epilepsy – MaTLE). In other cases, the cause remains of yet unknown etiology (paradoxical temporal lobe epilepsy – PTLE). In this article, an 'epileptogenic' (or 'epileptic') hippocampus is considered to be one having sclerosis.

The hippocampus, which is anatomically connected to many other brain regions within and outside the temporal lobe, is structurally complex. It is composed of two major components, the dentate gyrus and the hippocampus proper – the latter also referred to as Ammon's horn or *cornu Ammonis* (CA regions). The Ammon's horn region is divided into four subregions – area CA4 or the hilus, areas CA3, CA2, and CA1. Each of these regions has a distinctive organization, with defined afferents and efferents, and may make varying contributions to the epileptogenicity of the hippocampus. Area CA3 has been implicated in the synchronization of neuronal activity during the emergence of epileptiform activity in the hippocampus in animal models of epilepsy, although evidence for this role of CA3 in the epileptic human hippocampus is not available. The behavior of neurons in area CA3 of the hippocampus in the epileptic brain is determined, at least in part, by the circuitry of this region, including the neurotransmitters and their receptors within these circuits. Species differences in the distribution of receptors and receptor subtypes in CA3 have been reported. This article will review the receptor systems within area CA3, and particularly those localized on the CA3 pyramidal cells in the human brain. We will review the differences in the distribution of these receptors in the human epileptic brain as compared to nonepileptic control hippocampi.

Background

Pyramidal neurons are the principal neuron type in CA3, as in all areas of Ammon's horn. In contrast to CA1 pyramidal neurons, the CA3 pyramidal neurons in the primate often exhibit two apical dendrites arising directly from the triangular cell body, as well as several basal dendrites. A characteristic feature of the CA3 pyramidal neuron is the occurrence of a large number of large spines, or excrescences, on the proximal parts of the dendrites; in the primate, these excrescences are found not only on the apical dendritic shafts but also on the proximal part of the basal dendrites. These proximal spines are complex, having many branches emerging from a single dendritic origin. Distal dendritic segments show smaller and thinner spines. These pyramidal cells receive synaptic inputs from a variety of sources, but the most characteristic of these afferents is the input from the dentate granule cell axon, called the mossy fibers, which form complex synaptic connections with the proximal dendritic excrescences. In the rat hippocampus single mossy fiber boutons contact spines from only one parent dendrite of a CA3 pyramidal cell, and it is estimated that a single bouton can make up to 37 or more synaptic contacts with branched spines from a single CA3 dendrite. Each mossy fiber bouton contains thousands of small (~40 nm) clear vesicles (containing glutamate) and many large dense core vesicles (containing peptides). In addition to mossy fiber inputs, CA3 pyramidal neurons also receive synaptic inputs from the septal region and commissural fibers (that synapse on the apical dendrites in the area of the stratum radiatum), and from perforant path fibers (that synapse in the stratum moleculare).

Associated with the pyramidal neurons in area CA3 are several types of interneurons, some of which also receive mossy fiber inputs; the interneuron-associated mossy fiber boutons are smaller than those associated with the pyramidal neurons. Pyramidal inhibitory basket cells, whose axons innervate pyramidal principal cells are located immediately beneath the CA3 pyramidal cell layer. Another population of interneurons has somata within the stratum lucidum. All these interneurons receive mossy fiber inputs. While some of these interneurons in the stratum lucidum are spiny and contain glutamate, others are aspiny, and provide a GABAergic input to CA3 pyramidal cells.

Synaptic inputs into CA3 pyramidal neurons involve a variety of neurotransmitters. The major transmitter in the mossy fiber terminals is the excitatory neurotransmitter, glutamate. In addition, these mossy fiber terminals contain several peptides, including dynorphin, enkephalin, cholecystokinin (CCK), neuropeptide Y (NPY), and neurokinin-B. Many of the GABAergic interneurons in CA3 colocalize peptides (such as NPY and somatostatin) and are thought to release these peptides within the CA3 network; such colocalization is readily seen in interneurons in the stratum oriens. Given the variety of neurotransmitters that appear to be released at synapses onto CA3 pyramidal neurons, it seems clear that they must possess a broad range of neurotransmitter receptors and receptor subtypes. The localization of these receptors is reviewed later in the following sections.

Methods

Several approaches have been adopted to study the distribution of receptors and receptor subtypes in area CA3. At present, the method of choice is immunohistochemical localization of receptors using primary antibodies specific to a receptor or receptor subtype. Both light and electron microscopic analysis of tissue labeled with these antibodies can provide both the cellular and subcellular localization of the receptor molecules. In situ hybridization analysis for the localization of receptor-specific mRNA is also used to localize the cellular distribution of receptors. However, mRNA localization provides information only about the cell type (i.e., cell body) and does not yield information on the actual subcellular and membrane localization of the receptor proteins. In earlier studies, when specific antibodies were not easily produced or readily available, receptor localization and distribution was done using radioactively labeled molecules that were selective agonists or antagonists for the receptor site. The binding of these molecules to receptor sites was visualized by autoradiographic techniques. More recently, gene expression analysis in specific cell types has been used to compare the *levels* of receptor expression in specific cell types (such as the CA3 pyramidal neurons).

Results

Glutamate Receptors

The principal neurotransmitter of the afferents to CA3 from outside the region, including the mossy fiber terminals, is glutamate. Glutamate can act on several classes of receptors broadly divided into those that are directly linked to ion channels (ionotropic receptors) or those linked to ion channels via a second messenger system (metabotrophic receptors). Of the ionotropic receptors for glutamate, mossy fiber synapses have few *N*-methyl-d-aspartate (NMDA)-type receptors. The most abundant receptor at this mossy fiber synapse is α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-sensitive (AMPA) receptors. These are mostly located postsynaptically on the pyramidal neuron. Immunohistochemical localization of AMPA-type receptor subunits reveals that in the normal hippocampus, the GluR1 subunit proteins are diffusely expressed on dendrites of neurons; in contrast, the GluR2/3 subunit proteins are strongly expressed on the soma and proximal dendrites of pyramidal neurons, and clumps of punctate GluR2/3 immunoreactivity appear around smaller interneurons. Expression profile analysis performed on individual CA3 and CA1 pyramidal neurons, using a novel single cell RNA amplification methodology along with cDNA array analysis, shows that CA3 pyramidal neurons have significantly higher expression levels of AMPA receptor subunits GluR1 and GluR2 compared with CA1 pyramidal neurons. In the epileptogenic hippocampus of human TLE patients, while immunoreactivity is decreased due to neuronal loss, there is an increased expression of GluR1 on the dendritic excrescences on the proximal dendrites of CA3 pyramidal neurons that survive (**Figure 1**). Electron microscopic examination confirms that this increased GluR1 immunoreactivity in CA3 is postsynaptic and localized on the dendritic excrescences. Mossy fiber terminals make contact with these more intensely immunoreactive dendritic excrescences, suggesting that glutamatergic synapses on surviving CA3 pyramidal cells in the epileptogenic hippocampus probably have more robust responses to glutamate released by mossy fibers at the CA3 synapse.

The kainate family of glutamate receptors is made up of five subunit proteins – GluR5-7 and KA1-2. High-affinity receptor binding studies show that the KA receptors are localized in the mossy fiber pathway and appear to be located on the mossy fiber presynaptic terminal. Both sclerotic and nonsclerotic hippocampi from TLE patients have decreased GluR6 subunit mRNA hybridization densities per CA3 pyramid (as seen in in situ hybridization studies) compared with neurologically normal hippocampi (from autopsy tissue), while only the sclerotic hippocampi have decreased KA1 subunit.

The disposition of the metabotropic glutamate receptors in CA3 has also been studied. In the normal hippocampus, of the group 1 receptors, mGluR1b subunits are localized on the somata and proximal dendrites of pyramidal neurons; weak immunoreactivity for mGluR5 is reported in the CA3 molecular layer. Group 2 receptors mGluR2/3 are expressed in the stratum lucidum as distinct granular staining. Of the group 3 metabotropic receptors, mGluR4a is found consistently in the stratum lucidum, probably on mossy fibers, while mGluR8 is expressed on pyramidal neurons. In general, mGluR subtype expression in CA3 does not show any special pattern of reorganization in the epileptic brain; mGluR5 and mGluR8 subtypes are found on surviving CA3 pyramidal neurons at about the same density per neuron as in controls.

GABA Receptors

Area CA3 also has several GABA receptor subtypes. There are two major classes of GABA receptors – $GABA_A$ and $GABA_B$. Fast inhibitory action of GABA is mediated by the $GABA_A$ receptors. These receptors are composed from several subunits derived from multiple gene families (α1–α6, β1–β3, γ1–γ3, δ, ε π, θ), which are assembled as pentameric structures. The preferred pentameric structures in the hippocampus consist of two α-, two β-, and one γ- or δ-subunit. The localization of

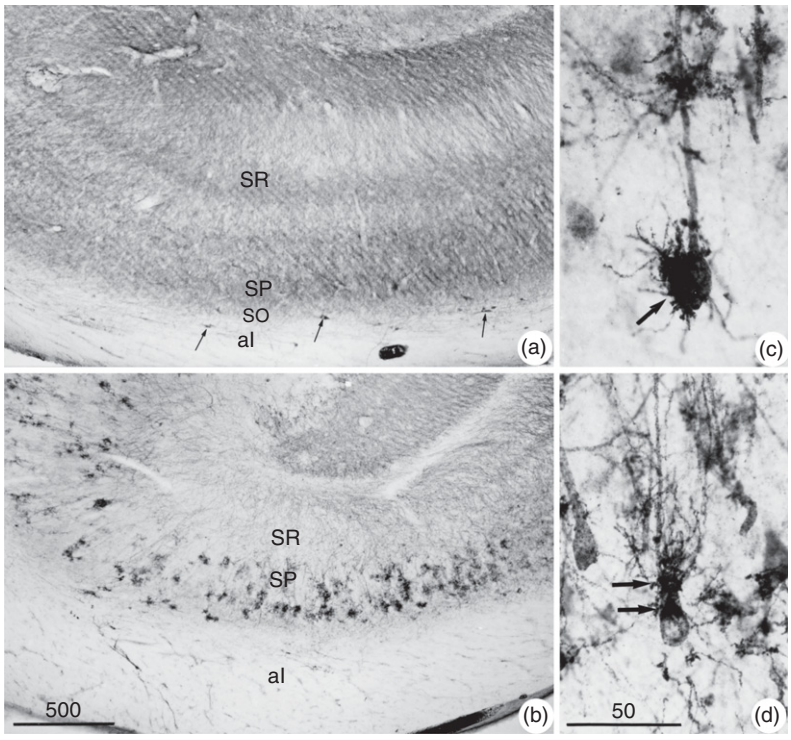


Figure 1 Photomicrographs from area CA3 showing GluR1-like immunoreactivity. (a) A portion of CA3 from a MaTLE (nonsclerotic) hippocampus showing a pattern of diffuse staining. Note the bands of darker staining that correspond to the stratum pyramidale (SP), a region of the stratum radiatum (SR) and the stratum lucidum. In the stratum oriens (SO) can be seen immunoreactive cell bodies (arrows). (b) Area CA3 from a sclerotic patient (MTLE) showing punctate clumps of staining on the cell body and proximal dendrites of neurons. (c) and (d) show individual neurons from area CA3 of MTLE, illustrating the pattern of punctate staining on these neurons. Al, alveus. Bar = 500 μm for (a) and (b), and 50 μm for (c) and (d). Adapted from de Lanerolle NC, Eid T, von Campe G, Kovacs I, Spencer DD, and Brines M (1998) Glutamate receptor subunits GluR1 and GluR2/3 distribution shows reorganization in the human epileptogenic hippocampus. *European Journal of Neuroscience* 10: 1687–1703.

some of these subunits – α1, α2, α3, β2, 3, and γ2 – has been studied in both the normal and epileptic human hippocampus. In both normal autopsy and nonsclerotic surgically removed hippocampi, there is abundant α2-subunit immunoreactivity in area CA3; immunoreactivity is most intense in the pyramidal layer, with lesser intensity in stratum lucidum and stratum radiatum. In sclerotic hippocampi, the intensity of expression of the α2-subunits parallels the degree of neuronal loss in CA3, showing no upregulation on surviving neurons. There is very little expression of the α3-subunit in CA3. Likewise, there is no α1-subunit staining on pyramidal cell bodies or dendrites. Most of the α1-subunit immunoreactivity in CA3 is in interneurons. In the pyramidal layer, these interneurons consist of various classes, including large multipolar cells with dendrites oriented perpendicular to the pyramidal cell layer, medium-sized oviform cells with dendrites that extend into the stratum radiatum, and small round interneurons. Alpha1-subunit expressing neurons with round to oval cell bodies and fine dendrites are seen in the stratum lucidum. In sclerotic hippocampi, there is a marked and significant loss of α1-subunit expressing interneurons in the CA3 pyramidal layer and stratum lucidum, whereas those in the stratum lacunosum-moleculare are preserved.

The $GABA_A$ beta subunits β1 shows comparable expression levels in CA3 of autopsy and sclerotic hippocampi, while increased expression is observed in nonsclerotic surgically removed hippocampi. The β2-subunit is expressed on pyramidal neurons and their processes in area CA3 of normal hippocampi; in sclerotic hippocampi, expression of this subunit is significantly stronger than in autopsy specimens, but similar to nonsclerotic specimens, thus suggesting that its expression may be related to a general effect of seizure activity. β3-Subunit expression is weak in CA3. The subunit γ2 shows diffuse but comparatively stronger expression than the β-subunits in the pyramidal layer of CA3. Expression levels are decreased in sclerotic hippocampus, parallel with neuronal loss. It is difficult, on the basis of published studies on $GABA_A$ β- and γ-subunit expression, to establish any distinctive pattern of change in expression of these subunits in CA3 pyramidal neurons that may be related to the causation or maintenance of seizures.

The $GABA_B$ receptors are metabotropic receptors, which generate long-lasting inhibitory postsynaptic potentials. Two $GABA_B$ receptors, $GABA_{B1}$ and $GABA_{B2}$, have

been identified; the functional receptors are dimeric, formed by isoforms of these two subunits. As presently understood, while both subunits are required for a functional membrane receptor, the $GABA_{B1}$ seems to provide the binding domain whereas the $GABA_{B2}$ is responsible for second messenger processing. Localization of $GABA_B$ receptors by receptor autoradiography with the receptor antagonist [^{3}H]-CGP62349 showed that while the overall binding density was decreased in CA3 of the sclerotic hippocampus compared with controls, $GABA_B$ expression per remaining neuron was increased, suggesting an upregulation of receptors in the epileptic focus. However, another study, which used a different ligand ([^{3}H]-CG54626A), while corroborating the decrease in binding in CA3 in sclerotic hippocampus failed to find an increase in binding when binding densities were adjusted per remaining neuron. Localization of mRNA expression of two $GABA_{B1}$ receptor isoforms ($GABA_{B1a}$ and $GABA_{B1b}$) and $GABA_{B2}$ subunit by in situ hybridization shows that expression levels for $GABA_{B1b}$ were decreased before correction for surviving neuron numbers, but not when per neuron levels are determined. However, $GABA_{B2}$ expression was increased per surviving neuron in area CA3 of the sclerotic hippocampus.

Other Receptors

There have been few published systematic studies on the localization of receptors for other neurotransmitters released by mossy fibers on their targets in area CA3. Whereas dynorphin is a prominent neurotransmitter in this system (being colocalized with glutamate in mossy fiber terminals projecting to area CA3), the expression of the κ1-opiate receptor (the presumed receptor for dynorphin) is only very weakly expressed in this area (though more pronounced in CA1 where there is no dynorphin detected immunohistochemically). The Y2 receptor for neuropeptide Y (NPY), examined by receptor autoradiography, is reported to be upregulated in the stratum radiatum of CA3 in sclerotic hippocampi but not in nonsclerotic hippocampi removed during epilepsy surgery. Y1 receptor levels have not been determined.

In summary, our present state of knowledge regarding the localization of receptor subunits in area CA3 of the epileptogenic (sclerotic) hippocampus in humans suggests that surviving pyramidal cells in these hippocampi show but a few significant receptor subunit changes. Primary among these is the AMPA glutamate receptor subunit GluR1, whose postsynaptic expression at proximal dendrite thorny excrescences is strongly upregulated at synapses with mossy fiber terminals. This change suggests the possibility of increased excitatory drive onto these neurons. There is also somewhat equivocal evidence for the upregulation of $GABA_B$ receptors, particularly the $GABA_{B2}$ subunit (with the $GABA_{B1}$ levels remaining the same per neuron), suggesting the possibility of increased slow but prolonged inhibition of these CA3 cells – although these effects will depend on the precise pre- or postsynaptic localization of these $GABA_B$ receptors. The net functional outcome of enhanced receptor expression for excitatory and inhibitory inputs to CA3 pyramidal cells needs to be elucidated. In addition, there are changes in the expression of presumptive presynaptic receptors on mossy fiber terminals; a decrease in the KA1 subunit on mossy fiber terminals would presumably facilitate glutamate release, whereas an increase in NPY receptor subtype Y2 would tend to inhibit glutamate release. These changes add a further level of complexity to understanding the overall level of excitability of CA3 pyramidal cells, and the underlying mechanisms, in the epileptic brain.

Challenges for the Future

A big challenge in understanding the role area CA3 plays in the human epileptic hippocampus involves identifying the time course of the changes in distribution and localization of receptors, and their subunits, in the transformation of a normal hippocampus into an epileptogenic one. Human hippocampi available for analysis are usually from patients who have many years of seizure disorder before coming to surgery. In many of these hippocampi, there is already considerable neuronal loss – and in some, very few neurons persist. It is difficult to imagine that in such specimens, surviving CA3 pyramidal cells play a significant direct role in the overall epileptic activity of the hippocampal seizure focus. The careful study of hippocampi when removed for seizure control from children with a shorter history of epilepsy may provide important information on pathogenesis. Furthermore, our knowledge of the organization of area CA3, even in the nonsclerotic human hippocampus, remains fragmentary. Knowledge of the exact subcellular localization of receptor subtypes, as may be elucidated from careful ultrastructural analysis of the synaptic organization of this region, is presently lacking and would be valuable in interpreting electrophysiological data obtainable from in vivo and slice electrophysiology of this region. Such electrophysiological studies carried out on the CA3 region in human surgical material are quite sparse at present. As a nodal point in the spread of excitability through the hippocampus, understanding receptor localization and other changes in area CA3 during pathogenesis of epilepsy remains important for a proper understanding of the role of the hippocampus in the epileptic brain. Since the neurochemical organization of the human hippocampus is considerably different from that of the commonly studied experimental models in the rat, elucidation of these details present a considerable challenge.

See also: **Glia**/**Astrocytes**: Properties of Glia in Epileptic Brain; **Glutamate**: Persistent Changes in Network Excitability Induced by Group I Metabotropic Glutamate Receptor Activation: Impact on Seizure Expression and Implications for Epileptogenesis; **Plasticity**: Axon Sprouting in Epilepsy; **Pyramidal Cells**: Membrane Properties and Synaptic Circuitry of CA3 Pyramidal Neurons that Promote Epileptiform Neuronal Synchronization; **Temporal Lobe Epilepsy**: Altered GABA Receptor Subunit Composition in Temporal Lobe Epilepsy.

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A New Look into Epilepsy as a Dynamical Disorder : Seizure Prediction, Resetting and Control

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Introduction

Epileptic seizures are manifestations of epilepsy, a common dynamical disorder second only to stroke among neurological disorders. Of the world's ~50 million people with epilepsy, about one-third have seizures that are not controlled by antiepileptic drugs (AEDs). One of the most disabling aspects of epilepsy is the seemingly unpredictable nature of seizures. If seizures cannot be controlled, the patient experiences major limitations in family, social, educational, and vocational activities. In addition, status epilepticus (SE), a life-threatening condition in which seizure activity occurs continuously, is often successfully treated only with extreme intervention. Until recently, the general belief in the medical community was that epileptic seizures could not be anticipated. Seizures were assumed to be abrupt transitions that occurred randomly over time. However, theories based on reports from clinical practice and scientific intuition, such as the 'reservoir theory' postulated by Lennox, have pointed to the possibility of seizure predictability. Various feelings of auras (patients' reports of sensations of an upcoming seizure) are common in medical literature.

The ability to predict epileptic seizures significantly before their occurrence (onset) may lead to novel diagnostic tools and treatments for epilepsy. Electromagnetic stimulation and/or administration of antiepileptic drugs during the preictal period may disrupt the observed dynamical entrainment of normal brain sites with the epileptogenic focus, and thus lead to a significant reduction of epileptic seizures. Evaluation of antiepileptic drugs and protocols, within the context of a patient's seizure susceptibility period and/or preictal period – as detected by seizure prediction algorithms – may lead to the design of new, more effective drugs with fewer side effects.

Background

The 1980s saw the emergence of new signal processing methodologies, based on the mathematical theory of nonlinear dynamics, for the study of the spontaneous formation of organized spatial, temporal or spatiotemporal patterns in physical, chemical, and biological systems. These methodologies quantify the complexity and randomness of the signal from the perspective of dynamical invariants (e.g., dimensionality of the attractor through correlation dimension, or entropy rate through Kolmogorov entropy), and represent a drastic departure from the signal processing techniques based on the linear model (e.g., Fourier analysis).

The dynamical hypothesis changed some long-held beliefs about seizures. Iasemidis, Sackellares, and colleagues reported the first evidence that the transition to epileptic seizures may be consistent with a deterministic process, and that the ictal electroencephalogram (EEG) can be better modeled as an output of a nonlinear system rather than a linear system. The existence of long-term preictal periods (order of minutes) was initially shown using nonlinear dynamical analysis of EEG from subdural arrays over the temporal lobe, and later from scalp and depth EEG; this demonstration raised the feasibility of developing seizure prediction algorithms by monitoring the temporal evolution of the short-term Lyapunov exponents (STL_{max}), measures of the chaos and instability of a system. The possibility of focus localization and seizure detection with these techniques was also reported.

Our group has described a progressive preictal increase of spatiotemporal entrainment (dynamical synchronization) between the critical sites of the brain as a precursor to epileptic seizures. The algorithm used in this demonstration was based on the entrainment (statistical convergence) of STL_{max} among critical electrode sites that are selected adaptively over time. This observation has been successfully implemented in the prospective prediction of epileptic seizures. Global optimization techniques were applied for selecting the critical groups of electrode sites. We have reported an average anticipation time of about 71.7 minutes prior to a predicted seizure, prediction of 80% to 90% of seizures (sensitivity), and a false prediction rate of 0.12 false warnings per hour. A by-product of this analysis was the finding that the majority of seizures (81%)

in patients with temporal lobe epilepsy (TLE) irreversibly reset (disentrain) the observed preictal dynamical entrainment postictally. This finding, combined with the observed nonresetting of brain dynamics during the preictal period, implies that seizures occur when there is a need to reset the brain's dynamics. Recent reports claim that what characterizes the preictal period is disentrainment rather than entrainment of brain dynamics. However, we have found that this 'reverse' concept of preictal disentrainment is either nonexistent or not consistent across seizures in the same patient, and/or across patients.

Employing neuronal population models that are capable of exhibiting seizurelike behavior, we have shown that entrainment (disentrainment) of the populations' STL_{max}, with increased (decreased) coupling between populations, resembles the observed preictal dynamical entrainment and postictal disentrainment of the STL_{max} at critical sites in the epileptic brain. In agreement with burst phenomena in adaptive systems, 'seizures' in these models occur if the existing (internal to the models) feedback loops are pathological so that they lack the ability to compensate soon enough for excessive increases in network coupling. This situation eventually leads to seizurelike transitions. Motivated by these findings, we postulated the existence of an internal pathological feedback action in the epileptic brain; subsequently, using a control-oriented approach, we developed a functional model for an external seizure controller. During periods of abnormally high synchrony (entrainment), the developed control scheme provides appropriate 'desynchronizing feedback' to maintain 'normal' synchronization levels between neural populations (homeostasis of dynamics). We have called this feedback control view of epileptic seizures 'feedback decoupling'; the developed seizure control strategies, were then validated on coupled chaotic oscillator models and biologically plausible neurophysiologic models.

Methods

The brain is inherently a nonlinear system. Therefore, it makes sense to move one step forward from linear methods of analysis (methods like Fourier transform and linear models), that can accurately analyze and characterize only linear systems, to methods that can analyze and correctly characterize linear and nonlinear systems. This move has progressively produced surprisingly refreshing results for epilepsy. Under certain conditions, through the method of delays, sampling of a single variable of a nonlinear system over time could determine all state variables of the system that are related to it. In the case of the EEG, this method can be used to reconstruct a multidimensional state space of the brain's electrical activity at a corresponding brain site. A dimension of 7 has typically been used to reconstruct the state space from EEG in epilepsy. This selection of dimension for the states (vectors) in the state space, together with a corresponding time delay of 20 msec between their components, has been justified in the literature.

Among the important measures of the dynamics of a linear or nonlinear system are the Lyapunov exponents that measure the average information flow (bits/sec) a system produces along local eigendirections through its movement in its state space. Positive Lyapunov exponents denote generation of information while negative exponents denote destruction of information. A chaotic nonlinear system possesses at least one positive Lyapunov exponent, and it is because of this feature that its behavior looks random, even though as a system it is deterministic. Methods for calculating these measures of dynamics from experimental data have been published. The estimation of the largest Lyapunov exponent from a chaotic system has been shown to be more reliable and reproducible than the estimation of the remaining exponents, especially when the actual value of the dimension of the state space is not known and changes over time. The brain, being nonstationary, is never in a steady state in the strictly dynamical sense, at any location. We have shown that, in the case of a nonstationary system with transients like epileptic spikes, using the short-term maximum Lyapunov exponent (STL_{max}) is a more accurate characterization of the average information flow than the one using the regular maximum Lyapunov exponent. STL_{max} is estimated from sequential EEG segments of 10 sec duration per recording site to create a set of STL_{max} profiles over time for all recording sites. Over the past 20 years, analyzing scalp, subdural, and depth EEG from patients with TLE and frontal epilepsy, we have shown that the STL_{max} profiles at brain sites systematically converge to similar values tens of minutes before a seizure. We have called these brain sites 'critical sites.' The seizure focus sites were found to be included in these sets of critical sites more consistently and more frequently than other sites; thus, a by-product of this methodology appears to be the localization of the epileptogenic zone. We have called this phenomenon *dynamical entrainment*, because measures of *dynamics* (like STL_{max}) appear to converge *progressively* over time and then typically stay entrained until the seizure's electrographic onset. We have also shown that this finding is very specific to seizures versus other interictal time points, and as such it has had wide implications – for example, the development of the first real-time online prospective seizure prediction algorithms. In short, the epileptic brain appears to be entrained by the focal sites, and therefore starts malfunctioning due to a loss of relative independence of processing information at normal brain sites, long before a seizure develops.

A statistical measure of entrainment between two brain sites i and j, with respect to a measure of their dynamics (e.g., STL_{max}), has been developed in the past. Specifically,

the T_{ij} between measures at electrode sites i and j and at time t is defined as

$$T_{ij}(t) = \frac{\hat{D}_{ij}(t)}{\left[\frac{\hat{\sigma}_{ij}(t)}{\sqrt{m}}\right]},$$

where $\hat{D}_{ij}(t)$ and $\hat{\sigma}_{ij}(t)$ denote the sample mean and standard deviation respectively of all the m differences $D_{ij}(t)$ between the STL_{max} values (one STL_{max} value is produced per 10 sec EEG segment) at electrodes i and j, within a moving window $w(t) = [t,\ t\text{-}m^*10\ \text{sec}]$. If the true mean $\mu_{ij}(t)$ of the differences $D_{ij}(t)$ is equal to zero, and $\hat{\sigma}_{ij}(t)$ are independent and normally distributed, $T_{ij}(t)$ is asymptotically distributed as the t-distribution with *(m-1)* degrees of freedom. We have shown that these independence and normality conditions are satisfied in EEG. Therefore, we define disentrainment (dynamical desynchronization) between electrode sites i and j when $\mu_{ij}(t)$ is significantly different from zero at a significance level α. The desynchronization condition between the electrode sites i and j, as detected by the paired t-test, is $T_{ij}(t) > t_{\alpha/2,m-1} = T_{th}$, where $t_{\alpha/2,m-1}$ is the $100\cdot(1\text{-}\alpha/2)\%$ critical value of the t-distribution with *m-1* degrees of freedom. If $T_{ij}(t) \leq t_{\alpha/2,m-1}$ (which means that we do not have satisfactory statistical evidence at the α level that the differences of values of a measure between electrode sites i and j within the time window w(t) are not zero), we consider that sites i and j are dynamically synchronized with each other (with respect to the utilized measure of synchronization) at time t. Using $\alpha = 0.01$ and $m = 60$, that is using a window $w(t)$ of 10 min in duration, the threshold $T_{th} = 2.66$. It is noteworthy that similar STL_{max} values at two electrode sites (and therefore low values of the T-index) do not necessarily mean that these sites even interact. However, when there is a progressive convergence (synchronization) over time of the measures at these sites, the probability that they are unrelated diminishes.

This is exactly what occurs before seizures, and it is illustrated in **Figure 1**. In this Figure, we plot the *average* T-index over time – before, during, and after a seizure of temporal lobe origin. Each average T-index value is estimated per moving 10 min window $w(t)$ and over selected pairs of sites. In particular, the average T-index shown in **Figure 1** (black line) is the average of all T_{ij} of selected pairs of sites (i, j) within $w(t)$ over time t. The selected pairs of sites are the ones that were significantly entrained (i.e., $T_{ij} \leq 2.66$) within a preictal window of 10 min in duration, positioned immediately before the seizure's electrographic onset. We observe significantly low values of T-index (i.e., convergence of the STL_{max} profiles of critical sites) long (90 min) before this seizure's onset. Moreover, the trend towards low values of T-index started 120 min before the seizure's onset; within the next 30 min, the critical pairs of sites became progressively

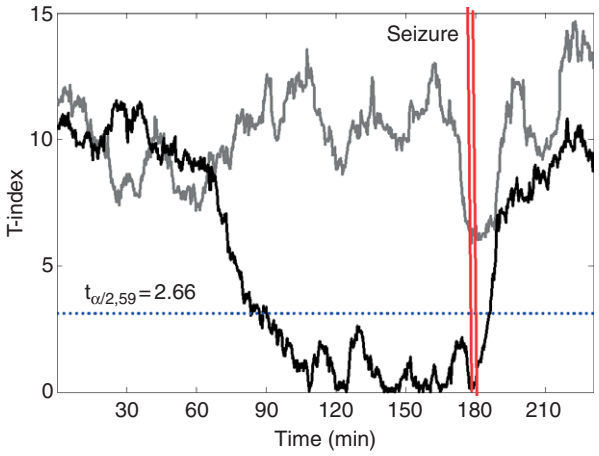


Figure 1 Dynamical resetting at seizures. Black line: Long-term, progressive dynamical entrainment preictally, and short-term disentrainment postictally, of all pairs of sites entrained 10 min prior to a seizure of temporal lobe origin (seizure occurs between the two vertical red lines). These critical pairs of sites get entrained ($T_{ij} \leq 2.66$) approximately 90 min before the seizure. Grey line: No trends indicative of neither the upcoming seizure nor resetting can be observed when pairs of maximally disentrained ($T_{ij} \geq 6.00$) sites are selected within 10 min prior to the seizure. The dynamical analysis was performed on subdural and depth EEG recordings from a patient with TLE admitted at the epilepsy monitoring unit (EMU).

entrained and stayed entrained thereafter, until the seizure's onset. The seizure lasted for 2.5 min (between the two vertical red lines in this Figure). After the seizure's onset, we observe a trend towards disentrainment of the preictally entrained pairs of sites (T-index moves rapidly towards higher values above T_{th}). Within 10 min postictally, disentrainment is established. We have called this reversal of dynamics *brain resetting*. While Morman and colleagues have proposed that preictal desynchronization may be a better precursor to seizures than preictal synchronization, this hypothesis does not seem to be valid. For example, the grey line in **Figure 1** is the counterpart of the black line; that is, it is generated from monitoring preictal desynchronization rather than preictal synchronization. In the preictal desynchronization scheme, maximally desynchronized ($T_{ij} \geq 6.00$) pairs of sites were analogously selected from the 10 min preictal window immediately before a seizure onset. No long-term preictal (or postictal) trends over time are observed in this scheme (see grey line), therefore it is ineffective for seizure prediction. We have shown in the past that the observed dynamical resetting is specific to seizures at the $\alpha = 0.05$ statistical significance level. This observation may reflect a passive mechanism (e.g., high electrical activity during a seizure might deplete critical neurotransmitters and thus deactivate critical neuroreceptors in the entrained neuronal network). An alternative explanation is that the release of neuropeptides in the brain, caused by seizure activity, may contribute to the temporary repair of a pathological feedback that allowed

the dynamical entrainment to occur in the first place, and then last for long time intervals.

These results demonstrate that seizures are not abrupt transitions in and out of an abnormal ictal state; instead they follow a dynamical transition that evolves over minutes to hours. During this preictal dynamical transition, multiple regions of the brain progressively approach a similar dynamical state. These observations are in agreement with observations in systems of coupled neural population models with internal (i.e., part of the overall system) pathological feedback. We have shown that by increasing the interpopulation coupling and detuning their internal feedback mechanism, these systems move from spatiotemporal chaotic states into spatially synchronized, quasiperiodic, 'seizurelike' states. These transitions are also characterized by spatial convergence of the maximum Lyapunov exponents of the individual populations. Such results directly address the process of ictogenesis and appear to support the hypotheses that: a) seizures may result from the inability of internal feedback mechanisms to provide timely compensation for/regulation of coupling between brain sites, and b) seizure control can be achieved by feedback decoupling of the 'pathological' sites via externally provided appropriate stimuli. Such stimuli are functions of both the EEG and the coupling of the respective brain sites. We have called such a closed-loop control scheme 'feedback decoupling.' The block diagram in **Figure 2** shows the basic elements of our hypothetical scheme of how a 'normal' brain works: a) brain in spatiotemporal chaos, b) stimulus enters the system, and changes the spatial coupling between two or more brain sites, c) spatial coupling produces spatial correlations, possibly storing the information about the stimulus and/or initiating action, d) spatial correlations also activate an internal compensating feedback mechanism, e) compensation removes (or assimilates) the developed spatial correlations by the stimulus, f) the system returns to spatiotemporal chaos. In the 'normal brain,' the correlations in the network must lie within a 'normal' range and be able to vary quickly in response to a stimulus – which implies that the internal feedback path should be well-tuned and be able to track changes of the coupling reasonably closely. Accordingly, we propose that the pathologic epileptic brain has pathologic (poorly tuned) internal feedback paths. Such pathology exhibits large errors in the estimates of the coupling, and can cause local destabilization and bursting of the network (seizures). Thus, the observed long-term dynamical entrainment prior to seizures can be interpreted as an indicator of pathology in the internal feedback of the network. This rationale can, of course, easily be applied to 'reflex' epilepsies too.

Recent Results

Given the phenomenon of resetting of brain dynamics associated with a seizure as described earlier, one would predict that status epilepticus (SE) is associated with the failure of seizures to reset the brain. That is what we have actually observed in EEG records analyzed from patients in the emergency room (ER), the intensive care unit (ICU) or the epilepsy monitoring unit (EMU). **Figure 3** illustrates this point in a 75 year old patient brought to ER after he was found unresponsive at home with his right arm twitching and his head deviated to his right. When the patient arrived, he was having rhythmic contractions in his right thigh adductor muscles, followed soon after by tonic–clonic movements on his right side accompanied by right gaze deviation and nystagmus. An EEG confirmed the diagnosis of status epilepticus. The etiology of the patient's seizures was noted as encephalomalacia in the left frontal cortex likely due to a prior stroke. The EEG

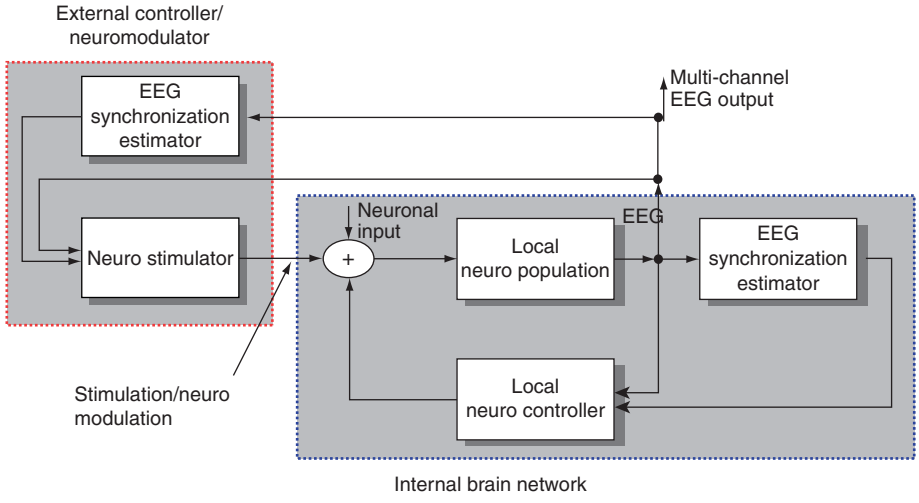


Figure 2 The concept of feedback decoupling for the control of epileptic seizures.

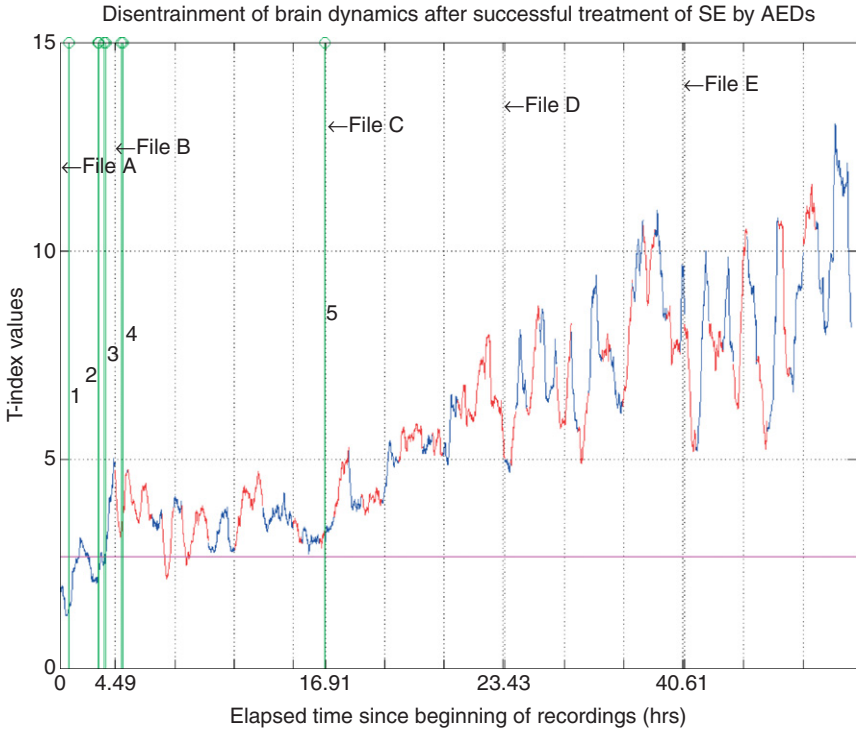


Figure 3 Long-term resetting of the brain dynamics in a patient with status epilepticus via successful administration of AEDs. The effectiveness of AEDs on the patient's recovery is correlated well with the exhibited trends of the T-index of the critical pairs of brain sites. The critical pairs for the analysis were selected 10 min before the beginning of the treatment of the patient with the first AED (a scalp EEG dynamical analysis). The vertical green lines 1 through 5 denote the times of AED administration (see below). The File vertical lines A through E mark the beginning of the five available EEG segments (of total duration of 13.51 hours) for the dynamical analysis (the existing gaps in time between the Files are not shown – the results from the dynamical analysis of the files are concatenated in time – Files A through E were of 1.10, 3.54, 3.03, 3.03 and 2.81 hours in duration respectively). The dynamical analysis shows that the patient was safe (high T-index values above $T_{th} = 2.66$) and stable (high T-index values for a long time) approximately 20 hours following admission to the emergency room. The patient was initially treated with 2 mg of Ativan IV followed by 20 mg/kg of phenytoin equivalence IV and a second fosphenytoin bolus of 5 mg/kg. Line 1 marks the completion of fosphenytoin infusion. Approximately half an hour after the beginning of the EEG recording, the patient was transferred to the ICU. The patient did not respond to the fosphenytoin so he was intubated and placed on a Propofol drip. Soon after, the EEG showed a burst suppression pattern. Line 2 marks the administration of Fentanyl and Etomidate. Line 3 marks when 50 mg of Propofol was administered and the beginning of a 30 mcg/kg/min propofol infusion. Line 4 marks a fosphenytoin 350 mg infusion and propofol administration at 50 mcg/kg/min. An additional dose of 1000 mg of levetiracetam IV was administered along with a propofol drip. Finally, line 5 marks where propofol is running at 35 mcg/kg/min. In the last EEG recording available to us (File E), the patient remained off from propofol.

was recorded using scalp electrodes at a sampling rate of 200 Hz. The patient recovered with AED treatment. The correspondence of the T-index values to the changing medical condition of the patient over time (see **Figure 3** legend) is remarkable, as is the correspondence of the effect (success or failure) of the different AEDs administered to the T-index; high T-index values were observed for successful resetting by an AED, and low values were observed when the influence of the AED was temporary (and therefore unsuccessful). The long-term recovery trend in the T-index over days indicates that this measure could be used for the detection of a patient's seizure susceptibility; that is, this measure could identify periods when a patient might be more vulnerable to seizures.

While seizures can currently be anticipated with relatively good sensitivity and specificity, the question is whether we can effectively intervene to change the pathological brain dynamics (long-term entrainment) in a timely manner and prevent a seizure from occurring. Towards this end, we first showed that impulsive electrical stimulation can temporally disentrain the dynamics of a rat's epileptic brain (larger T-index values post- than prestimulus). Second, we utilized our adaptive seizure prediction algorithm in the feedback external branch of a closed-loop electrical stimulation scheme for seizure control. In the baseline mode (no stimulation, just spontaneous occurrence of seizures), the sensitivity and specificity of the seizure prediction algorithm were tested on epileptic rats with chronic epilepsy, and very similar values to the ones we have reported from patients with focal epilepsy were found. In the control mode, the seizure prediction program was used to analyze the recorded EEG from the rat's brain (continuous real-time estimation of STL_{max} and T-index every 10 sec), issuing seizure

warnings and triggering the stimulator to stimulate the brain at each warning.

In this study, the lithium-pilocarpine (LP) model of acute SE in rats was chosen as the animal model for chronic epilepsy due to the similarities it exhibits with human TLE. Male Sprague–Dawley rats were used for the study. Three to four weeks after PI-induced SE, rats were implanted with a six-microwire monopolar electrode array targeted to four cortical and two hippocampal locations; two Teflon-coated tungsten bipolar twisted stimulating electrodes were implanted in the centromedial thalamic nucleus. A stimulator/seizure prediction program was developed in-house to control an A-M Systems Model 2300 stimulator unit (Calsborg, WA). At a seizure warning, a train of square pulses of amplitude 600 μA, pulse-width 100 μsec, and frequency 130 Hz was administered across the centromedial thalamic electrodes. The train of pulses was 1 min in duration and was delivered in a charge balanced bipolar cathodic fashion.

Some results from this experiment are shown in Figure 4. The seizure rate reduced sharply within 1 day following the onset of the closed-loop control scheme described earlier. The rat became seizure-free during the third and fourth days, but seizures slowly started to reappear thereafter. As can be observed in this Figure, the seizurefree state was attained only when resetting by the externally provided stimulus was achieved. Subsequent open-loop periodic stimulation (that is, without the parallel running of the seizure prediction software) in the same rat, over 10 days with the same stimulus parameters, and rate of stimulation equal to the mean rate of stimulation by the closed-loop control scheme, did not reduce the rat's seizure rate at all. This result implies that the timing of stimulation is critical for seizure control (keeping the same rate of stimulation is not enough). There are two very important observations from this study. First, the T-index values are inversely proportional to the seizure rate, denoting that entrained dynamics increase the probability of seizure occurrence (and thus increase the seizure rate), whereas disentrained dynamics do not (and thus decrease the seizure rate). This point is in agreement with our previous results and concepts. Second, in the portion of the record in which seizure control was not achieved (days 5 to 11), the electrical stimulation protocol we utilized failed to stop the recurrence of seizures – as in SE with unsuccessful AED administration. We suggest that this

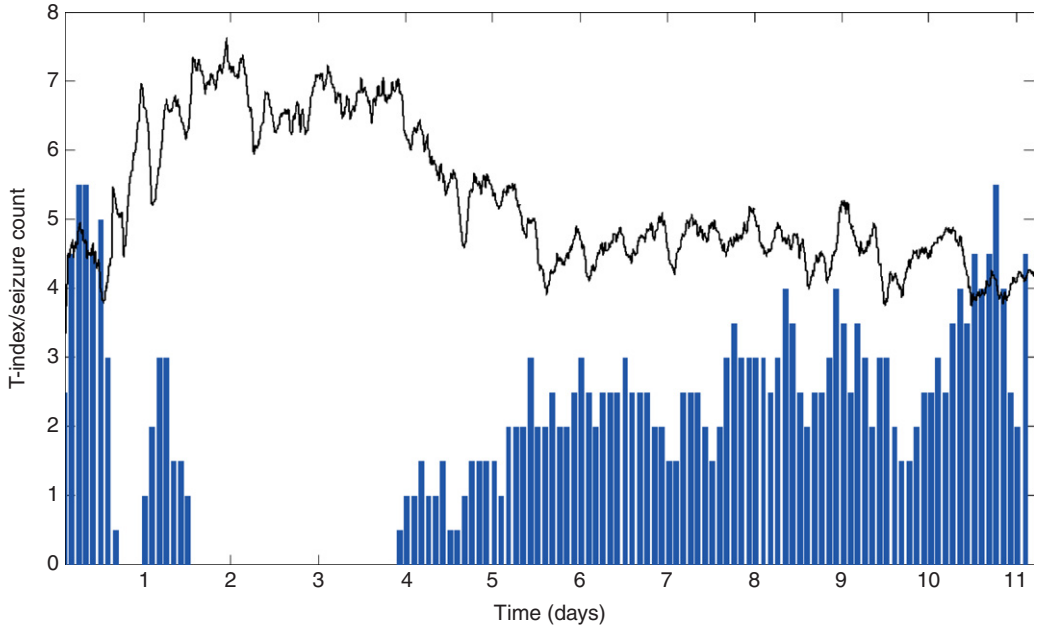


Figure 4 Real-time closed-loop control of seizures in an epileptic rat: On-line seizure prediction algorithm in conjunction with short electrical stimulation delivered to the thalamus when warnings were issued by the seizure prediction algorithm. The T-index profile of critical sites selected from baseline (black line) and the number of seizures exhibited per 2 hour of recording (blue bars) are plotted together (time resolution of the calculations for the T-index curve is 10 min, and for the rate of seizures is 2 hours). In accordance with our hypotheses, it is observed that successful control of seizures (e.g., almost no seizures in days 3 and 4) corresponds to high T-index values, that is, to brain's dynamical disentrainment. Respectively, low values of T-index (high level of entrainment) correspond to failure of seizure control and occurrence of seizures. All EEG data in this study were recorded on a Beehive Millenium® LTM EEG machine (Grass-Telefactor Inc., West Warwick, RI). The EEG machine included two 32-channel preamplifiers with front end digital sampling at 800-Hz which was multiplexed to a PAN-Isocoder unit that down-sampled and saved the data digitally at 200-Hz. Recorded EEG data were bandpass filtered from 0.3—70-Hz (including a 60 Hz notch filter) and written to a network hard disc in 1-min file segments. All recordings were done in a referential montage.

second observation calls for a new type of stimulation that should be incorporated into a more advanced and successful closed-loop control. One such candidate scheme can be the feedback decoupling scheme for seizure control we have proposed. Its application to biologically plausible simulation models with 'seizures' has generated results that can explain the successes and failures of both the open and closed-loop control schemes presented above. In addition, it was shown that the feedback decoupling scheme outperformed both these experimental control schemes presented above with respect to the control of 'seizures' in those models.

Future Goals

In summary, from our group's past and ongoing research on the dynamics of epilepsy, the following three central concepts about epileptic seizures have emerged. First, we have shown that seizures are manifestations of the recruitment of brain sites in an abnormal hypersynchronization. The onset of such recruitment occurs long before the onset of a seizure (on the order of minutes with respect to seizure prediction, on the order of hours and days with respect to seizure susceptibility), and progressively culminates in a seizure. Second, time-irreversible resetting of the observed preictal dynamical recruitment occurs postictally (via a hysteretic loop). Preictal and postictal periods could be mathematically (statistically) defined and detected from dynamical analysis of the EEG. Partial or no resetting of the preictal entrainment of the epileptic brain after a seizure may affect the route to the subsequent seizure (an example of such a situation is the existence of status epilepticus). The partial resetting may also explain the observed nonstationary nature of the seizure occurrences. Third, through control-oriented modeling, a feedback control view of epileptic seizures has been postulated, wherein epileptic seizures are hypothesized to be a result of the inability of the internal feedback/regulatory mechanisms of the brain to track excessive synchronization changes between the epileptogenic focus and other brain areas prior to a seizure.

All these concepts are interrelated, and constitute the basis for the development of seizure prediction algorithms, epileptogenic focus localization algorithms, and first generation closed-loop control algorithms. These concepts may provide the key ingredients for the development of robust brain pacemakers for the treatment of epilepsy in the near future. In addition, these concepts can be used for the evaluation of the efficacy of existing antiepileptic drugs, as well as for the design of novel drugs that would target the resetting of the pathological dynamics of the epileptic brain. Finally, as shown in a case of SE, the developed measures and methodology could be used as monitoring tools incorporated in medical devices for the evaluation of seizure susceptibility.

Acknowledgements

We would like to acknowledge the support from USA National Institutes of Health (EB002089 BRP Grant on Brain Dynamics), the Barrow Neurological Foundation, the Epilepsy Research Foundation of America and Ali Paris Fund for LKS Research, the National Science Foundation (Grant No. 0601740), and the Arizona Science Foundation (a Competitive Advantage Award grant, 2008).

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; **Models**: Ictogenesis in the in vitro Isolated Guinea Pig Brain; **Neurophysiology**: Seizure Detection and Prediction; **Seizures**: Seizure Prediction.

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Seizure-Induced Gene Expression

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Introduction

Seizures, defined as a pathological increase in neuronal synchronization, are not just the characteristic symptoms of epilepsy, but can also have direct effects on the brain. In particular, prolonged seizures can produce neuronal damage and trigger cascades of biochemical events that can have long-term effects. These seizure events activate second messengers, transcription factors and gene expression, plastic phenomena (such as mossy fiber sprouting), neurogenesis, and altered neurotransmission – all of which may underlie consequent sensory-motor, developmental, and behavioral and cognitive deficits. Seizure-induced gene expression is of particular importance, since production of new proteins (or proteins at altered levels) provides the means for long-lasting alterations in brain functions. Analysis of alterations in gene expression can single out particular molecular mechanisms involved in such long lasting, potentially harmful, effects of seizures.

Background

Regulation of Gene Expression

In this article we focus on seizure-induced gene expression. While approaching this subject, one should be aware that the regulation of gene expression is a very complex phenomenon. By definition, regulation of gene expression refers to the control of the amount and timing of appearance of functional gene products, that is, RNAs and proteins. The process includes transcription as well as posttranslational modification of protein, which can be modulated at any step via any number of mechanisms.

The first level of regulation of gene expression relies on modification of DNA. Chemical modification refers to methylation of CpG islands by methyltransferase, a common way for silencing a gene. Structural modifications of DNA arise from phosphorylation or methylation of histones; histone acetylation is of special importance for regulation of transcription, since histone deacetyltransferases enable the dissociation of DNA from the histones and therefore allow transcription to proceed.

Progression of transcription and the efficiency of RNA synthesis depend on several components of the RNA polymerase machinery, including several regulatory proteins that bind to regulatory sites, usually located near the promoter. The regulatory proteins can be categorized according to their function as: (1) specificity factors that alter specificity of RNA polymerase for given promoter and repressors that bind to noncoding DNA sequences in the vicinity of the promoter and impede RNA polymerase's progress along the DNA strand; (2) basal factors which position RNA polymerase at the start of transcription, and activators that enhance RNA polymerase interaction at a given promoter either by interaction with RNA polymerase subunits or changing the structure of the DNA; and (3) enhancers that lead to changes in the three-dimensional structure of DNA and bring specific promoters into the vicinity of the initiation complex.

When RNA is synthesized, it undergoes further modifications which influence its stability, and therefore determine the yield of translation into protein. Capping protects mRNA from 5′exonuclease, splicing removes introns – noncoding regions – from RNA and allows translation into proper protein, and addition of poly(A) tail increases mRNA's stability by slowing down 3′exonuclease action.

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SEIZURES

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Autonomic Consequences of Seizures, Including Sudden Unexpected Death in Epilepsy

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Introduction – Seizures Cause Autonomic Disturbances

Seizure-induced autonomic dysfunction could cause serious clinical consequences, yet may occur only transiently and escape detection. Signs of autonomic nervous system dysfunction are common in epileptic patients, but are often overshadowed clinically by more apparent motor and cognitive effects of seizures. Small focal (i.e., simple partial) seizures may produce flushing, sweating, and piloerection, while complex partial seizures can produce a broader spectrum of autonomic signs, including changes in heart rate and rhythm (often bradycardia; complete AV block has been reported), blood pressure (usually hypertension), and increased or decreased gastrointestinal motility (usually vomiting) and secretion. Complex partial seizures may also induce relative hypoxia, though the mechanism of this change is not understood. Generalized tonic-clonic seizures may be associated with severe increases in blood pressure and changes in heart rate and conduction, such as complete nodal block. In a subgroup of epileptic individuals, however, seizures may trigger more ominous cardiovascular derangements such as marked bradycardia or asystole. At autopsy, pulmonary edema is the most common finding. Although still poorly understood, the causes of sudden unexpected death in epilepsy (SUDEP) are strongly suspected to involve cardiovascular and/or respiratory dysfunction provoked by seizures spreading into the autonomic nervous system.

Mortality among epileptic individuals is 2–3 times greater than among their nonepileptic counterparts. Among individuals with epilepsy, SUDEP is the single most common cause of death, accounting for as much as 10–15% of mortality in this group and exceeding the mortality attributable to status epilepticus. Estimates of the annual incidence of SUDEP among individuals with epilepsy range from 1 in 1000 to 1 in 500. The average age for SUDEP is 28–35 years, and men in their third or fourth decade have the highest annual risk of SUDEP, as high as 1 in 200. SUDEP occurs in different seizure types, and does not show a consistent predilection for localization-related or primary generalized epilepsy. Further, SUDEP is not restricted to individuals with the most severe seizures. SUDEP incidence, however, is highest in individuals with medically refractory seizures and lowest in individuals with well-controlled seizures. The relative

risk of SUDEP in individuals with two or more seizures per year has been reported to be 20 times greater than the risk in epileptic individuals who are seizure-free, and the increase in risk surpasses 50-fold when the number of seizures in a year is more than 50.

Most studies of the autonomic consequences of seizures have focused on cardiovascular consequences, primarily because these changes are seen as the most significant clinically, but also because considerable information can be extracted from analyses of the ECG. Several analyses of the R-R interval (RRI) – the period of time separating successive heartbeats – reveals RRI fluctuations at the respiratory frequencies that are parasympathetically-mediated, and RRI fluctuations at higher frequencies that are mediated by the combined effects of sympathetic and parasympathetic inputs to the heart. A smaller number of studies monitor other parameters, e.g., blood pressure monitoring by tonometry. Using continuous monitoring of various types, autonomic disturbances have been reported during both ictal and interictal periods. Patients with complex partial seizures had large fluctuations in heart rate during interictal periods that were not seen in controls, and acute deterioration of seizure control was accompanied by increased parasympathetic activity (including bradycardia) before seizure onset. Additionally, some of the dynamic complexity of the autonomic consequences, including baroreceptor reflex function, has been explored. Temporal lobe seizures are frequently associated with bradycardia, but the development of tachycardia during temporal lobe seizures can be proportional to the spread of seizure activity to other cortical areas. Complex-partial seizures were found to be associated with a pattern of parasympathetic activity that fell rapidly about 30 s before seizure onset, and sympathetic activity that continued to rise and peaked at seizure onset.

Although it is clear that the autonomic impact of seizures is broad and complex, it is also clear that clinical studies alone cannot entirely assess the connection between seizures and autonomic dysfunction. Clinical investigations are limited because some phenomena of interest are too infrequent for practical prospective studies, and there are limits to the invasiveness of autonomic monitoring that can be performed with human patients.

Background

Animal Studies of Autonomic Nervous System Dysfunction During Seizures

Animal models offer the advantage of invasive monitoring, but come with the disadvantage of perhaps involving seizure activity unlike that in human epilepsy patients. This disadvantage has been addressed principally by the development of various animal models. Seizures have been caused in different animal species (from rodents to primates) by different means (such as electrical stimulation or chemical convulsants) in different parts of the brain under different conditions (anesthetized or awake).

Seizure induction mechanisms fall into two broad categories: drugs or techniques that enhance excitatory synaptic transmission, and drugs or techniques that reduce inhibitory synaptic transmission. High frequency trains of electrical stimulus pulses can elicit a seizure if the stimuli are large enough in amplitude and given for a sufficient period. Kindling is a technique whereby trains of relatively low amplitude electrical stimuli are repeatedly applied to a region of the brain. Initial stimulation episodes may have no detectable effect, but after repeated presentations of stimuli, each presentation triggers increasingly severe seizures. Chemical convulsants are generally agonists (or modulators) for the glutamate receptor or antagonists (or modulators) of the GABA receptor. Kainic acid is an example of a convulsant that is a glutamate receptor agonist. After a single systemic dose, seizures recur for hours, and a chronic condition can develop after weeks, during which seizures occur spontaneously. Kainic acid-induced seizures appear to begin in the limbic cortices and have led investigators to consider this approach as a model for temporal lobe epilepsy.

Most invasive monitoring is performed in acute anesthetized animal experiments. Some anesthetics permit seizure activity and some abolish seizures. Pentobarbital is a potent anticonvulsant. The combination of ketamine and xylazine permits seizure activity, including motor convulsions. Urethane is interesting as an anesthetic because it permits limbic seizure activity, but not neocortical seizure activity. The hemispherectomized rat is used for acute studies without anesthesia. The entire cortex is removed under short acting anesthesia, and the anesthesia is stopped. In the absence of neo- and limbic cortex, sensory perception and motor activity, including motor convulsions, are abolished. Each animal model offers access advantages for autonomic nervous system studies that can be impossible to obtain when studying patients. Some examples of autonomic consequences of seizures are given here, and more details appear in the listed readings.

Amygdala-kindled seizures in rats were found to activate both sympathetic and parasympathetic systems and produced ictal hypertension and bradycardia that could each be blocked pharmacologically. Left insular cortex stimulation in rats produced an increasing degree of heart block and resultant ventricular escape rhythms that eventually resulted in asystole. In our own rat model, left-sided limbic seizure activity was associated with a more pronounced vagus nerve activity increase and associated blood pressure decrease.

Systemic pentylenetetrazol seizures in cats were shown to be associated with cardiac sympathetic and parasympathetic activity that was intermittently synchronized with interictal spike activity ('lockstep' phenomenon). Unstable

lockstep periods, when interictal spikes and synchronized autonomic activity occurred at irregular intervals, were associated with large fluctuations in blood pressure.

Focal penicillin-G application to the hypothalamus, in hemispherectomized, unanesthetized rats, produced bradyarrhythmias and a small decline in systolic blood pressure. Creating an additional penicillin focus at the mesencephalic level increased paroxysmal activity, and produced severe conduction anomalies (including AV block). Coactivation of hypothalamic and mesencephalic centers by focal injection of penicillin often resulted in death in rats.

Hypoventilation during status epilepticus, in a sheep model of SUDEP, correlated strongly with seizure-induced death; the incidence of benign cardiac arrhythmias, catecholamine levels, seizure type and seizure duration did not differ between animals that died and animals that lived. Mice that exhibit audiogenic seizures frequently display respiratory arrest that can be fatal – and can be prevented by increasing the quantity of oxygen in the environment.

Pathways for Spread of Seizures to the Autonomic Nervous System (ANS)

Neocortex and limbic cortices are heavily connected with structures that constitute the ANS, or with regions that project into autonomic structures. The central nucleus of the amygdala and bed nucleus of the stria terminalis are major inputs to hypothalamic, pontine and medullary structures that control preganglionic autonomic neurons of both the sympathetic and parasympathetic branches of the autonomic nervous system. They are positioned to relay cortical activity into the ANS. Neocortex and limbic cortices, especially subiculum, also have direct projections to hypothalamus. Autonomic pathways are integrated in the hypothalamus and influence all visceral systems, including cardiovascular, genitourinary, endocrine, and digestive, as well as peripheral involuntary muscles such as pilomotor and pupillary sphincters.

Projections of hypothalamic, including pontine and medullary neurons to preganglionic parasympathetic and sympathetic neurons controlling cardiac and respiratory function, have been studied anatomically and physiologically. Hypothalamic cells project onto medullary structures (ventrolateral medulla and periaqueductal gray) that project directly to sympathetic preganglionic cell bodies located in the intermediolateral horn of the spinal cord. Activity of hypothalamic and medullary neurons correlates with sympathetic nerve activity. Some hypothalamic cells themselves project directly into the spinal cord.

For the parasympathetic system, central nuclei of the amygdala and hypothalamus (PVN and SON) project directly into the dorsal motor nucleus of the vagus and the nucleus ambiguus, the sites of parasympathetic preganglionic cell bodies. Furthermore, the sympathetic and parasympathetic paths can influence each other. For example, efferents of the nucleus ambiguus and dorsal nucleus of the vagus (preganglionic parasympathetic neurons) project to the nucleus of the solitary tract which has a relayed projection to intermediolateral horn (sympathetic preganglionic neurons).

It is not surprising, then, that thalamic/hypothalamic discharges elicited by penicillin-G application would be associated with bursts of activity in vagus nerve recordings. We also found bursts of activity in vagus nerve recordings during the slow spiking period at the end of the limbic seizure, although the total amount of vagus nerve activity at these times was much less than we found earlier in each seizure episode. Interestingly, while the types of seizures in these two animal models are quite different, a pathway from hypothalamic areas through the medulla and into the periphery is common, and can account for similarities in vagus nerve discharge.

Methods – Our Acute Rat Model

Our rat model for acute studies of autonomic consequences replicates many features of autonomic disturbances seen in epilepsy patients, and offers some unique advantages. Using kainic acid as the convulsant drug on a background of urethane anesthesia, we produce a prolonged period of recurring limbic seizures that do not significantly involve the neocortex. As a result, animals can be kept in a stereotaxic frame without paralytic agents. The lack of motor convulsions permits stable recordings from the brain, spinal cord, and peripheral nerve, as well as accurate, invasive monitoring of systemic parameters such as blood pressure. We have also developed a simple method for reversibly suppressing seizures in one or both hemispheres repeatedly. The preparation is described briefly here, and more details are given in the readings.

We use male Sprague-Dawley albino rats for most experiments. Six to 8 week old animals (200–350 g) are commonly used. Animals are anesthetized with urethane ($1.5\ g\ kg^{-1}$ ip), fitted with an endotracheal tube, and maintained at 37 °C. Blood pressure is monitored with an intra-arterial line (polyethylene tubing, 0.5 mm id, 0.8 mm od, inserted into the femoral artery) and pressure transducer. An increase in mean arterial pressure, systolic or diastolic pressure (with no increase in pulse pressure) indicates an increase in sympathetic vasoconstrictor tone. Likewise, a decrease in blood pressure reflects a decrease in sympathetic tone. Increases in pulse pressure reflect increased cardiac stroke volume, which occurs physiologically through two mechanisms: At slow heart rates, stroke volume increases through increased diastolic filling time. At increased heart rates, increased stroke volume reflects increased cardiac emptying from increased cardiac contractility produced by

increased sympathetic input to the cardiac muscle and increased circulating catecholamines.

ECG is recorded with parasternal electrodes. Heart rate will increase in response to increased sympathetic and/or decreased parasympathetic outflow to the heart. Decreases in rate are mediated by decreased sympathetic and/or increased parasympathetic outflow. Heart rate changes can occur as a direct response to descending inputs to sympathetic or parasympathetic preganglionic neurons, or changes can be reflexively driven by baroreceptor activity. The presence of abnormally shaped QRS complexes indicates ectopic beats (i.e., patterns of contraction not following the typical conduction pathways). Rates are calculated from the number of beats per unit time and rhythm is assessed by looking at P waves and associated QRS complexes for variations in beat-to-beat intervals and atrial-ventricular coupling. Successive R-R intervals will increase if the heart is under predominant parasympathetic control. Successive R-R interval decreases indicate relative sympathetic nervous system control of the heart. An added benefit of using anesthetized animals is that transthoracic echocardiography can be used to study the mechanical properties of the heart during seizures. The force of contraction, filling and emptying properties, and chamber sizes, are some of the measurements that can be readily made in an echocardiographic study. Ultrasound probes are now quite good for small animal imaging studies, even at the high heart rates seen in mice or rats.

For peripheral nerve recordings, we use a linear array electrode that consists of 4 platinum/iridium wires embedded in a small epoxy trough. Wires pierce large nerves as they lay in the trough. Smaller nerves (e.g., renal nerve) are 'woven' between electrodes. Excellent multi-unit recordings, occasional single unit recordings, and even simultaneous stimulation and recording are possible with this electrode. Vagus or cervical sympathetic electrodes are placed through a ventral neck incision. Renal sympathetic nerve electrodes are placed through a retroperitoneal incision near the kidney. The renal sympathetic nerve contains postganglionic sympathetic fibers to the renal vascular supply. Splanchnic nerve exposure is also through a flank incision on the left side. Greater splanchnic nerve recordings give access to sympathetic preganglionic fibers. Cardiac sympathetic nerves coming off the stellate ganglion are exposed under the second rib through a parasternal incision.

Baroreceptor reflex function is conveniently assessed by recording peripheral autonomic nerve and systemic parameter variations to bolus injections of a vasodilator (e.g., sodium nitroprusside – 0.1 mg i.a.) or a vasoconstrictor (e.g., phenylephrine – 0.25 mg i.a.).

By using a stereotaxic frame, multiple electrodes can be implanted for recording seizure activity from limbic cortical structures. These electrodes are placed using stereotaxic coordinates through craniotomy holes. Depending on the experiment, we use dorsal and/or ventral hippocampal electrode placements. Electrodes are generally 75 μm teflon-insulated stainless steel wires (tip impedances ~100 kΩ). Typical stereotaxic coordinates for EEG electrodes are 5.2 mm anterior to lambda, 3 mm lateral to midline, 2–3 mm below skull surface (dorsal CA1); and 2.7 mm anterior to lambda, 5 mm lateral to midline, 7 mm below skull surface (ventral CA1, ventral subiculum).

Seizures are induced with systemic kainic acid (10–12 mg/kg). A single dose of kainic acid causes a period of recurring seizures that lasts more than 4 h. We found that unilateral common carotid artery occlusion will suppress seizure activity ipsilaterally, and our setup allows for brief occlusion episodes to be repeated and alternated. Our carotid occluder is built from a 0.5 ml syringe, the needle of which is removed and a narrow notch is cut near the top of the syringe. The barrel is shortened and the plunger is removed, but the rubber top of the plunger is left inside the barrel. The short barrel is closed by fusing it to the top of a second syringe, leaving the needle to connect to a length of PE tubing. Air movements in the PE tubing (by a remote syringe) cause the rubber plunger tip to press against the top of the syringe, compressing the artery.

Recent Results

Findings Using Our Model

Kainic-acid-induced seizures were associated with massive increases in parasympathetic (vagus nerves) and sympathetic (cervical sympathetic ganglion, renal sympathetic nerve, splanchnic nerve, cardiac sympathetic nerve) activity. Peripheral nerve activity increases during seizures were much greater than increases that were induced by administration of nitroprusside or phenylephrine, which produced mean arterial pressure changes of >50 mm Hg. Increases in c-fos expression have been found in both sympathetic and parasympathetic medullary regions (as well as hypothalamic areas). Baroreceptor reflex function was impaired during seizures: Changes in nerve activity induced by blood pressure increases or decreases were smaller or absent during tests made with phenylephrine or nitroprusside in the seizure state compared with pre-seizure conditions. In echocardiographic studies, striking acute cardiac dilatation accompanied a profound bradyarrhythmia. Finally, a significant fraction of the animals die during seizures, and the mechanism of death was defined – through ECG, BP, and echocardiographic measures – to be profound mechanical dysfunction coupled with sinus bradycardia and AV nodal block, leading to hypoperfusion of the brain and finally hypoperfusion of the heart itself.

Our preparation has an advantage in that the consequences of severe limbic cortical seizures can be studied in animals that breathe spontaneously. This is a critical

feature for observing and manipulating respiratory activity. We have been able to define the additive roles of seizure-induced autonomic activity and respiratory dysfunction in this seizure model. We have proposed that the massive parasympathetic and sympathetic outflow that occur during a seizure can be compounded by respiratory distress (driving both ANS divisions in the same direction) to impair mechanical function and slow the heart sufficiently to hypoperfuse the brain. The time scale for seizures to impact the heart is seconds, and the development of cardiac dilatation with bradyarrhythmia and eventual death occurs on a time scale of minutes. We suggest that this sequence of events is the most common mechanism for sudden death in epilepsy.

Both Divisions of the ANS are Overactive

The notion that autonomic dysfunction arises from differences in activity of the two hemispheres pre-dates suggestions that asymmetric seizure activity may contribute to sudden death in epileptic patients. The 'brain-laterality hypothesis' was proposed to account for cases of sudden cardiac death, wherein patients died during extreme stress. The brain-laterality hypothesis suggested that emotional arousal could trigger ventricular fibrillation and sudden death by inducing a net lateralized imbalance of activity in some brain areas, leading to uncompensated sympathetic outflow to the heart. Hemispheric inactivation studies have supported this idea.

Differences in activity in the two hemispheres have also been proposed as a contributor or cause for autonomic disturbances – including death – in seizure patients. Controlled lateralized differences in activity are difficult to find (or cause) in studies of seizure activity because seizures can spread rapidly in the brain, and most examples of death during or after a seizure involve generalized seizure activity. A different kind of asymmetry involves differential activation of dorsal and ventral portions of the limbic cortices. Part of this notion comes from data that ventral hippocampal areas (in rodent) have a greater tendency to generate seizure activity than dorsal areas. The connectivity of hippocampal formation with brain regions having autonomic impact – e.g., insular cortex – is also heaviest from ventral areas.

Other studies in people and animals suggest that the extent of the cortical involvement, not lateralization of seizure activity, is important for significant heart rate changes. The study by Epstein and colleagues describes predominantly heart rate increases in patients, but also mentions patients that deviated from the majority, showing heart rate slowing and sinus pauses. Ictal bradycardia was found to most often occur in patients with temporal lobe seizures when the seizure activity was bilateral. In kindling animal models, however, arrhythmias were often seen early in the kindling progression (e.g., stage 2 seizures), when seizure spread is limited; however, none of these arrhythmias were fatal.

Although we have seen differences in ANS activity depending on the hemisphere exhibiting seizure activity, our nerve recordings and c-fos data indicate that both divisions of the ANS are strongly activated during kainic acid-induced limbic seizure activity in rats. Further, our data indicate that death results from massive combined ANS overactivity, with the overall result being a parasympathetic-dominated cardiovascular state. In our opinion, cardiovascular complications depend on the degree and duration of increased autonomic activity, and therefore likely depend on the extent and duration of the seizure.

Death is Mainly a Parasympathetic Phenomenon that Resembles Asphyxia

Death in our animal model does not resemble most of cases of sudden cardiac death. Although the literature spans some three decades, there is almost universal agreement that 80–85% of cases of sudden cardiac death are due to ventricular arrhythmias. Sudden death from fatal arrhythmia is most commonly a ventricular tachyarrhythmia. In some patients, a ventricular tachyarrhythmia is triggered by an acute myocardial ischemic event, and in other patients, a ventricular tachyarrhythmia is related to an anatomical substrate (e.g., scarring from a previous myocardial infarct). Prevention is aimed at preventing the heart disease that can precipitate cardiac events, and treatment is mainly defibrillation. There are fewer treatment options, other than a pacemaker, for the bradyarrhythmias.

It is interesting that the picture we find during seizures resembles the picture found during asphyxiation. In fact, the respiratory distress during a seizure is a form of asphyxiation, and works pathophysiologically in the same direction as the seizures to drive the sympathetic and parasympathetic divisions simultaneously. Respiratory distress causes an increase in vagal activity because of the increase in arterial carbon dioxide, and it increases sympathetic activity (and blood pressure), which also favors an increase in vagal outflow via any functional baroreceptor activity. The combination clearly can be rapidly lethal. Some intense bursts of parasympathetic activity are likely to be large enough to make nodal blockade the initial or predominant first event. In the absence of sustained seizure activity or respiratory distress, a transient arrhythmia may be all that occurs.

In light of these data, one obvious question is whether seizures alone are enough to cause death. In our acute experiments and kindling studies in freely-moving rats, seizures sometimes cause serious arrhythmias, but each episode is usually transient, and therefore not life-threatening. We have not seen evidence for apneic periods caused by seizures in our urethane-anesthetized animals or in animals that have motor convulsions (animals

anesthetized with ketamine/xylazine or unanesthetized animals). The very sudden death that has been described in some mice makes us wonder whether complete respiratory arrest – in addition to or leading to cardiac arrest – is possible. More severe autonomic disturbances might result when seizures occur on the background of a damaged brain, rather than the background of a normal brain. Another possibility is that episodes of arrhythmia predispose to more serious arrhythmias.

Another question that remains unanswered is whether the massive sympathetic outflow that occurs in parallel with the vagal outflow is somehow protective, if it makes matters worse, or if it is irrelevant. Koizumi and others have suggested that there are times when parallel ('non-reciprocal') activation of the sympathetic and parasympathetic outflow to the heart occurs physiologically, e.g., in response to decreased arterial oxygen (in the presence of constant arterial carbon dioxide). They found a similar parallel activation during hypothalamic stimulation, and co-activation actually enhances cardiac output. In rat and rabbit models of asphyxiation, epinephrine and vasopressin have been used with some success to improve resuscitation. These findings suggest that the sympathetic outflow may be a physiological attempt to promote survival of the organism. However, a background of sympathetic activity can enhance cardiac responses to parasympathetic innervation, either directly or via complex activity within the intrinsic cardiac plexus. Such a synergy could enhance the chronotropic and dromotropic actions of the parasympathetic neuronal transmitter, acetylcholine. Favoring this view of harmful consequence of parallel activation is the fact that we never saw ventricular escape beats, even when heart rates fell to what were lethal levels. Finally, strong coactivation of parasympathetics and sympathetics contributes to the impairment of the baroreceptor reflex. Baroreceptor reflex function was shown to be impaired in patients with temporal lobe epilepsy that were refractory to drug treatment. The interaction of sympathetic and parasympathetic innervation at the heart is complex and this question will not be easily settled.

Future Directions

The ultimate goal of our research is to construct a complete picture of the ANS activity during seizures. The afferent pathways used by different types of seizures need to be fully defined. The long-term consequences of seizures should be studied, including (a) the lasting effects on ANS activity (not during the seizure itself) of multiple seizures, in chronically altered brains; (b) the mechanisms by which changes in the network that generates seizures (e.g., as regions sustain damage or by conversion of previously less active areas) impact the ANS; and (c) the possibility that a newly epileptogenic region is more disruptive for ANS function. These questions must be pursued with a combined approach that involves acute and chronic experimentation in animals as well as corollary studies in patients.

Animal studies will not only be essential for defining the pathophysiology of autonomic consequences in people, but also serve a role in identifying the most significant variables that can and should be monitored in patients. Some of these variables may be critical for identifying a specific immediate risk (e.g., an arrhythmia that will have the potential to be life-threatening); other ANS parameters may even aid in seizure detection. Other approaches, such as functional studies of cardiac function with echocardiography, may be more suitable for defining broader periods of risk, or significant changes in risk during the life of a seizure patient.

See also: **Models**: Models of Generalized Seizures in Freely Moving Animals; **Treatments (non-Antiepileptic Drugs)**: Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy.

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In the case of many novel seizure-induced genes, it is still a challenge to define the consequences of altered expression because of the lack of information about the function of protein products. Even if we know the localization of transcription, in many cases the only other available information is gene sequence. The information available about the function of a gene (or gene product) in other tissues or cell lines cannot always be easily translated to the brain. Therefore, much basic research is needed before we understand the net effect of global alterations of gene expression in the brain, or even attempt to determine if a given alteration in gene expression is beneficial of harmful.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; Gene Expression in Immature and Mature Hippocampus After Status Epilepticus; Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; Surrogate Markers for Epileptogenesis; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Post-Traumatic Epilepsy**: Posttraumatic Models.

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Seizure Initiation and Termination

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Introduction

A seizure is defined as an episode of excessive, synchronized neuronal activity associated with disturbance of sensation, loss of consciousness, convulsive movements, or combinations of these clinical signs. The precise combination is thought to reflect the brain regions that are synchronized in the discharge. Ever since electrical activity has been recorded in the brain, there has been reference to 'spontaneous' activity, the most marked form of which is seizure activity. Normally, brain activity does not appear to be highly synchronized. However, during a seizure, there is abnormal synchronization of brain circuits. Importantly, however, the occurrence of a seizure does not mean that the brain is abnormal. For example, seizures can be triggered in 'normal' brain by metabolic abnormalities such as hyponatremia and hypoglycemia, or by infection or inflammation. Every brain has a capacity to express a seizure.

The brain produces a variety of transient oscillations – synchronized activity in populations of neurons – that do not initiate seizures. This observation can be viewed in two ways. One is that there is a specific type of 'trigger' necessary to initiate a seizure. This trigger – such as an electrical shock – may itself produce excessive synchronization or may lower some threshold for seizure initiation. Alternatively, one could view the brain as having mechanisms that control the degree of synchronization; if these limiting factors fail, a seizure may begin. An example of such a process would be loss of inhibitory function in the cortex. Interestingly, if both excitation and inhibition are blocked, neurons tend to fire at a higher rate and in a more rhythmic pattern compared with activity during baseline conditions. This finding supports the idea that there are intrinsic mechanisms that determine neuronal excitability in brain networks, and that these intrinsic mechanisms could limit seizure initiation. Certainly, an active input (a trigger) could interact with loss of an

intrinsic brake to result in the excessive synchronization that characterizes a seizure discharge. The discharge provides an increase in excitation that when coupled with other mechanisms, such as strong recurrent synaptic connections, may allow spread of the activity from a single cell to a few cells, and then from the few cells to a larger population.

Epilepsy is generally defined as chronic, recurrent seizures. The mechanisms behind the recurrent seizures are presumably similar to those behind seizures in normal brain, except that the 'trigger' is now present in the brain. Alternatively, or in addition, there is a loss of the intrinsic mechanism that controls/limits synchronization. Yet, despite all of the possible changes in an epileptic brain, the seizures remain episodic. They still start and stop. The changes underlying the epileptic state may make it more likely that the neurons will synchronize to initiate a seizure, but do not explain why the seizure actually starts. Why should it start a one particular moment? Why not 5 or 73 min later? We still have an incomplete understanding of the details of initiation of seizures. Further, a seizure (once started) can vary from a few seconds in duration to tens of minutes. Why the seizure stops is largely unknown. Seizures are followed by a refractory period, both absolute and relative, during which another seizure cannot be elicited. When considering possible mechanisms of seizure onset and termination, the presence of a refractory period needs to be taken into account.

Background

Specific mechanisms have been proposed to contribute to the onset of single seizures. These mechanisms can be distinguished from the more chronic changes that underlie the epileptic condition. The mechanisms proposed for seizure onset can be roughly divided into network mechanisms (which include changes in synaptic connections and in neurotransmitter systems), and cellular/molecular mechanisms. Hormones and other factors are also likely triggers for seizure onset, but will not be discussed here.

The network mechanism that has received much support over the years is a 'fading' of inhibition due to increased neuronal activity. Inhibitory systems in the brain normally function to control neuronal activity and reduce the likelihood of excessive synchronization. Thus, a loss of inhibition would be predicted to increase the chance that a seizure might begin. In support of this idea, GABA antagonists can cause seizures in experimental animals. In addition, in epileptic animals, reduced recurrent inhibition has been found to contribute to neuronal hyperexcitability and hypersynchrony. However, in brain slices, if nothing else is done, complete (or near complete) blockade of GABA receptors is needed to achieve synchronized burst firing in the hippocampus. This observation suggests that, most likely, more than one mechanism underlies seizure onset. Indeed, even in these animal models of reduced inhibition, the seizures are episodic – they start and stop. Reduced inhibition would not be predicted to contribute to seizure termination.

Increases in excitation, either intrinsic excitability or synaptically-mediated excitability, could contribute to seizure initiation. Seizures depend on circadian rhythms as well as minute fluctuations of ionic concentrations, such as increases in extracellular potassium and decreases in calcium, both of which increase excitability. Since astrocytes are thought to play a major role in the regulation of the extracellular environment, an increase in excitability due to changes in the ion concentrations could be due to glial dysfunction. Consistent with this idea, gliosis and/or reactive astrocytes are frequently found in epileptic tissue, and other investigators have described various abnormalities in the normal function of these astrocytes. Other ways in which an increase in excitation might contribute to seizure initiation include an acute increase in glutamate release, a transient inhibition of glutamate transporters on glial cells, or a transient increase in neuronal firing, which could result in recruitment of surrounding neurons and, ultimately, in excessive synchronization.

Changes in the characteristics of a number of ion channels could increase intrinsic excitability, which could in turn contribute to seizure initiation. For example, the presence of a small persistent sodium current has been proposed (based on computer modeling) to be necessary for the generation of spontaneous activity in the CA1 region of hippocampal slices exposed to zero-calcium perfusing solutions. The sodium current is necessary for the generation of an after-depolarizing potential, and prolongs the neuronal firing in an individual neuron. A number of modifications in ion channels (channelopathies) have been described in inherited epilepsies. It is possible that a change in expression of genes that encode the same (or different) ion channels could account for other types of epilepsy that do not now appear to be inherited. However, it seems unlikely that a change in channel function that makes it more likely that a neuron will fire an action potential can be the sole mechanism behind seizure initiation. Seizures require multiple neurons to fire synchronously.

Most seizures begin in a small group of neurons and then spread. One theory has been proposed that irregular action potential discharge, which may or may not be synchronized across a small population of neurons, progresses into rhythmic synchronized discharges. These low-frequency discharges, often expressed as interictal activity, then 'fuse' into larger aggregates. This process can then spread locally and/or to synaptically-connected brain regions. Recurrent excitatory connections, such as in the CA3 region of the hippocampus, provide a

mechanism for synchronizing neuronal activity when only one, or a few, neurons fire. Noteworthy, however, in an animal model of temporal lobe epilepsy, an increase in recurrent excitation in the temporal lobe was not found to contribute to neuronal excitability. If a population of neurons in a brain region begins firing spontaneously, then the probability that 2 or 3 neurons might fire simultaneously is dependent on the frequency of firing. Once a small group of neurons begins to fire simultaneously, then these cells will be more likely to fire together again – and to recruit local or synaptically-connected neurons into the discharge. The presence of interictal activity is also proposed to lead to the formation of new, or abnormal, excitatory connections, promoting further changes in the brain. However, the idea that interictal activity actually begins the process of synchronization is debated. Indeed, interictal activity could reduce the likelihood of synchronization. The relative anatomy and physiology of the brain region in which the interictal activity is present probably determines the outcome.

Our understanding of the mechanisms underlying seizure termination is minimal. One of the earliest theories was that the intense neuronal activity depleted the stores of readily available ATP. Essentially, the theory suggests that running out of energy results in loss of the ion gradients across the membrane, making it impossible to generate action potentials. Such a condition would effectively end the synchronous discharge, and would account for the refractory period seen after a seizure. Short of a complete loss of ATP, there is also the possibility that the ion gradients are lost, or sufficiently reduced, that action potential firing is limited until the gradients can be restored. For this theory to hold true, action potential discharge during seizures would need to be frequent enough that the ion gradients cannot be regenerated. A less extreme hypothesis is that there is inactivation of ion channels underlying action potentials, resulting in blockade of action potential generation – and explaining the refractory period. Other theories for seizure termination include the proposals that the elevated extracellular potassium produced by the seizure activity causes a depolarizing block of neuronal activity, and that the sodium/potassium ATPase activity is enhanced by the seizure. Inactivation of sodium channels by the neuronal activity would also be predicted to terminate action potential firing. It has also been suggested that pacemaker failure is responsible for termination of ictal events in the cortex.

Before and during seizures there is an increase in the concentration of glutamate in the extracellular space. Such an increase could result in desensitization of the glutamate receptors. If the receptors were no longer sensitive to stimulation by glutamate, then neuronal excitability would be reduced. Cyclothiazide, a compound that inhibits desensitization of ionotropic glutamate receptors, has been shown to increase the frequency and prolong epileptiform discharges induced in rat hippocampal slices with low magnesium. This observation suggests that receptor desensitization could contribute to seizure termination. However, in the absence of synaptic transmission, epileptiform activity is also periodic, suggesting that receptor desensitization is not the only mechanism.

In the CA3 region of the hippocampus, the recurrent collateral synaptic connections are postulated to underlie the epileptiform activity that can be easily initiated by reduction of inhibition. Investigation of the burst discharge in this region has shown that exhaustion of the releasable pool of glutamate is a mechanism behind the termination of the individual bursts. When extracellular calcium is replaced with strontium, there is a reduction in the rate of glutamate release and prolongation of the bursts in disinhibited CA3. Replenishment of this pool would account for the interval between the short bursts of action potentials that characterize this epileptiform activity. Another possible mechanism that could contribute to termination of a burst of action potentials is a short-term depression of synaptic function induced by the electrical activity in the burst.

Methods

The major approach to the study of initiation and termination of seizure activity has been electrophysiological – to record from individual neurons and from populations of neurons in specific brain regions. This approach can be combined with pharmacological or genetic manipulation of excitability. Because of the ease of precise localization and identification of cell type in vitro, this type of experiment is typically carried out in acute brain slice preparations.

Epileptiform activity can be produced in hippocampal slices by a variety of methods. Short (<1 s) bursts are readily produced by raising extracellular potassium concentrations, lowering extracellular calcium or magnesium, adding blockers of GABAergic transmission, adding drugs that increases excitability, or a combination of these manipulations. Prolonged epileptiform activity (>5 s), which more closely mimics seizures in vivo, can be produced in vitro in the absence of synaptic transmission in both CA1 and the dentate gyrus. The discharges can be quite long, and consist (at the extracellular level) of a negative shift of the DC potential upon which population spikes are superimposed (indicating synchronous neuronal activity). The bursts recur at fairly regular intervals, suggesting the presence of some type of pacemaker activity. Epileptiform activity can be induced in cortical areas using many of the same manipulations.

There are some limitations of the currently available in vitro models for the study of seizure initiation and termination. In the intact brain, synaptic transmission is rarely, if ever, completely blocked. Therefore, the mechanisms that underlie onset and termination of the prolonged bursts in the absence of synaptic transmission may or may not be the mechanisms behind seizures in vivo. Similarly, the short bursts seen in in vitro models are more characteristic of interictal spikes than of seizures in vivo, leaving open the question of what allows, or stimulates, the more prolonged discharges in vivo. It is also possible that isolation of tissue from the whole brain alters the mechanisms that work to initiate and provide early synchronization of epileptiform activity. Mechanisms behind termination of synchronized activity are also probably different in the presence and absence of synaptic transmission.

Recent Results

Mechanisms of seizure initiation under various conditions have been studied, but relatively few studies have addressed seizure termination. In particular, it is unclear how seizures are terminated in the absence of synaptic activity, where there is no loss of excitatory drive or augmentation of inhibitory inputs. We studied dynamic activity-dependent changes of intracellular pH in the dentate gyrus, in the absence of synaptic transmission, in acute hippocampal slices, using a fluorescent pH indicator. Slices were incubated in 40 μM SNARF-1 AM for 3 h. SNARF-1 is taken up into cells, where it is hydrolyzed by an endogenous esterase. Recurrent epileptiform activity was initiated by perfusing the slices with 0 mM-added calcium and 8 mM potassium solution. After 20–30 min in this solution, prolonged epileptiform activity (20–40 s discharges) recurs at regular intervals in the dentate gyrus. The epileptiform activity consists of a negative shift of the DC potential, with superimposed population spikes. Each burst of epileptiform activity was found to be associated with intracellular acidification, while between seizures the intracellular pH recovered (Figure 1). The average magnitude of the change in intracellular pH was 0.025 pH units; this average includes intracellular pH changes in nonbursting cells. These pH changes begin early in the discharge, suggesting that a particular level of intracellular pH during the prolonged epileptiform activity is associated with termination of the activity. This hypothesis was tested with antidromic stimulation during the recovery period. Early in the recovery period, stimulation could not initiate a prolonged burst. When the stimulation was applied in the middle of the recovery period, it could evoke a burst, but the duration of this burst was shorter than the regularly recurring bursts. This experiment

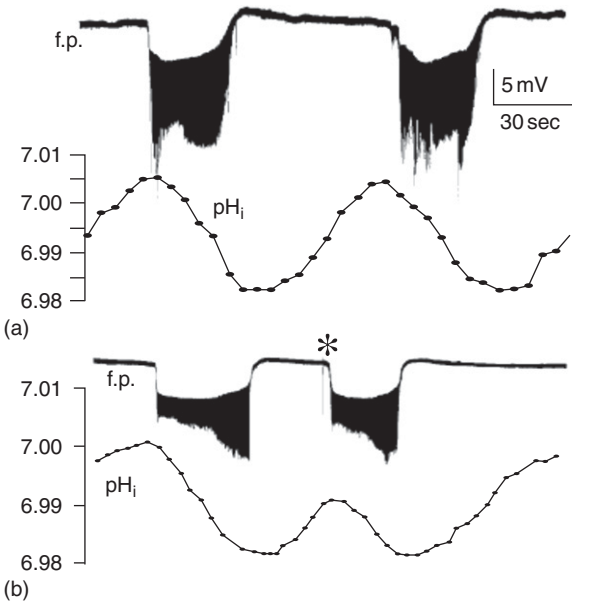


Figure 1 Changes in intracellular pH correlate with duration of prolonged field bursts in the dentate gyrus of hippocampal slices. Cells were loaded with SNARF 1-AM and then the slices were perfused with 0-added calcium and 8 mM potassium. Recurrent epileptiform field bursts were accompanied by intracellular acidification, which gradually recovered between the bursts (a). If an antidromic stimulation was administered between the spontaneous bursts (*), the subsequent field burst was shorter than the previous spontaneous one. The duration correlated with the degree of recovery of the intracellular pH.

demonstrated that the duration of the burst correlated with the degree of recovery of the intracellular pH. A recovery of the intracellular pH to at least ~50% of the baseline was required before the antidromic stimulation could initiate a prolonged burst.

To further explore this relationship, a variety of conditions were tested. Experimental conditions that shortened the epileptiform discharge correlated with more rapid intracellular acidification, and experimental manipulation of intracellular pH altered the duration of the seizure discharge; acidification resulting in early termination of the epileptiform activity. Altogether, these data show a direct relationship between the level of intracellular acidification and the duration of the seizure-like discharge, suggesting that an intracellular pH-dependent process can terminate nonsynaptic neuronal synchronization. Many cellular processes are dependent on pH, but it is not known which process terminates the seizure-like activity in this model.

When considering possible mechanisms of seizure termination, these data demonstrate that neuronal excitability is not reduced at the end of a prolonged discharge. Immediately after termination of the bursting, an antidromic stimulation can elicit a population spike whose amplitude is not reduced when compared with control conditions. Thus, loss of ATP, enhanced sodium pump activity, inactivation of sodium channels or depolarizing

blockade are unlikely to be involved in terminating the burst discharge activity in this model. There is clearly no decrease in neuronal excitability and, since there is no synaptic transmission in this model, there is no change in synaptic function, which would explain burst termination. It would appear that the intracellular acidification inhibits cellular processes that are necessary for continued neuronal firing or bursting.

As suggested earlier, this model of epileptiform activity, in the absence of synaptic transmission, does not accurately model seizures in vivo. However, there are some significant similarities which make this correlation between intracellular pH and seizure termination quite compelling. First, the dentate gyrus provides a gating function in the transmission of seizure activity into and through the hippocampal circuits. The dentate gyrus appears to have a high seizure threshold and to be resistant to the initiation of seizure activity. However, once the seizure activity in the dentate gyrus has begun, all parts of the hippocampal circuit discharge as one. Early in the synchronization of the activity in the dentate gyrus, there is no alkalinization of the extracellular space suggesting that the seizure onset does not involve synaptic activity. In addition, maneuvers that increase or facilitate nonsynaptic mechanisms of synchronization increase the likelihood that discharges will begin in the dentate gyrus. Finally, the only way to mimic the prolonged discharges in an in vitro system is to block synaptic transmission. Together, these observations suggest that nonsynaptic mechanisms are likely to be critical in the initiation and termination of epileptic discharges in the dentate gyrus, a structure that is thought to 'gate' the spread of seizure activity throughout the hippocampal circuit.

Future Goals

If intracellular acidification in the dentate gyrus represents a critical mechanism in control of seizure activity, in at least one brain region important in seizure spread, then determining the source of the acidification – and the cellular processes that are influenced or modulated by the acidification – will be important steps in understanding seizure control. Augmentation of the process, or processes, underlying seizure termination might help control seizures. In addition, understanding how changes in pH affect neuronal function would contribute to our insight into cellular control of neuronal excitability.

One thing that is not clear, and will need to be investigated, is whether the mechanisms that allow initiation and those that terminate synchronized neuronal activity are the same in normal and epileptic brains. Further, it will be important to investigate whether the mechanisms that underlie seizure initiation are the same in all brains with epilepsy – no matter what the type of epilepsy. One would guess that the mechanisms are, in fact, quite diverse. There could also be multiple mechanisms involved in seizure termination. Finally, one must ask whether we can therapeutically reduce the likelihood of seizure onset and augment the mechanisms underlying seizure termination. One could argue (based on what we do know about their mechanisms of action) that the currently available antiepileptic drugs reduce the likelihood of seizure onset. However, whether they (or other drugs) augment termination mechanisms is unknown.

See also: **Models**: Ictogenesis in the in vitro Isolated Guinea Pig Brain; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; Changes in Extracellular Ion Composition; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; Seizure Termination and the Postseizure Refractory Period.

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Seizure Prediction

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Introduction

Epilepsy affects ~1% of the population worldwide. Two-thirds of affected individuals achieve effective seizure control from anticonvulsive medication, and another 8–10% could benefit from resective surgery. Thus, in about 25% of individuals with epilepsy, seizures cannot be controlled by any available therapy. In most individuals with epilepsy, seizures appear to occur unpredictably, with no discernible pattern; in other patients, seizures appear to be entrained to biological rhythms. Only few individuals can identify periods when seizures are likely to occur, and even these patients can rarely specify an exact time when a seizure will happen. From Lennox's 'reservoir' theory, several seizure-facilitating factors are known, but these factors are rather nonspecific and highly variable; they depend on individual habits, susceptibility, and daily routine. Research in experimental models of focal epilepsy has identified a variety of potential ictogenic (seizure-generating) mechanisms. However, it is still a matter of debate how these mechanisms give rise to seizures, and to date they have not been useful in constructing a seizure prediction algorithm.

Epileptic seizures are commonly believed to begin abruptly, just a few seconds before their clinical manifestations. The motivation for research into the predictability of seizures is thus straightforward. A question of particular interest is whether characteristic features that reliably herald an impending seizure can be extracted from continuous electroencephalographic (EEG) recordings. In recent years, a number of new analytic techniques have been developed that appear to be capable of predicting seizures from EEG recordings. Studies on seizure prediction have advanced from preliminary descriptions of preseizure (preictal) phenomena and proof-of-principle studies, to studies on continuous multiday recordings. Nevertheless, it is still a matter of debate whether seizures are predictable by prospective algorithms. Given the potential impact of seizure prediction methods to expand knowledge of seizure-generating mechanisms, and to move treatment concepts from preventive strategies toward an EEG-triggered 'on-demand' therapy, it is important to understand potential influencing factors, to improve seizure prediction performance, and to develop reliable methods ready for clinical deployment.

Background

If epilepsies are considered as dynamic diseases of brain systems, then one could postulate two primary scenarios of how a seizure could evolve. Initiation of seizures in primary generalized epilepsy could be caused by a sudden and abrupt transition, in which case the seizure would not be preceded by detectable dynamic changes in the EEG. In focal epilepsies, the transition could be a gradual change (or a cascade of changes) in EEG dynamics, which could in theory be detected. A large number of studies have provided clinical and physiological support for the idea that at least certain types of focal seizures are predictable.

Non-EEG-Based Evidence for a Preictal Period

According to Lennox, seizure facilitation can be defined as the input of sensory, metabolic, emotional, or other yet unknown factors that '...fill up some reservoir until it overflows,' which in turn results in a seizure. Factors known to potentiate seizures, particularly in sensory-evoked and reflex epilepsies, include the following: state of consciousness, sleep deprivation, anxiety, disturbances of electrolytes and acid–base balance, sensory stimulation, and exposure to certain drugs. Studies based on questionnaires or seizure dairies have revealed that individual patients can identify periods when seizures are most likely to occur. These periods are characterized by vague sensations, called clinical prodromes, which include mood changes, irritability, sleep problems, nausea, and headache. There are also physiological studies in small numbers of patients who support the existence of a preictal period. In these studies, an increase in blood flow and in cerebral oxygen availability in the epileptic temporal lobe, or changes in brain perfusion, was observed in the minutes to hours prior to seizure onset. Preictal changes in the R–R interval on the electrocardiogram may also have predictive value in some types of epilepsy.

EEG-Based Evidence for a Preictal State – Early Approaches

A large number of studies on the level of neuron networks suggest that focal seizures are initiated by abnormally discharging neurons that recruit and entrain neighboring

neurons into a critical mass. This phenomenon might be mediated by an increasing synchronization of neuronal activity that is accompanied by a loss of inhibition, or by other seizure-facilitating processes. The concept of a critical mass, however, might be misleading, since it implies an increasing number of neurons that are entrained into an abnormal firing pattern. Such a mass phenomenon would be easily accessible to conventional EEG analysis, which, to date, has failed to detect it. Early investigations of the relationship of interictal spike occurrence to seizure onset revealed a decrease (or even total cessation) of spikes before seizures, but a reexamination in a larger sample did not confirm this phenomenon. Moreover, findings obtained from applying linear analysis techniques, such as pattern recognition, analytic procedures of spectral data, or autoregressive (AR) modeling of EEG data indicated that EEG changes characteristic of preictal states may be detectable, at most, a few seconds before the actual seizure onset.

With the availability of so-called nonlinear analysis techniques, a number of laboratories have continued to face the challenge of developing algorithms to detect unknown patterns associated with seizure generation, and have shown characteristic changes minutes to hours prior to seizure onset on the intracranially-recorded EEG. Nonlinear time series analysis techniques involve univariate measures, such as Lyapunov exponents, dimensions, entropies, or correlation densities (see Methods). Additional methods developed to define preictal states include the following: extraction of neurophysiologic features from the EEG associated with epileptiform activity (e.g., bursts, slowing, chirps, and changes in signal energy), recurrent neural networks, simulated neuronal cell models, and techniques developed in catastrophe theory or in the field of self-organized criticality. In addition, some laboratories have focused on bivariate or, more generally, multivariate measures that are based on analysis concepts for synchronization phenomena in nonlinear dynamical systems. These analysis techniques permit the assessment of synchronous activity from multiple sites and include nonlinear interdependence, measures for phase synchronization and cross-correlation, difference of the largest Lyapunov exponents of two or more channels, nonlinear causality, as well as classification approaches based on a fusion of multiple EEG features from multiple sites.

Results obtained from preliminary descriptions of preictal phenomena, and from early proof-of-principle studies, indicated that seizures are not random events, but rather are related to ongoing dynamical processes that may begin minutes to hours to days beforehand. They also indicated that the seizure-initiating process might better be visualized as a process in which an increasing number of critical interactions between neurons in a focal region, and connected units in an abnormal functional network, unfold over time. The different prediction horizons were thought to reflect different aspects of ictogenesis as captured by the different approaches, but also indicated that none of these techniques appears to depict the process fully. Since the observed patterns appeared to be patient-specific, it was conjectured that different approaches may be required to predict seizures with clinically useful accuracy in different individuals or in different epilepsy syndromes. No method, however, convincingly demonstrated unequivocal seizure prediction in blinded, prospective, randomized clinical trials, and with appropriate statistical validation.

EEG-Based Evidence for a Preictal State – Recent Approaches

In 2000, the International Seizure Prediction Group (ISPG) was established with the specific goal of moving the field of seizure prediction forward from proof-of-principle experiments into validated, well-understood methods that could be applied to both basic science and clinical applications. With the wide acceptance of digital electroencephalography, the maturation of methods for recording the EEG from intracranial electrodes, and the availability of powerful computers, members of the ISPG began to store and to analyze complete, long-duration, multichannel EEG datasets acquired during presurgical monitoring from a large number of patients implanted with intracranial electrodes. This approach helped to avoid a potential bias resulting from analyses of highly selected EEG data that was restricted with regard to seizure type, patient state, recording sites, signal to noise ratio, duration of recordings, artifacts, etc. Moreover, since many past studies merely concentrated on sensitivity issues (i.e., ignoring the specificity of preictal changes), techniques were developed that allow one to evaluate the performance of seizure prediction algorithms. These techniques go beyond the mere estimation of sensitivity and specificity (using, e.g., receiver-operating characteristics (ROC)) and include analytical, stochastic, and surrogate-based approaches. With these approaches, it is only recently that some laboratories reevaluated the performance of seizure prediction algorithms previously considered suitable for prediction and showed that many of them (including correlation dimension, Lyapunov exponents, and signal energy) do not perform better than a random predictor. Parenthetically, some of these techniques – particularly nonlinear algorithms – do provide an improved characterization of the spatial distribution of the epileptic process during the interictal period. Evidence has accumulated, however, that particularly seizure prediction algorithms that are based on a quantification of relations between recording sites appear to perform significantly better than random prediction, even if rigorous statistical validation is applied.

Evidence for Preictal Alterations from Studies in Animal Models of Epilepsy

Although there is a considerable bulk of literature on animal models of epilepsy, the phase of transition to the seizure state has not yet been fully explored. Some earlier studies indicated that discernible preictal patterns can be detected, at least in some experimental models of epilepsy, but these studies must be regarded as proof-of-principle studies since most lack a sound statistical validation. In addition, a number of these studies have focused on acute seizure models, whose behavior is not clearly analogous to spontaneous seizures in humans. More recently, alterations in interictal activity were shown to precede the transition to ictal-like activity in a hippocampal–entorhinal brain slice preparation exposed to high extracellular potassium and to low magnesium concentrations. Preictal changes were characterized by alterations in the origin and spread of CA3 epileptiform discharges and by a prominent spectral power increase in the 200–400-Hz frequency range. Similar oscillations were also observed in microelectrode studies in vivo in the hippocampus of kainate-treated rats and in depth EEG recordings from patients with mesial temporal lobe epilepsy. These so-called 'fast ripples' have been hypothesized to identify microscopic regions important to seizure generation, and may reflect pathological hypersynchronous population spikes of bursting pyramidal cells. Fast ripples are also seen during the latent period (i.e., between the occurrence of an initial epileptogenic insult and the onset of recurrent spontaneous seizures) in rat models of chronic epilepsy, and may thus be a possible marker of alterations in interictal activity preceding the transition to ictal activity.

Methods

Methods employed in seizure prediction have been developed to identify unknown patterns on the EEG associated with seizure generation. Many methods are mathematically complex and not easily accessible to those outside of the world of physicists, mathematicians, and engineers. Some of them may be more difficult to relate to the neurophysiology of epilepsy than are others. Many techniques place great demands on the recorded EEG with respect to the precision of the data, the absence of noise, and stationarity. In practice, however, none of these requirements can be exactly fulfilled. Moreover, numerous studies have identified factors related to the EEG recording system that might all have a strong influence on the findings obtained from applying the different methods. Finally, potential pitfalls specific to the individual algorithms have to be taken into account. Thus, although a number of toolboxes are already available, analysis techniques must be painstakingly applied, and the results obtained should be interpreted with great care.

Major Methodological Steps

In a retrospective seizure prediction study, three major methodological steps are involved (see **Figure 1**). The first step consists of preprocessing the EEG. This typically consists of artifact detection and removal, filtering, and normalization – depending on which methods are used in the second step, which consists of scanning the EEG for predictive features. This scanning permits reduction of large amounts of EEG data to a small number of parameters (important for downstream processing), and may consist of an automated detection of epileptiform patterns, such as spikes or slowing. In the majority of studies, however, a certain measure, which is thought to characterize specific aspects of the ictogenic process, is calculated from the EEG using a moving-window technique. The length of the time window is chosen so that there is a reasonable trade-off between approximate stationarity of the EEG signal and sufficient number of data points to characterize the EEG dynamics. The resulting temporal evolution of the measure (measure profile) is then scanned for prominent characteristics that can be related to the actual seizure times. These features might be drops or peaks (e.g., quantified as threshold crossings) or any other distinct pattern in the measure profile, and can be detected automatically with pattern recognition methods or with a variety of novel classifiers, such as probabilistic artificial neural networks. In a third step, the measure's capability to distinguish the preictal state from the interictal interval is evaluated with test statistics quantifying the occurrence of these features relative to the seizure times, which results in some kind of performance value. A high performance value is assumed to reflect the existence of a preictal state and the capability of the applied measure to detect it.

EEG Analysis Techniques and Characterizing Measures

Analysis techniques currently in use to calculate a characterizing measure from continuous EEG recordings can be divided into two main categories: linear and nonlinear. Depending upon whether EEG data from one, two, or more recording sites are analyzed independently, or for possible interactions, these techniques can further be divided into univariate, bivariate, and multivariate approaches. A more detailed description of the physical and mathematical concepts underlying these techniques, along with their implementation details for EEG analysis, can be found in a number of textbooks and review articles.

Linear methods comprise analysis techniques in the time domain such as evaluation of amplitude, interval or period distributions, or estimation of auto- and cross-correlation functions, as well as analysis in the frequency domain (using the fast Fourier transform, the wavelet transform, or other time–frequency transformations) such as power spectral

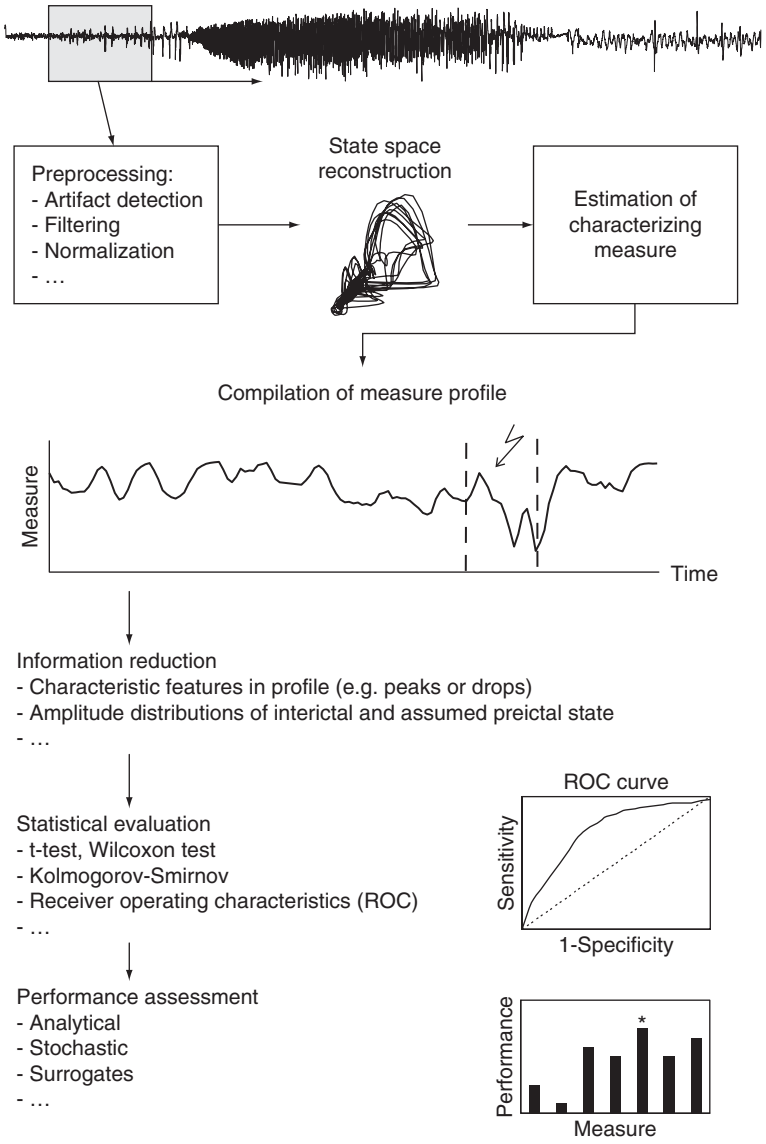


Figure 1 Flow chart of the major methodological steps involved in retrospective seizure prediction studies. The chart is shown for a univariate measure but can be extended easily for bivariate and multivariate measures. An EEG recording is partitioned into segments of a given duration (typically tens of seconds). EEG data of a given segment is preprocessed (if necessary) and the dynamics is reconstructed in state space in case of a nonlinear EEG analysis approach. A characterizing measure is estimated for this segment, and analysis steps are repeated for the next segment, after moving forward in time. The resulting measure profile (i.e., time-dependent changes of a characterizing measure) is then scanned for prominent characteristics that can be related to the actual seizure times. Separability of preictal and interictal characteristics is evaluated with statistical methods to yield estimates for sensitivity and specificity. In case of a multichannel recording, all steps are repeated for each channel (univariate approach) or combinations of channels (bi- or multivariate approaches). It is advisable to assess and to validate the prediction performance, particularly with respect to possible clinical applications.

estimates, cross-spectral functions, or linear coherence. AR and AR moving average (ARMA) models provide an alternative way to estimate properties of the power spectrum.

Nonlinear analysis techniques were originally developed within the physical–mathematical theory of nonlinear dynamics (colloquially termed 'chaos theory') and aim at an improved characterization of the complicated, apparently irregular behavior of complex nonlinear dynamical systems such as the brain. They provide a methodologically different approach to EEG analysis, which in general is based on the reconstruction of the dynamics in a so-called 'state space' – e.g., via the so-called method of delays (time-delay embedding). The Cartesian state space is spanned by all state variables (i.e., the number of degrees of freedom) of a dynamical system, and the system dynamics generates a trajectory through this space. Properties of the trajectory in state space can then quantitatively be characterized by nonlinear measures. It should be noted that the embedding

of an EEG time series in state space is regarded as a crucial point in nonlinear EEG analysis. An improper state space reconstruction is a common source of errors and can lead to a mischaracterization of the dynamics.

A number of univariate and bivariate nonlinear measures are now available, and their ability to detect predictive features in the EEG has already been evaluated in a number of studies. Univariate nonlinear measures comprise an effective correlation dimension, correlation density, entropy-related measures, or Lyapunov exponents, which allow one to draw inferences about the number of degrees of freedom (or complexity), the amount of order or disorder, or about the degree of chaoticity or predictability in a single EEG time series. Other measures provide an estimate of the amount of nonstationarity or allow one to discriminate between deterministic and stochastic dynamics. Bivariate nonlinear measures such as similarity index, phase synchronization, nonlinear interdependency, and other measures for generalized synchronization allow one to estimate dynamical interactions between two EEG time series. Some of these approaches can provide information about the direction of interdependence.

Assessing the Performance of a Seizure Prediction Algorithm

Usually, algorithm performance requires optimizing numerous computational parameter choices and sometimes the choice of an estimation algorithm. These choices often rely on a posteriori knowledge, which interposes significant risk of in-sample overtraining, resulting in seemingly good results just by chance. To avoid the potential for fitting results to the data and to enable independently reproducible results, application of control tests and independent validation is essential. The currently available statistical methods for assessing the performance of seizure prediction techniques comprise analytical, stochastic, and surrogate-based approaches. The analytical approach makes use of the so-called seizure prediction characteristic and compares the sensitivity of a method under investigation with the performance of an unspecific, naïve (random or periodic) predictor. The stochastic approach incorporates an underlying stochastic model for seizure generation and allows for modeling of a so-called permissive preictal state that is able to return to a baseline state without giving rise to a seizure. Surrogate-based approaches test the null hypothesis of the nonexistence of a preictal state, do not assume specific models for the development of a preictal state, and can be applied to any type of analysis, algorithmic or statistical. Seizure time surrogates are artificial seizure onset times generated by randomly shuffling the original interseizure intervals. With surrogate seizure onset times, the EEG data are subjected to the same analysis algorithms and prediction statistics that were used for the original onset times, including any in-sample optimization carried out in the process. Measure profile surrogates are derived from a constrained randomization of the time profile of a characterizing measure with seizure onset times kept fixed. With these approaches, the null hypothesis can only be rejected if the performance of the algorithm for the original data is better than the performance for a number of independent realizations of the surrogates, indicating that a given algorithm can detect a preictal state with a performance above chance level. It is important to keep in mind, however, that the fact that the null hypothesis cannot be rejected does not prove its correctness, since there may be alternative explanations for this result.

Recent Results

Despite the proof-of-principle evidence for the existence of a preictal state, many previous studies provided an overoptimistic view on the performance of prediction algorithms. More recent studies showed that findings obtained by applying highly optimized algorithms to small, selected data sets could not be reproduced on unselected, more extended EEG recordings that are more closely related to the challenge of predicting seizures prospectively from the continuous EEG. The overt initial enthusiasm has now given way to careful introspection. There is now a focus on meticulous data acquisition, annotation, study design and rigorous statistical design of prediction studies, and validation of results. Seizure prediction is now defined as reliably identifying periods of time in which there is increased probability of seizure onset.

The state-of-the-art of the field does not allow us to definitely answer the question of whether seizures can be predicted using prospective algorithms – despite the availability of intracranial, continuous, multiday, multichannel EEG recordings, a multitude of refined EEG analysis techniques, and the more rigorous methodological design in recent studies using statistical methods for performance assessment. Although a few studies have used prediction algorithms in a quasi-prospective manner, these studies have not included a statistical validation or have not allowed conclusions based on statistical validity.

From a number of retrospective studies that included rigorous statistical validation, strong evidence has accumulated that particularly measures quantifying relations between recording sites appear to perform significantly better than do random prediction. These measures allowed identification of a state of increased seizure likelihood lasting up to several hours, but seizures could not be predicted with exact timing. Whether this accuracy is sufficient for a prospective clinical application will need to be decided on an individual basis. Interestingly, some studies reported that recording sites carrying relevant information were not in close vicinity to the epileptic focus, but could be located in remote or even contralateral

brain structures. This finding supports the notion of an epileptic network whose interactions extend over large regions of the brain rather than the concept of a localized and well-defined epileptic focus.

Future Goals

A major challenge in working on seizure prediction is to establish convincing evidence for the existence of a preictal state in prospective studies. In addition, seizure prediction studies must offer an appropriate model for its characteristics in human epilepsy (both in adults and children), and take into account the heterogeneity of the disease. This approach may help to single out candidate epilepsies or candidate seizure types, for which prediction-based intervention techniques can be considered as an alternative therapy option. One should keep in mind, however, that such studies require large, high-quality, meticulously collected and annotated data archives that are well-characterized and represent the heterogeneity of patterns and patients found in human epilepsy.

To gain deeper insights into mechanisms underlying ictogenesis, future studies should address the following questions: What is the period of time over which seizures are generated? What components of the epileptic network are necessary for clinical events to occur? What is the role of high-frequency oscillations in ictogenesis? How are precursors distributed temporally and spatially in brain? What are effective intervention strategies for modulating this process? Together with an improved understanding of the interictal period and all of its confounding variables, answers to these questions might help to refine already existing approaches in order to increase predictive performance, to develop new analysis concepts and measures, and to guide basic science investigations to specific mechanisms. Such studies, however, have to be designed with great care, and with an acute awareness that as we delve deeper into seizure generation, we may be confronted with other, yet unforeseen problems. Along these lines, there is a great need for new analysis methods, using multivariate techniques, to analyze data streams from multiple sensory sites simultaneously, and even to sample at multiple temporal and spatial scales (from single neurons to ensembles, from local to global networks), and to investigate the physiological substrate for larger-scale quantitative observations. In this context, more work on spontaneously seizing animal models of epilepsy will allow access to deep brain structures and other locations in the epileptic network that cannot be explored in human studies because of safety concerns.

Finally, another issue of potential importance is to improve neurocomputational modeling of the dynamics of neuronal networks underlying the transition to seizures. Models currently available are mostly used to investigate epileptic phenomena at different levels of complexity within specific situations. They share, however, the common feature of providing insight into mechanisms involved in the generation of epileptic activity either at a microscopic or a macroscopic level. Research along this line may help to integrate the plethora of experimental data available, to improve analysis concepts and measures, and to test various hypotheses concerning preictal brain dynamics and its relation to endogenous and exogenous control parameters.

It is clear that these investigations require interdisciplinary research and collaborations.

See also: **Models**: Computer Models of Seizures and Epilepsy: Understanding Epileptogenesis using Complex Large-Scale Models; **Networks**: Recurrent Synaptic Actions and the Genesis of Epileptiform activity; **Neurophysiology**: Seizure Detection and Prediction; Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings; **Oscillatory Activity**: Cellular and Network Oscillations in Epilepsy; Cellular Mechanisms of Network Oscillations Occurring Prior to, and during, Seizures; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; Seizure Initiation and Termination; **Treatments (non-Antiepileptic Drugs)**: Control of Seizure Activity with Electrical Stimulation.

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http://www.mpipks-dresden.mpg.de/~tisean – A software project for the analysis of time series with methods based on the theory of nonlinear deterministic dynamical systems.

http://en.wikipedia.org/wiki/Computational_neuroscience – A very complete overview of the rapidly growing field of computational neuroscinece with many links to standard text books and review articles, software tolls, journals, conferences, and research groups.

http://www.scholarpedia.org/article/Encyclopedia_of_Computational_Neuroscience – Scholarpedia is a peer-reviewed encyclopedia written by the leading experts in their respective fields. It does not publish ''research'' or ''position'' papers, but rather ''living reviews'' that will be maintained by the future generation of experts via the process of curatorship.

http://www.scholarpedia.org/article/Encyclopedia_of_Dynamical_Systems – The above mentioned articles/links provide excellent overviews of the diverse fields needed to improve understanding of epileptic phenomena.

http://www.scholarpedia.org/article/Encyclopedia_of_Computational_Intelligence – An understandable and useful encyclopedic reference for scholars of different levels, highly recommended.

Seizure Termination and the Postseizure Refractory Period

R F Berman, University of California Davis, Davis, CA, USA

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Introduction

Seizures are typically self-limiting, synchronous electrical discharges of neurons within circuits or systems of the brain. A great deal is known about the epidemiology, pathophysiology, and the treatment of seizures and seizure disorders. However, much less is known about the cellular mechanisms that stop seizures once they begin (Lado and Moshe, 2008), or that may underlie the postseizure refractory period when the brain shows increased resistance to subsequent seizures. Indeed, most seizures stop on their own, with typical durations in the range of tens of seconds. **Figure 1** shows an example of a brief, self-limiting seizure episode recorded from a rat hippocampus 30 s after an i.v. injection of 0.5 $mgkg^{-1}$ bicuculline, a $GABA_A$ receptor antagonist. The initial burst of seizure activity lasted approximately 50 s, followed after a delay by a lower frequency secondary afterdischarge, and then a period of postictal EEG flattening when the hippocampus is typically partially refractory to further seizures activity.

The self-limiting nature of most seizures can also be seen in clinical epilepsy. Video-EEG monitoring of five major seizures types in hospitalized patients shows an average duration of 18.5 s for tonic seizures, 28 s for simple partial seizures, 66 s for primary generalized tonic–clonic seizures, 78 s for complex partial seizures, and 130 s for secondarily generalized tonic–clonic seizures (Jenssen *et al.*, 2006). Prolonged life-threatening catastrophic seizures can occur as in *status epilepticus*, reflecting a failure of normal seizure-terminating mechanisms. The fact that most seizures are self-limiting suggests the operation of some cellular mechanism, or mechanisms, to terminate ictal activity. The purpose of this review is to summarize some of what is known about why seizures stop once started. Research on the cellular mechanisms that contribute to the termination of the ongoing seizure activity is important, because such information may provide new insights into the mechanisms of seizures (and the patterning of seizures over time), as well as indicate new strategies for developing effective therapeutics agents for seizure control.

Background – Problems in Studying How Seizures Stop

While the question of why seizures stop is clear, it is less evident how to answer this question experimentally. This deceptively simple question about why seizures stop is unavoidably tied to questions about why seizures occur. That is, part of the difficulty lies in the fact that seizures can be caused by one (or more) of several brain mechanisms (e.g., channelopathies, mis-wiring of brain circuits, abnormal metabolism, neurochemical disturbances, etc.). Further, there are many different types of seizures, each associated with different, and sometimes overlapping, cellular mechanisms. There are also technical difficulties in determining which neural mechanisms stop seizures that are independent of those mechanisms initiating and maintaining seizures. This challenge arises because seizure-sustaining and seizure-ending cellular mechanisms can occur together during the development and the progression of seizures.

A second type of problem is that models of epilepsy typically model only specific aspects of epilepsy, or are specific to a subtype of epilepsy. For example, the application of penicillin to the cortex has been used to model a localized cortical seizure focus, while intraperitoneal injections of γ-hydroxybutyrate (GHB) are commonly

Relevant Websites

http://www.mpipks-dresden.mpg.de/~tisean – A software project for the analysis of time series with methods based on the theory of nonlinear deterministic dynamical systems.

http://en.wikipedia.org/wiki/Computational_neuroscience – A very complete overview of the rapidly growing field of computational neuroscinece with many links to standard text books and review articles, software tolls, journals, conferences, and research groups.

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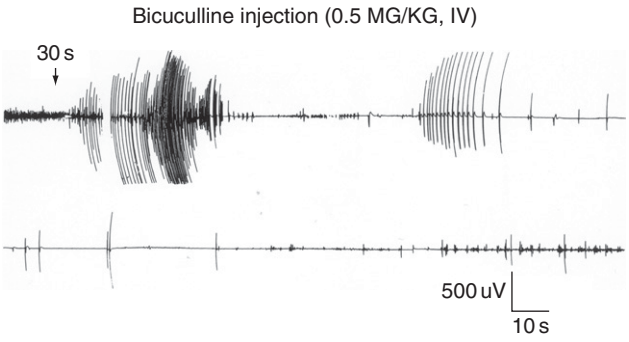


Figure 1 Seizures recorded from the hippocampus induced by 0.5 mg kg^{-1} i.v. bicuculline injection. Seizures began approximately 30 s after injection, resulted in a primary afterdischarge, and a secondary discharge followed by a period of postictal EEG flattening where the EEG was isoelectric. Reproduced from Berman *et al*. (2000).

used to produce spike and wave discharges (SWD) that model absence seizures. Answers about why seizures stop in one model may not apply to other models or types of epilepsy. For example, in vitro models, including low Mg^{2+}, low Ca^{2+}, or elevated K^+ in brain slices, are particularly well suited to discover the cellular mechanisms underlying seizures (and by extension, epilepsy), but results from such studies may not readily generalize to clinical seizures/epilepsy. In vivo animal models, including those developed in nonhuman primate, cat, and rodent, while more realistically modeling clinical epilepsy, still require assumptions that may not be valid when generalizing results to clinical epilepsy. Finally, research carried out directly in human epileptic patients poses its own set of difficulties, including ethical concerns associated with human experimentation, difficulties in controlling the onset and patterning of seizure in a clinical research setting, and obvious difficulties in obtaining tissue for cellular and molecular studies.

Even when such challenges are overcome, the fact that seizures can result in global disturbances in brain activity and metabolism makes it particularly difficult to isolate specific mechanisms central to seizure termination. For example, measurements of specific changes in brain levels of neurotransmitters (e.g., GABA) or neuromodulators (e.g., adenosine) that may be important for stopping seizures can easily be masked or overshadowed by global, and often massive, changes in brain chemistry or metabolism that are involved in triggering and maintaining the seizure activity itself. As an additional example, in vivo microdialysis and ion selective microelectrodes can measure neurochemical and ionic events during either the triggering or stopping of seizures, but are limited to measuring extracellular molecules and ions. As a result, these techniques cannot directly measure intracellular processes that may be particularly relevant to understanding why seizures stop.

Methods

Several in vitro and in vivo models of epilepsy are used to try to understand why seizure stops. Studies of epileptiform spiking in neurons are often carried out in vitro in 400–500 μm thick brain slices cut from the hippocampus or cortex and maintained in culture for hours, or sometimes days. A particularly elegant preparation is the organotypic slice preparation which can be maintained in culture for several weeks. Such slices retain synaptic circuitry capable of supporting seizure activity. Importantly, seizures can be evoked in such brain slices by electrical stimulation of specific fiber pathways (e.g., perforant path axons; Schaffer collateral axons), pharmacological blockade of inhibitory circuitry (e.g., GABA receptor antagonists such as bicuculline), increasing (e.g., K^+) or decreasing (Ca^{2+}, Mg^{2+}) extracellular ion concentrations in the bathing media surrounding the slice, or by activating excitatory circuitry with specific agonists (e.g., glutamatergic agents such as kainic acid). In vivo methods for producing seizures in animal models include the use of chemical convulsants (e.g., fluorothyl; pentylenetetrazol, kainic acid), electrical stimulation (e.g., electroconvulsive shock, electrical kindling, etc.), and the generation of an epileptic focus using focal injections of alumina gel or penicillin into the cortex.

While typically used to study the genesis of seizures, these models are also used to understand why seizures stop, as well as to describe the endogenous anticonvulsant mechanisms in the brain. The research strategy in these model systems is to evoke seizure activity and study the electrophysiological and molecular events that occur in the tissue over the course of seizure activity, concentrating on those events that occur coincident with the cessation of electrographic seizure activity. For example, limbic seizures can be gradually induced by daily electrical stimulation of the amygdala, in a process known as kindling. Implanted microdialysis probes can then be used to sample the chemical environment (i.e., ions, neurotransmitters, neuromodulators) in brain regions showing kindled seizure activity, before, during, and after a seizure episode. Such studies have already provided strong evidence for an endogenous anticonvulsant activity of adenosine as described later in this article.

An alternate approach is to modify some aspects of synaptic circuitry (such as receptor density, second messenger signaling systems, neurotransmitter levels or gene expression) to see how this modification changes the threshold for producing a seizure, or the duration or severity of the seizure. In animal models, this approach often involves the use of genetically altered mice (e.g., receptor knockout or knock-in mice), in which a specific change, sometimes in a specific brain region, can be studied for its effect on seizures. In contrast, studies in humans rely

mainly on relatively noninvasive procedures such as EEG recordings, and MRI and PET imaging procedures to try to understand the electrical, chemical, and metabolic changes that occur in the brain during and after seizures.

Recent Findings

Cellular Mechanisms

Mechanisms that terminate the ongoing seizure activity have been described at the cellular level, and involve changes in sodium, potassium, and calcium channel activity. Specifically, sustained excitatory input during seizures can generate large (20–40 mV) and prolonged (50–200 ms) depolarization of neurons and trigger trains of action potentials. This pattern of prolonged depolarization and high frequency firing of neurons has been called the paroxysmal depolarizing shift (PDS), and epileptiform cortical EEG activity appears to reflect this underlying cellular depolarization. Large and sustained depolarization during seizures also activates calcium- and sodium-dependent potassium channels resulting in substantial potassium efflux into the extracellular space. Extracellular $[K^+]_0$, normally around 3 mM, rises during a PDS to 5–7 mM, and during prolonged seizure activity to as high as 10–12 mM. This large increase in $[K^+]_0$ initially contributes to further membrane depolarization and increased firing. However, prolonged depolarization and the rise in extracellular potassium can cause depolarization blockade, resulting in the cessation of firing, at least in part, through the inactivation of sodium channels. In a recent computer simulation of model neurons, repetitive firing of action potentials did not result in seizure activity, and ion shifts were relatively minor (Kager *et al.*, 2007). However, when the simulated Na^+/K^+ pump current or the glial membrane conductance were depressed, potassium accumulated in the extracellular space and produced regenerative seizurelike electrical discharges. Such studies emphasize the importance of nonsynaptic (i.e., ionic) mechanisms for seizure generation. Decreased extracellular calcium levels during seizures may also play a role in stopping seizures. Measurements with ion-selective electrodes, in brain slices or in vivo, also show a large decrease in extracellular Ca^{2+} beginning with the onset of the seizure discharge, indicating calcium influx into neurons at the seizure focus. Such a decrease in extracellular calcium could limit calcium-dependent release of excitatory neurotransmitters (e.g., glutamate) and contribute to the stopping of seizure activity.

In one of the few studies directly examining the cellular mechanisms underlying the termination of epileptic afterdischarges in the hippocampus (see **Figure 2**), investigators observed that afterdischarges produced in the hippocampus by electrical stimulation of the perforant path or commissural fibers were followed by a slowly spreading (0.1–0.2 mm s^{-1}) negative DC potential that began in the distal apical dendrites of CA1 pyramidal cells (e.g., around the hippocampal fissure) and slowly spread to the pyramidal cell layer.

A similar slowly spreading DC potential occurred with the termination of the afterdischarge in the dentate gyrus/CA3 region, after which the EEG became isoelectric (i.e., postictal EEG depression). The authors suggested that stimulation-induced epileptiform afterdischarges were terminated by a

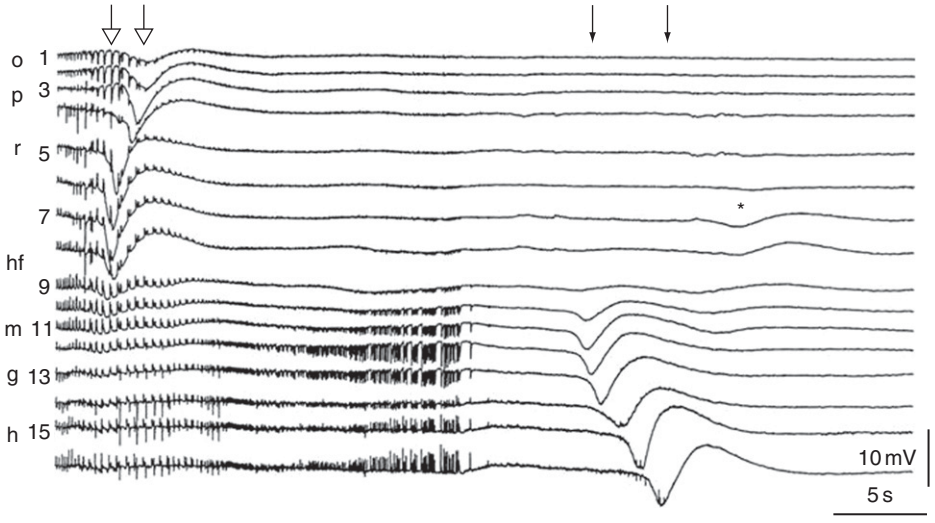


Figure 2 Extracellular recordings of a propagating epileptiform afterdischarge in the CA1-dentate gyrus in the awake rat. Multielectrode recording shows the large DC potential that began in the apical cell layer near the hippocampal fissure (electrode 8) and spreading to the CA1 pyramidal cell layer (electrode 3) at 0.28 mm s^{-1} (open arrows) and later into in the dentate gyrus (filled arrows) at 0.12 mm s^{-1}. Reproduced from Bragin A, Penttonen M, and Buzsaki G (1997) Termination of epileptic afterdischarge in the hippocampus. *Journal of Neuroscience* 17(7): 2567–2579, with permission from Society for Neuroscience.

depolarization blockade of principle cells, followed by a pervasive spreading depression of neuronal activity.

In summary, prolonged and intense seizures can generate large changes in ion homeostasis, including up to a three- to fourfold increase in extracellular potassium with a subsequent depolarization blockade of neuronal firing and termination of epileptiform activity.

Energy Metabolism and Seizure Termination

Altered energy metabolism can also influence the duration of seizures. The cerebral metabolic rate is higher during seizures than during virtually any other state of brain activity, as reflected in a large increase in oxygen uptake, glucose uptake, glucose metabolism, and elevated ATP levels. Increased cerebral blood flow and vasodilation also occur during seizures. In rodents a single electroconvulsive seizure can elevate glucose metabolism for up to 1 h throughout the postictal period.

In general, if arterial blood pressure, arterial oxygenation, and blood glucose concentrations are maintained, enhanced metabolism during seizures can be sustained in excess of one hour.

In contrast, prolonged seizures, as in *status epilepticus*, produce a dramatic fall in energy stores in the brain, depleting levels of brain glucose, glycogen, phosphocreatine and ATP, and decreasing intracellular pH. This finding has contributed to the radical hypothesis that seizures may actually be an adaptive response of the brain to energy failure, particularly failure of microchondrial respiration, with seizures producing vasodilation, increased cerebral blood flow, and greater glucose availability. According to this hypothesis, when energy balance is restored, seizure activity stops. Indeed, hypoglycemia can cause seizures in humans and animals and can potentiate flurothyl-induced generalized seizures, indicating that low systemic levels of glucose are proconvulsive. Interestingly, [^{18}F]fluorodeoxyglucose imaging during the interictal period in humans shows hypometabolism in brain regions showing EEG seizure activity. Such hypometabolism may be a hallmark of an epileptic focus, although neuronal loss due to seizures may also contribute to reduced regional brain metabolism.

Extracellular adenosine triphosphate (ATP) levels are also elevated during intense seizure activity, and elevated ATP levels have been suggested to activate the inwardly rectifying K_{ATP} channel Kir6.2, hyperpolarizing neurons in the substantia nigra pars reticulata (SNr). The SNr has been suggested to serve as a gateway for the propagation of cortical seizure activity, with decreased firing of SNr GABAergic neurons associated with a decreased spread of seizure activity. Because the SNr has been argued to function as a central gating system for the propagation of seizures, its inactivation by Kir6.2 channel activity could represent a mechanism for protection against the development and the spread of generalized seizures.

GABAergic Inhibition

γ-Aminobutyric acid (GABA) is one of the major rapidly acting inhibitory transmitters in the nervous system, and the loss of GABAergic inhibition has been argued to underlie many types of epilepsy. Therefore, the activation of GABA-mediated inhibition may also play a role in the stopping of seizures. GABA mediates the inhibitory activity through opening GABAergic Cl^- ($GABA_A$) or K^+ ($GABA_B$) ion channels, producing hyperpolarization of target neurons. Extracellular levels of GABA rise with the seizure onset in rodents and in human epilepsy, and feed-back and feed-forward inhibition through GABAergic circuitry has been hypothesized to create a powerful inhibitory zone that serves as a barrier to the seizure activity and the seizure spread. In general, such synaptic modulation via the GABAergic system is thought to provide tonic inhibition in the brain, providing a barrier to the pathological excitatory activity that occurs during a seizure.

The focus on GABA is also based on the fact that the enhancement of GABAergic inhibition in the brain is the primary molecular mechanism of action of many, if not most, antiepileptic medications, including the benzodiazepines and barbiturates. Other anticonvulsants, such as γ-vinyl GABA (vigabatrin) and tiagabine potentiate GABA-mediated inhibition of neuronal activity by inhibiting GABA-transminase or GABA uptake, respectively. Conversely, proconvulsant drugs such as picrotoxin, pentylenetetrazol, and *t*-buryl bicyclophosphorothionate (TBPS) decrease the mean $GABA_A$ channel open time and reduce the inhibitory activity.

Changes in pH and Seizure Termination

Neuronal activity elicits substantial changes in intracellular and extracellular pH, and it has been proposed that changes in intracellular pH (pH_i) may have a role in terminating seizures. Paroxysmal epileptiform afterdischarges evoked in rat dentate gyrus cause acidification (0.07–1.5 pH units) in the interstitial fluid of the dentate. The acidification has been attributed to the production of CO_2 and other acid metabolites such as lactate. Extracellular acidification, produced experimentally by increasing CO_2 levels or directly acidifying the bath media to pH 6.7 in the in vitro 0-Mg^{2+} model of epilepsy in hippocampal brain slices, increases the interval and decreases the maximal amplitude of seizurelike events, and lowers the rate of seizure propagation. Intracellular acidification also occurs during the paroxysmal activity evoked in hippocampal slices by incubation in 0 Ca^{2+} and high K^+; acidification occurs with each spontaneous field burst, with the lowest pH_i levels seen at the end of each burst and recovering to resting values over

approximately 20 min. Importantly, when pH_i decreases below a certain value, the paroxysmal burst stops, and the neurons are refractory to further burst activity until pH_i recovers by approximately 50%. Thus, intracellular acidification may contribute to the cessation of epileptiform discharges.

Because many cellular processes are pH-sensitive, intracellular acidification may be an important mechanism preventing neuronal activity from reaching levels that may threaten normal brain. The mechanisms underlying pH effects on epileptiform field bursts are unclear, but could involve proton sensitivity of the NMDA channel via an intrinsic proton sensor. When extracellular pH decreases, such as can occur during the epileptiform activity, NMDA receptors are inhibited and this could inhibit glutamate-associated seizure activity. Gap junction coupling has also been shown to be sensitive to pH_i with closure occurring at a low pH. Therefore, intracellular acidification following seizure activity could reduce gap junction coupling and prevent the development of astrocytic calcium waves that may contribute to the initial spread of seizure activity, thus facilitating seizure termination.

Adenosine

Several lines of evidence indicate that adenosine is important for terminating the ongoing seizure activity (Boison, 2005). Adenosine release has been shown to occur during seizures; adenosine agonists suppress seizures and raise seizure thresholds; and adenosine antagonists are proconvulsant. These actions are not surprising in view of the powerful inhibitory effect that adenosine has on synaptic activity and neurotransmitter release. The proposed mechanism for seizure termination by adenosine involves increased adenosine synthesis from ATP that is coupled to the dramatically increased metabolic demand generated by the seizure activity. Adenosine is synthesized by the hydrolysis of nucleotides (ATP, ADP, AMP) by several endo- and ectonucleotidases. Efflux of adenosine into the extracellular space, through a bidirectional transporter, allows adenosine to interact with cell-surface receptors (i.e., A_1, A_{2A}, A_{2B}, and A_3) to modulate the synaptic activity. Adenosine acts both presynaptically to inhibit the release of excitatory and inhibitory neurotransmitters (including glutamate, acetylcholine, norepinephrine, and GABA) and postsynaptically by hyperpolarizing neurons through an increase in potassium conductance.

As indicated earlier, extracellular levels of adenosine have been shown to increase during seizures in animal models of epilepsy and in human epileptic tissue. Specifically, in vivo microdialysis studies show substantial increases in the hippocampus of rats undergoing pharmacological seizures produced by kainate, bicuculline or pentylenetetrazol. Brief seizures produced by bicuculline can produce increases of up to twofold, while more severe and prolonged seizures produced by pentylenetetrazol can generate 4–5-fold increases. The anticonvulsant activity associated with the adenosine receptor activation appears to be primarily mediated via activation of the A1 receptor subtype. The increase in extracellular adenosine could therefore contribute to the termination of the ongoing seizure activity.

Systemic treatment with adenosine receptor agonists, or direct injection of adenosine agonists into a seizure focus, has been shown to block seizure activity, and to prolong postictal EEG flattening seen during the postseizure refractory period in the kindling model of epilepsy. **Figure 3** shows a typical epileptiform afterdischarge recorded from an amygdala-kindled rat pretreated with saline (a), an adenosine receptor antagonist, adenosine 5′-ethylcarboxamide (NECA) (b), or caffeine, an adenosine receptor antagonist (c). Compared to saline, NECA reduced the seizure duration and prolonged the period of postictal EEG flattening, while caffeine prolonged the seizure and eliminated the postictal EEG flattening.

The combination of *N*6-cyclohexyladenosine, an adenosine A1 agonist, with major antiepileptic drugs (e.g., diazepam, sodium valproate, diphenylhydantoin, phenobarbital, carbamazepine, etc.) shows an enhanced anticonvulsant

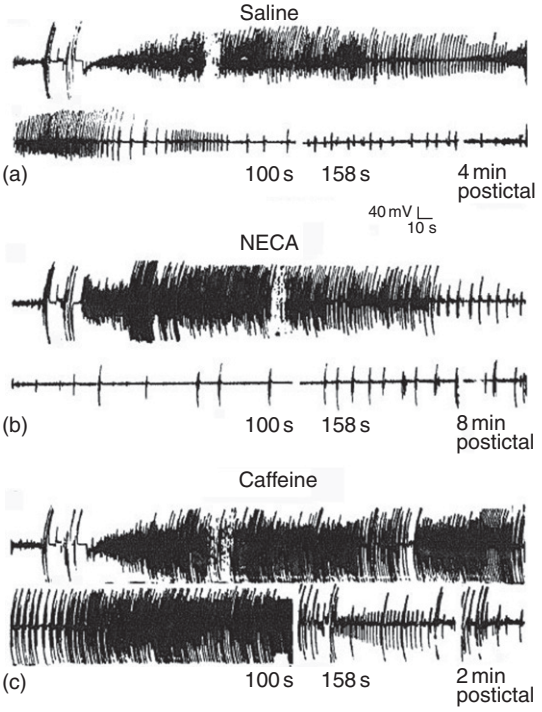


Figure 3 Prolonged epileptiform discharges recorded from the amygdala of an amygdala-kindled rat. (a) Preseizure treatment with saline, (b) 0.032 mg kg^{-1} NECA, or (c) 10 mg kg^{-1} caffeine. Compared to saline treatment, NECA shortened the seizure duration and prolonged the period of postictal EEG flattening. Caffeine prolonged the seizure and eliminated the posticatal EEG flattening. Reproduced from Whitcomb *et al.* (1990).

activity in mice against seizures produced by electroconvulsive shock or pentylenetetrazol. Adenosine reduces seizure activity in the rat hippocampus in the in vitro 0-Mg^{2+} model of epilepsy, and similar effects have been observed in slices of human epileptogenic cortex. Finally, inhibitors of adenosine kinase, which increase brain adenosine levels, are also anticonvulsant.

These studies are consistent with the hypothesis that adenosine release during a seizure can initiate a powerful adenosine-mediated inhibition of neuronal firing that would be expected to terminate seizures and render the brain refractory to further seizures, as long as adenosine levels remain elevated.

Neuropeptides as Endogenous Anticonvulsants

Several neuropeptides, including neuropeptide Y (NPY) and somatostatin (SS) have been shown to have anticonvulsive properties and could play significant roles in the termination of seizure activity. NPY is a 36 amino-acid neuropeptide that is preferentially expressed in GABAergic interneurons and is widely distributed in the mammalian nervous system. Recent findings indicate an important role for NPY in inhibiting the seizure activity. Specifically, mice lacking the NPY gene develop spontaneous seizures and show heightened susceptibility to evoked seizures. Administration of exogenous NPY suppresses seizures produced by a variety of chemical convulsants, suppresses absence seizures in genetically seizure-prone rats, and reduces epileptiform activity in hippocampal and cortical brain slices. The potent anticonvulsant effects of NPY are thought to be due to the activation of presynaptic Y_2 receptors which reduces calcium influx into presynaptic terminals and thereby inhibits glutamatergic synaptic transmission. A possible endogenous anticonvulsant role for NPY is also supported by the findings that it is released following the seizure activity, that the expression of NPY is increased after electrically or chemically induced seizures, and that overexpression of NPY reduces seizure susceptibility in rats. In a rat model of febrile seizures, the most common seizure type in infants and young children, a single prolonged (~24 min) febrile seizure increases the expression of NPY. This observation has led to the hypothesis that the enhanced NPY activity following a febrile seizure inhibits further seizure activity, resulting in the 'single seizure per febrile seizure' phenomenon seen in most children with febrile seizures. In brain tissue specimens from patients with severe drug-resistant mesial temporal lobe epilepsy, abundant sprouting of NPY fibers occurs in the hippocampus along with upregulation of Y_2 receptor, possibly representing an endogenous homeostatic anticonvulsant mechanism. Considered together, these findings suggest that the release of NPY may contribute to the termination of the seizure activity.

Somatostatin (SS) is a tetradecapeptide that was first identified as the anterior pituitary factor that inhibited growth hormone. Since that discovery, SS has been found in several brain regions, including the hippocampus, amygdale, and neocortex, where it is frequently found colocalized with GABA (in GABAergic neurons). Evidence for the fact that SS plays a prominent role in epilepsy includes demonstration of its release during seizures, modulation of SS gene expression and transcription by seizures, and a powerful inhibitory influence on neuronal firing, including anticonvulsant activity. The initial link between SS and epilepsy came from studies showing a loss of SS-containing neurons in the hilus of the dentate gyrus after seizures, and this loss is considered a common characteristic of an epileptic hippocampus. SS inhibits epileptiform activity in in vivo and in vitro hippocampal brain slices. Direct injection into the cerebral ventricles, hippocampus, or amygdala of rats reduces the severity and duration of seizures, and injection into the hippocampus of an antibody directed against SS accelerates the development of hippocampal kindled seizures. In the in vitro hippocampal brain slice preparation, SS inhibits epileptiform bursting in the 0-Mg^{2+} seizure model. The inhibitory effects appear to be mediated via hyperpolarization of neurons through an increase in two types of postsynaptic K^+ currents: a voltage-sensitive current and a voltage-insensitive potassium leak current. Finally, seizures cause a large and prolonged enhancement of SS mRNA expression in models of epilepsy such as kindling. As with NPY, the release of SS during seizures and its powerful inhibitory influence on neuronal activity suggest that SS could be involved in reducing discharge during seizures and thus contributing to seizure cessation.

Endocannabinoids and Seizures

Cannabis extracts have been used for centuries to treat epilepsy, and it is now known that these effects are due to the activity of a family of compounds known as cannabinoids in the extracts. Recent research suggests that endocannabinoids produced in the brain may have anticonvulsant and neuroprotective activity against excitotoxic seizures. Endocannabinoids (EC) are endogenous small lipid molecules, structurally related to the eicosanoids. The two major EC's are arachidonyl-ethanolamide (anandamide) and 2-arachidonyl glycerol (2-AG). They are synthesized by enzymatic activity in neurons and are released postsynaptically in an activity-dependent manner to retrogradely activate presynaptic G-protein coupled receptors. The principal cannabinoid receptor subtype in the nervous system is CB1R, which is localized on or near synaptic terminals. CB1R receptors are widely distributed throughout the brain, and the activation of the CB1R receptor inhibits the release of glutamate and

GABA by blocking presynaptic N-type voltage-gated Ca^{2+} channels. A clear role for EC and their receptors in seizure disorders has not yet been firmly established, but cannabinoid compounds such as Δ^9-tetrahydrocannabinol and WIN 55,212-2 (that activate CB1 receptors) have potent anticonvulsant properties against seizures produced by maximal electroshock in rats. The endogenous ligands anandamide and 2-AG also attenuate low-Mg^{2+} epileptiform activity in the rat in vitro hippocampal slice preparation. The enhancement of endogenous EC activity can be achieved by inhibition fatty acid amid hydrolase (FAAH), the primary mediator of hydrolysis of endocannabinoids. The inhibition of EC hydrolysis, using the FAAH inhibitor AM374, significantly reduces seizure severity as well as cellular markers of neuronal and synaptic damage in kainic-induced seizures in rats. Additional evidence for a neuroprotective function of endocannabinoids comes from studies of human postmortem hippocampal tissue from epileptic patients. The level of mRNA for the CB1 cannabinoid receptor in epileptic tissue, measured by RT-PCR, is reduced by up to 60% compared to control tissue, and the ratio of CB1-positive to CB1-negative axon terminals is reduced in glutamatergic axon terminals, but not in GABAergic terminals. These findings, while not proving that endocannabinoids are involved in seizure termination, do indicate that the endocannabinoid system can modulate seizure activity.

Summary and Directions for Future Research

Our current understanding of why seizures stop is incomplete, and the search for unifying principles continues in this area of research. It is probable that no single mechanism will be found capable of fully explaining the self-limiting nature of most seizures. Multiple inhibitory mechanisms appear to be engaged during seizures, and modulation of seizure activity occurs at several levels, from individual neurons to networks of neurons to more global regulation across brain regions. In spite of such challenges, the self-limiting nature of most seizures has been linked to several mechanisms, including depolarization blockade, pH changes, metabolic state, and the inhibitory actions of neurotransmitters (GABA) and several neuromodulators (e.g., neuropeptide Y, somatostatin, endocannabinoids, etc.). At the single cell level, it is likely that major shifts in ionic equilibria for K^+ and Ca^{2+} trigger nonsynaptic membrane events leading to depolarization blockade and inhibition of neuronal firing. At the synaptic level, several endogenous substances have been clearly implicated in limiting seizure activity. The best evidence to date may revolve around adenosine. Adenosine synthesis and release are linked to enhanced metabolic activity such as occurs during seizures; the powerful pre- and postsynaptic inhibitory actions of adenosine in the CNS are consistent with an endogenous seizure terminating anticonvulsant role. The neuropeptides NPY and somatostatin are also released during seizures and inhibit neuronal activity, albeit by quite different molecular mechanisms. Finally, the endocannabinoids, which are also thought to presynaptically block neurotransmitter release, have received a great deal of recent attention as endogenous anticonvulsants.

Future research should be directed to better isolate and define the critical events that occur at the time seizures stop, with the goal of identifying those events that are necessary and sufficient for the termination of seizures. If depolarization blockade and spreading depression play a central role in stopping seizures, as indicated by current research, then it will be necessary to better understand the cellular mechanisms underlying these phenomena. An important role for glia in regulation of brain excitability is also becoming clear, and their involvement in seizures, and stopping of seizures, should also become a focus of future research. Newer technologies will be needed that can measure, in real-time, changes in putative endogenous anticonvulsants during seizures activity, so that chemical events in the brain can be mapped directly on to the electrophysiological indices of seizure activity and seizure termination. Finally, it will be necessary to develop new pharmacological tools to study some of the more recently discovered substances that appear to have endogenous anticonvulsant activity.

See also: **Non-Synaptic Mechanisms**: Changes in Extracellular Ion Composition; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; **Peptides**: Endocannabinoids, Opioids and the Regulation of Hippocampal Inhibition; Tachykinins and Epilepsy; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis; **Seizures**: Seizure Initiation and Termination.

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SLEEP

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Brain Mechanisms Linking Epilepsy to Sleep

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Introduction

Even before proclaiming epilepsy independent of any malevolent deity, the Hippocratic school had already demystified sleep (God Hypnos in Greek mythology). The physicians of antiquity used sleep in their medical practice in various ways, and the relationship between sleep and epilepsy was recognized. Extensive clinical observations in the nineteenth century distinguished a large percentage of seizures (>20%) that occurred only at night (nocturnal), and a particular vulnerability to the occurrence of other types of epilepsy at awakening and sleep onset (i.e., transition times). Advances were made with the advent of electroencephalography (EEG) in the 1930s and with the widespread availability of polysomnography in the following decades, which led to the seminal series of studies that associated 'awakening seizures' to idiopathic generalized epilepsy (IGE) with tonic–clonic seizures. More recently, video-polysomnography and presurgical EEG monitoring have expanded and confirmed these associations, establishing the statistical likelihood of specific types of epilepsy to express their signs during particular sleep stages.

Aggravation of seizures by sleep would sound perhaps incompatible with the old ideas of sleep being a state of brain inhibition and restoration. Today, we know that sleep contains periods of intense neuronal activity, and that even when thalamic gates are closed to the outside world, intracortical dialogue is maintained. We distinguish three states of existence: awake, rapid eye movement sleep (REMS), and non-REM sleep (NREMS). Typically, as one goes to sleep, the low-voltage fast EEG pattern of wakefulness gradually gives way to slower frequencies, as NREMS goes from stage 1 (decrease in alpha) to stage 2 (spindles, K-complexes) to stage 3 and 4 (increasing amplitude and regularity of delta rhythm – see **Figure 1**(**a**)); stages 3–4 are therefore referred to as 'slow-wave sleep.' This pattern of change is the NREMS descending phase, which is followed by a more abrupt ascending phase (from stage 4 to stage 1) and an episode of REMS. Besides the characteristic saccadic eye movements, REMS is characterized by a high level of brain activity, the presence of low-voltage fast EEG activity (desynchronized EEG), the suppression of muscle tone, vivid dreaming, and the highest awakening threshold (therefore, 'paradoxical sleep'). In contrast, during NREMS, brain activity is reduced in most brain areas and thinking is usually nonvisual and ruminative. As displayed in the hypnogram in **Figure 1**(**b**), every night's sleep progresses through several such ~90-min-long NREMS–REMS cycles, with NREMS predominating in

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Seizure-Induced Gene Expression

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Introduction

Seizures, defined as a pathological increase in neuronal synchronization, are not just the characteristic symptoms of epilepsy, but can also have direct effects on the brain. In particular, prolonged seizures can produce neuronal damage and trigger cascades of biochemical events that can have long-term effects. These seizure events activate second messengers, transcription factors and gene expression, plastic phenomena (such as mossy fiber sprouting), neurogenesis, and altered neurotransmission – all of which may underlie consequent sensory-motor, developmental, and behavioral and cognitive deficits. Seizure-induced gene expression is of particular importance, since production of new proteins (or proteins at altered levels) provides the means for long-lasting alterations in brain functions. Analysis of alterations in gene expression can single out particular molecular mechanisms involved in such long lasting, potentially harmful, effects of seizures.

Background

Regulation of Gene Expression

In this article we focus on seizure-induced gene expression. While approaching this subject, one should be aware that the regulation of gene expression is a very complex phenomenon. By definition, regulation of gene expression refers to the control of the amount and timing of appearance of functional gene products, that is, RNAs and proteins. The process includes transcription as well as posttranslational modification of protein, which can be modulated at any step via any number of mechanisms.

The first level of regulation of gene expression relies on modification of DNA. Chemical modification refers to methylation of CpG islands by methyltransferase, a common way for silencing a gene. Structural modifications of DNA arise from phosphorylation or methylation of histones; histone acetylation is of special importance for regulation of transcription, since histone deacetyltransferases enable the dissociation of DNA from the histones and therefore allow transcription to proceed.

Progression of transcription and the efficiency of RNA synthesis depend on several components of the RNA polymerase machinery, including several regulatory proteins that bind to regulatory sites, usually located near the promoter. The regulatory proteins can be categorized according to their function as: (1) specificity factors that alter specificity of RNA polymerase for given promoter and repressors that bind to noncoding DNA sequences in the vicinity of the promoter and impede RNA polymerase's progress along the DNA strand; (2) basal factors which position RNA polymerase at the start of transcription, and activators that enhance RNA polymerase interaction at a given promoter either by interaction with RNA polymerase subunits or changing the structure of the DNA; and (3) enhancers that lead to changes in the three-dimensional structure of DNA and bring specific promoters into the vicinity of the initiation complex.

When RNA is synthesized, it undergoes further modifications which influence its stability, and therefore determine the yield of translation into protein. Capping protects mRNA from 5′exonuclease, splicing removes introns – noncoding regions – from RNA and allows translation into proper protein, and addition of poly(A) tail increases mRNA's stability by slowing down 3′exonuclease action.

All these regulatory mechanisms determine which genes are transcribed and at what efficiency. The number of mRNA copies of a given gene determines the translation into the protein and protein content in the cell. Additionally, translation and protein stability and trafficking are tightly regulated. The ultimate outcome is the tight control of the cell's structure and metabolism at a given moment in time.

It is, therefore, reasonable to ask whether gene transcription in the brain is regulated by seizures. Are there any regulatory mechanisms triggered by seizures? Studies have now shown that seizures can influence the transcriptional events on several levels. On the level of DNA modification, seizures regulate histone acetylation and phosphorylation on several promoters. There are also data showing that seizures affect the transcriptional machinery by activation of transcription factors, including transcription factors linked to maintenance of CpG islands like SP-1 or transcription factor complexes that provide means of tight control of transcription AP-1, cAMP response element-binding protein (CREB), the activating transcription factor-4 (ATF4), DREAM (downstream response element antagonistic modulator), NF-kappa-B, Nurr1, and others.

The fact that transcriptional events can be regulated by seizures leads us to the next question: What are the target genes which expression is regulated? We address this question in the coming sections, following a brief description of the methods used to study regulation of gene expression.

Methods

Methods Used for Studies of Activation of Gene Expression

Quantitative analysis of the mRNA content of selected genes of interest

One way of approaching the transcriptional activity of genes of interest is simply to evaluate the amount of its mRNA in the tissue. Through the years a number of methods have been mastered to achieve this goal. One of the oldest methods is Northern blot. For this application, RNA is isolated from the sample and then is separated by size, using gel electrophoresis. The RNA is transferred to a membrane which is then exposed to a radiolabeled, gene-specific probe. If the mRNA of interest is in the sample, the probe will specifically hybridize to the mRNA and produce a signal at the predicted size on the autoradiogram.

In situ hybridization provides a means of determining the pattern of mRNA expression at the cellular level. For in situ hybridization, fixed tissue sections with preserved cellular anatomy are used. The tissue sections are mounted on slides and prepared in a way that allows the labeled, gene-specific probe to have access to the cellular compartments and to hybridize with the mRNA present in the section. Radioactively labeled probe is then detected with X-ray film. To obtain cellular details, the slides can be coated with photographic emulsion; following development, the signal will be seen as silver grains (black dots) over the area containing the mRNA of interest. If fluorescently labeled probes are used, the results of hybridization are analyzed using standard fluorescent microscopy techniques.

Another powerful technique for quantifying the mRNA content is reverse transcription polymerase chain reaction (RT-PCR). The principle of this method rests on the propagation of mRNA species of interest from the minute quantities found in the starting material. For gene expression analysis, the first step is synthesis of cDNA on the basis of mRNA extracted from the studied tissue, using the enzyme called reverse transcriptase. This cDNA serves then to amplify cDNA representing the gene of interest; the procedure involves the application of a thermosTable DNA polymerase and gene-specific oligonucleotide primers which hybridize to cDNA of interest and allow the enzyme to attach to and synthesize DNA. The PCR reaction is cycled trough a series of temperature changes which provide the conditions for repeated cycles of: separation of DNA strands (e.g., at 94 °C), primer annealing (usually at 42–65 °C), and synthesis (at 72 °C). PCR results in very efficient amplification of DNA and is successfully used for evaluation of the level of mRNA expression, especially if real-time PCR is used, which allows quantification of amplification product in each reaction cycle.

Global analysis of gene expression

During the last decade, we have witnessed the rapid development of methods for the analysis of the whole transcriptome. Such methods do not require preselection of a gene of interest, and thus provide a means for unbiased analysis of global gene expression – which may result in detection of expression level alterations of previously unknown genes.

Historically, one of the first approaches to study a large fraction of the transcriptome was Differential Display. It is a RT-PCR-based method, used to identify differentially expressed genes. In differential display, mRNAs from the specimen are first reverse transcribed and then amplified using nonspecific primers. The array of bands obtained from a series of such amplifications is separated on a sequencing gel, and compared with analogous arrays from different samples. Any bands unique to a particular sample represent differentially expressed genes. The bands can be purified from the gel and sequenced.

SAGE (serial analysis of gene expression) and MPSS (massively parallel signature sequencing) are methods that involve creation of large number of short cDNA tags

(produced on the basis of studied mRNA), their subsequent sequencing, counting, and assigning to corresponding genes using bioinformatics tools. The frequency of a specific tag is related to the abundance of the corresponding mRNA in the cell, and this information allows for a comparison of mRNA expression of both known and novel genes in different samples. No a priori knowledge of the transcript sequence or mRNA abundance is required.

DNA microarrays are increasingly popular in studies of gene expression. They enable evaluation of the expression levels of thousands of genes at the same time, and thereby provide global insight into transcriptional events taking place in a studied phenomenon. The principle of DNA microarray technology is the hybridization of labeled cDNA synthesized on the basis of mRNA derived from the tissue of interest, to a microarray consisting of a large number of gene probes placed with high density on a solid surface. The strength of the hybridization signal associated with a particular gene probe reflects the abundance of the respective mRNA in the sample. There are currently two main types of microarrays: spotted microarrays and Affymetrix Gene Chips. In the case of spotted microarrays, the probe is usually spotted on a surface and is hybridized with fluorescently labeled cDNA. It is possible to perform simultaneous hybridization with cDNA from two different conditions (for example control vs. treated), with each condition labeled with a different fluorochrome. Detection of transcript using GeneChips, produced by Affymetrix (Affymetrix, Inc., Santa Clara, CA), relays on perfect match versus mismatch oligonucleotide probe pairs; several probes for each gene increase the specificity and reproducibility of the quantitative results. Microarray experiments require specialized bioinformatic tools for analysis of raw hybridization data as well as for extracting biological meaning from such data. Tools that suggest assignment of experimental data to known cellular and molecular pathways are readily available, and new possibilities for in-depth analyses are constantly emerging.

Promoter activity assays

An alternative method to study regulation of transcription is evaluating the transcriptional activity of the promoter of a gene of interest (instead of quantifying its mRNA level). This approach is usually carried out by introducing into the cells under study a plasmid or viral vector containing the reference gene under control of a promoter of interest. The reference gene usually codes for fluorescent protein (luciferase or green fluorescent protein), so that it can be easily measured by fluorescence or luminescence counter. The activity of the promoter is directly related to the amount of the detected reference protein. In this way the influence of a stimulus (e.g., a seizure) on transcriptional activity of a selected promoter can be assayed.

Animal Models of Seizures

The prerequisite for undertaking any experimental study on seizure-induced gene expression is identification of a proper animal model. Although human tissue coming from resections or autopsy is widely used in epilepsy research, it is of limited use in search for seizure-induced events. The molecular state of the tissue is heavily influenced by disease history and medication, which would be indistinguishable from the effects of seizures alone in such tissues.

Selection of the animal model depends on the specific questions being asked, and a wide range of characterized models is available (see Pitkänen and coworkers, 2006 in Further Reading). In some models, seizures can be evoked in otherwise normal animals by application of proconvulsant. Many such convulsive substances have been tested; the most frequently used are $GABA_A$ receptor blockers (pentylenetetrazol, bicuculine, picrotoxin, penicillin), excitatory amino acid receptor activators (kainic acidquisqualic acid, NMDA), acetylocholine-related substances (pilocarpine), strychnine, flurothyl, topical application of metals, and many others. Another way of inducing seizures in normal, nondiseased brain is electrical stimulation of the whole brain (electroshock) or defined brain structures (hippocampus, amygdala). Depending on the specific protocol and method of seizure induction, one can produce short seizures, or status epilepticus (which has much more pronounced consequences for the brain).

Seizures can also be studied in experimental animals that generate seizures spontaneously – that is, that are 'epileptic.' There are a number of rat (e.g., GAERS and WAG/Rij) and mouse (e.g., EL, tottering, staggerer, weaver) models that are genetically prone to develop seizures. A number of genetically modified mice have also been created that express an epileptic (i.e., spontaneous seizure) phenotype. Epilepsy can also be induced experimentally – by chemically – or by electrically induced status epilepticus, stroke, traumatic brain injury, or extensive kindling. Such animals become epileptic with time and express spontaneous seizures.

Some of these models have been successfully used to study seizure-induced gene expression.

Recent Results

The expression of a large variety of genes has been studied in response to seizure activity. Most of the data come from studies using status epilepticus as the seizure model, and only a few studies have monitored gene expression after single induced or spontaneous seizures. Selected papers describing changes in gene expression following brief, chemically or electrically induced seizures or kindling are summarized in **Table 1**. Recently, reviews of status epilepticus-induced gene expression as well as of

Table 1 Selected studies demonstrating seizure induced changes in gene expression

Gene	*Model*	*Author*
Transcription factors		
c-fos	Audiogenic seizures	Shin *et al*. Brain Res. 1990;518:308–12. Simler *et al*. Neurosci Lett. 1994;175:58–62.
	Kindling	Dragunow *et al*. Nature. 1987;329:441–2. Clark *et al*. Mol Brain Res. 1991;11:55–64. Teskey *et al*. Mol Brain Res. 1991;11:1–10. Simonato *et al* Mol Brain Res 1991;11:115–24.
	Metrazol	Morgan *et al*. Science 1987;237:192–7. Kanter *et al*. Exp Neurol 1994;129:290–8. Retchkiman *et al*. Neurobiol Aging 1996;17:41–4. Del Bel Ea *et al*. Cell Mol Neurobiol 1998;18:339–46. Saffen Proc Natl Acad Sci USA 1988;85:7795–9. Yount *et al*. Mol Brain Res 1994;21:219–24.
	ECS	Winston *et al*. J Neurochem 1990;54:1920–5. Marcheselli *et al*. J Neurosci Res 1994;37:54–61.
	Electrical stimulation	Ebert *et al*. Brain Res 1995;671:338–44.
	Febrile seizures	Kim *et al*. Neuroreport 2001;12:3243–6.
	Febrile seizures	Kim *et al*. Neuroreport 2001;12:3243–6.
	Cocaine-induced seizures	Clark *et al*. Brain Res 1992;582:101–6. Helton *et al*. Mol Brain Res 1993;20:285–8.
c-jun	Metrazol	Saffen Proc Natl Acad Sci USA 1988;85:7795–9. Yount *et al*. Mol Brain Res 1994;21:219–24.
	Kindling	Simonato *et al*. Mol Brain Res 1991;11:115–24.
	ECS	Winston *et al*. J Neurochem 1990;54:1920–5.
jun-B	Metrazol	Saffen Proc Natl Acad Sci USA 1988 Oct;85(20):7795–9.
zif/268	Metrazol	Saffen Proc Natl Acad Sci USA 1988;85:7795–9. Yount *et al*. Mol Brain Res. 1994;21:219–24.
	Kindling	Simonato *et al*. Brain Res Mol Brain Res. 1991 Sep;11(2):115–24.
	ECS	Marcheselli *et al*. J Neurosci Res 1994;37:54–61.
	Cocaine-induced seizures	Helton *et al*. Mol Brain Res 1993;20:285–8.
ICER, CREM	ECS	Fitzgerald *et al*. J Neurochem 1996;66:429–32.
CREB	Metrazol	Pi *et al*. Brain Res. 2004;127:60–7.
NeuroD-related factor	Metrazol	Konishi *et al*. Brain Res. 2001;97:129–36.
DREAM	Metrazol	Matsu-ura *et al*. Mol Brain Re. 2002;109:198–206.
Zac1	Metrazol	Valente *et al*. Neuroscience 2004;128:323–36.
Receptors, channels, transporters		
GluR1	ECS	Wong *et al*. Epilepsy Res 1993;14:221–7.
	Hilar lesion	Gold *et al*. J Comp Neurol 1996;365:541–55.
GluR2	Metrazol kindling	Lason *et al*. Brain Res 1998;785:355–8.
	Hilar lesion	Gold *et al*. J Comp Neurol 1996;365:541–55.
GluR3	Hilar lesion	Gold *et al*. J Comp Neurol 1996;365:541–55.
KA1 and KA2	ECS	Porter *et al*. Brain Res 1996;710:97–102.
NMDAR1	Metrazol	Jensen *et al*. Mol Brain Res 1997;44:157–62.
	Metrazol kindling	Lason *et al*. Brain Res 1998;785:355–8.
GABAA receptor alpha 1	Kindling	Kokaia *et al*. Mol Brain Res 1994;23:323–32.
GABAA receptor alpha 4	Kindling	Clark *et al*. Mol Brain Res 1994;26:309–19.
GABAA receptor beta 1	Kindling	Clark *et al*. Mol Brain Res 1994;26:309–19.
GABAA receptor beta 3	Kindling	Kokaia *et al*. Mol Brain Res 1994;23:323–32. Clark *et al*. Mol Brain Res 1994;26:309–19.
GABAA receptor gamma 2	Kindling	Kokaia *et al*. Mol Brain Res 1994;23:323–32.
GABA(A)-receptor delta	Metrazol	Penschuck *et al*. Mol Brain Res 1997;51:212–9.
GABAA receptor alpha 1, alpha 4, beta 2, and gamma 2	γ-Hydroxybutyric acid induced seizures	Benerjee *et al*. Exp Neurol 1998;154:213–23.
Kv1.2, and Kv4.2	Metrazol	Tsaur *et al*. Neuron 1992;8:1055–67.
m1 and m3 muscarinic cholinergic receptor	Kindling ECS	Mingo *et al*. Brain Res 1998;810:9–15.
HCN1 and HCN2	Febrile seizures	Brewster *et al*. J Neurosci 2002;22:4591–9.
voltage-dependent calcium channel alpha1	Kindling	Hendriksen *et al*. Mol Brain Res 1997;50(1–2):257–66.
T-type low-voltage-activated calcium channels alpha1G	Absence seizures	Talley *et al*. Brain Res Mol Brain Res 2000;75:159–65.

Continued

Table 1 Continued

Gene	*Model*	*Author*
GLUT1 and GLUT3	Metrazol	Nehlig *et al*. Brain Res 2006;1082:32–42.
Peptides and receptors		
Prodynorphin/dynorphin	Kindling	Xie *et al*. Brain Res 1989;495:156–60. Moneta *et al*. Neurosci Lett 1990;110:273–8.
	Metrazol	Yount *et al*. Brain Res 1994;21:219–24.
	Cocaine-induced seizures	Helton *et al*. Mol Brain Res 1993 Nov;20(3):285–8.
Proenkephalin/enkephalin	ECS	Hong *et al*. Neuropeptides 1985;5:557–60.
	Cocaine induce seizures	Przewlocka *et al*. Neurosci Lett 1994;168:81–4.
	Kindling	Moneta *et al*. Neurosci Lett 1990;110:273–8. Rosen *et al*. Mol Brain Res 1994;27:71–80.
	Cocaine-induced seizures	Helton *et al*. Mol Brain Res 1993;20:285–8.
dynorphin	Metrazol	Yount *et al*. Mol Brain Res 1994;21:219–24.
neuropeptide Y	Metrazol	Yount *et al*. Mol Brain Res 1994;21:219–24.
	ECS	Mikkelsen *et al*. Mol Brain Res 1994;23:317–22. Madsen *et al*. Neuroscience 2000;98:33–9. Mikkelsen *et al*. J Psychiatr Res 2006;40:153–9.
	Kindling	Kopp *et al*. Mol Brain Res 1999;72:17–29. Rosen *et al*. Mol Brain Res 1994;27:71–80.
Neuropeptide Y receptor Y1 and Y2	ECS	Madsen *et al*. Neuroscience 2000;98:33–9.
Neuropeptide Y receptor Y1, Y2 and Y5-R	Kindling	Kopp *et al*. Mol Brain Res. 1999;72:17–29.
somatostatin	Kindling	Shinoda *et al*. Mol Brain Res 1989;5:243–6.
	ECS	Mikkelsen *et al*. J Psychiatr Res 2006;40:153–9.
Oxitocin	Metrazol	Carter *et al*. Neurosci Lett 1993;160:135–8.
vasopresin	Metrazol	Carter *et al*. Neurosci Lett 1993;160:135–8.
	Kindling	Greenwood *et al*. Mol Brain Res. 1994;26:286–92. Greenwood *et al*. Mol Brain Res 1994;24:20–6. Greenwood *et al*. Neurosci Lett 1997;224:66–70.
cholecystokinin	Kindling	Zhang *et al*. Mol Brain Res 1996;35:278–84. Burazom *et al*. Neurosci Lett 1996;209:65–8.
Trophic factors and receptors		
NGF (nerve growth factor)	Hilar lesion	Rocamora *et al*. Mol Brain Res. 1992;13:27–33.
	ECS	Follesa *et al*. Exp Neurol 1994;127:37–44.
	Kindling	Bengzon *et al*. Neuroscience 1993;53:433–46.
bFGF (basic fibroblast growth factor)	ECS	Follesa *et al*. Exp Neurol 1994;127:37–44.
BDNF (brain derived nerve growth factor)	Kindling	Bengzon *et al*. Neuroscience 1993;53:433–46. Bregola *et al*. Epilepsia 2000;41 S6:S122–6. Morimoto *et al*. Brain Res Bull 1998;45:599–605.
	ECS	Jacobsen *et al*. Brain Res 2004;1024:183–92.
	Febrile seizures	Kim *et al*. Neuroreport 2001;12:3243–6.
	Hilar lesion	Rocamora *et al*. Mol Brain Res 1992;13:27–33.
FGF-2 (fibroblast growth factor 2)	Kindling	Bregola *et al*. Epilepsia. 2000;41 S6:S122–6.
neurotrophin-3	Kindling	Bengzon *et al*. Neuroscience 1993;53:433–46.
	Hilar lesion	Rocamora *et al*. Mol Brain Res. 1992;13:27–33.
trkB	Kindling	Bengzon *et al*. Neuroscience 1993;53:433–46.
trkC	Kindling	Bengzon *et al*. Neuroscience 1993;53:433–46.
	Intracerebroventricular injection of bicuculline	Mudo *et al*. J Mol Neurosci 1995;6:11–22.
Other		
glucocorticoid and mineralocorticoid receptor	Kindling	Clark *et al*. Neuroendocrinology 1994;59:451–6.
thyrotropin-releasing hormone	Kindling	Rosen *et al*. Mol Brain Res 1994;27:71–80.
neuronal nitric oxide synthase	Kindling	Elmer *et al*. Neuroreport 1996;7:1335–9.
tPA (tissue plasminogen activator)	Kindling	Qian *et al*. Nature 1993;361:453–7.
	Metrazol	Popa-Wagner J Gerontol A Biol Sci Med Sci 2000;55:B242–8.

Continued

Table 1 Continued

Gene	*Model*	*Author*
cyclophilin	Hilus lession	Yount *et al*. Mol Brain Res 1992;14:139–42.
HSP73 (heat shock protein 73)	ECS, kindling	Wong *et al*. Mol Brain Res. 1992 Mar;13(1–2):19–25.
Hsc70 (heat shock cognate protein 70)	ECS	Kaneko *et al*. Neurosci Lett 1993;157:195–8.
pannexin1	4-aminopyridine-induced seizures	Zappala *et al*. Neuroscience 2006;141:167–78.
agrin	Hilus lesion	O'Connor *et al*. Mol Brain Res 1995;33:277–87.
MAP 1B (microtubule-associated protein 1B)	Metrazol	Popa-Wagner *et al*. Exp Neurol 1997;148:73–82. Popa-Wagner *et al*. Neuroscience. 1999;94(2):395–403.
synapsin I	Kindling	Morimoto *et al*. Seizure 1998;7:229–35.
ferritin heavy chain	Absence seizures	Lakaye *et al*. Exp Neurol 2000;162:112–20.
glycerol phosphate dehydrogenase	Metrazol	Link *et al*. J Neurochem 2000;75:1419–28.
sialyltransferase isoenzymes ST3Gal IV, ST6GalNAc II, ST3Gal I, ST8Sia IV	Kindling	Okabe *et al*. J Neurochem 2001;77:1185–97.
cyclooxygenase-2	Kindling	Tu *et al*. Exp Neurol 2003;179:167–75.
G protein signaling protein subtypes-2,-4,-7,-8 and -10	ECS	Gold *et al*. J Neurochem 2002;82:828–38.
Claudin 8	Kindling	Lamas *et al*. Mol Brain Res 2002;104:250–4.
orphanin FQ/nociceptin	Kindling	Bregola *et al*. Epilepsia 2002;43 S5:18–9.
calbindin-D28k	Kindling	Sonnenberg *et al*. Mol Brain Res 1991;9:179–90.
GAD (glutamate decarboxylase)	Kindling	Sonnenberg *et al*. Mol Brain Res 1991;9:179–90.

global analyses of gene expression have been published (see Further Reading section). In the coming sections, we summarize some of the genes that are most studied.

Transcription Factors

Seizures not only influence the activity of preexisting transcription factor proteins but also act by inducing their mRNA transcription. A number of seizure-induced transcription factors are immediate early genes. By definition, induction of immediate-early genes (IEGs) mRNA expression is rapid and does not depend on protein synthesis. Kindling, electroconvulsive seizures and chemoconvulsants have all been shown to markedly induce the expression of transcription factor mRNAs in the hippocampus and/or cerebral cortex. Among the best studied are components of AP-1 transcription factor (c-fos, c-jun, c-myc, jun-B, fra-1), krox-20, erg-1, erg-2, erg-3, Nurr 1 or CREM/ICER. Genes coding for transcription factors, following translation, can regulate the expression of other sets of genes. Targets genes for some transcription factors have been identified. For example, ATF4 and CREB regulate the expression of $GABA_BR1a$ and $GABA_BR1b$ isoforms of $GABA_B$ receptors, thus influencing neuronal excitability. The DREAM transcription factor regulates genes related to neuronal plasticity such as c-fos and preprodynorphin. The AP-1 transcription factor has been shown to regulate the expression of nerve growth factor (NGF), proenkephalin, and prodynorphin. Changes in the expression of such transcription factors can have long-lasting and pronounced consequences.

Neurotransmitter Receptors, Transporters and Ion Channels

Alterations in the expression of proteins related to the ligand and voltage-gated ion channels would influence the electrophysiological properties of neurons and glia, and therefore represent obvious candidates for studies of network excitability. Many changes in the expression of mRNAs coding for neurotransmitter receptors and ion channels have been described. For example, changes in mRNA expression for selected $GABA_A$ receptor (ligand-gated chloride channels that mediate fast inhibitory synaptic transmission) subunits have been reported following kainic acid, pilocarpine, or electrically induced status epilepticus as well as following kindling-induced seizures. Changes in the expression of mRNAs encoding metabotropic $GABA_B$ receptors occur following chemically or electrically induced status epilepticus or kindling. Expression of genes coding for the glutamate receptors, the major excitatory receptors in the brain, has been extensively described. Seizures induce alteration in the expression of several glutamate-gated ion channels, including NMDA, AMPA, and kainate receptor subunits, as well as metabotropic mGLUR5 and mGLUR1 glutamate receptors. Finally, expression of genes coding for sodium channels, calcium channels, hyperpolarization-activated cyclic nucleotide-gated cation channels, and glutamate transporters can be regulated by seizures. All alterations in the gene expression of neurotransmitter receptors and ion channels will lead to changes in the quantity or subunit composition of receptors and channels, at the synapse or

on other parts of cell membranes, and thus, influence synaptic function. Alteration in neurotransmitter transporter will modify neurotransmitter metabolism and the availability of neurotransmitter for synaptic transmission. Thus, such seizure-induced changes can have pronounced effects on neuronal excitability, and these changes can lead to functional abnormalities.

Neuromodulators

In addition to channels and receptors, many other factors – such as neurotrophins or growth factors – can affect neuronal excitability. Since neurotrophins can influence neuronal network excitability, their expression following seizures has been extensively studied. For example, seizures can induce neuropeptide Y (NPY) expression, which in turn can have a significant role in suppression of seizure activity. Seizures can also regulate the expression of BDNF (increase or decrease), NGF or NT-3, as well as neurotrophin receptors. Seizures have also strong potential for regulating expression of neuropeptides like dynorphin, somatostatin or prohormone convertase. All these changes in expression levels can contribute to seizures-induced plastic changes in the neuronal circuits.

Defense Proteins

Upregulation of the expression of genes coding for immune and defense proteins has been described repeatedly, mainly following status epilepticus. The proteins most often identified include interleukin-1 and-6 (IL-1, Il-6), tumor necrosis factor (TNF-a), leukemia inhibiting factor (LIF), and numerous heat shock proteins (Hsp) or heat shock cognate proteins (Hsc). These expression changes may be caused by status epilepticus-induced neurodegeneration, may reflect cell death, or may reflect the cell's attempt to switch on tissue repair mechanisms. Some of the immune proteins, like IL-6, have been shown to have a direct influence on neuronal excitability.

Structural Proteins

Seizures can influence the expression of number of genes which, by different means, can influence structural features of neurons, including structure of the synapse. A few of the many such genes include homer-1 (which is induced as an immediate early gene and codes for a scaffold protein that anchors metabotropic glutamate receptors to the cytoskeleton and regulates pyramidal neuron excitability) and immediate early gene Arc (an activity-regulated cytoskeletal-associated gene; Arc mRNA is targeted to recently activated synapses and locally translated into protein). Seizures also regulate cytoskeletal protein like α-tubulin or GFAP; vesicle proteins like chromogranin, secretogranin or clathrin; structural synaptic proteins like dystrophin; or extracellular proteins like tenascin or extracellular metaloproteinases and their inhibitors.

Insight from Global Analysis of Gene Expression Studies

Recent technologic developments allow the analysis of gene expression at the level of nearly the whole transcriptome, an approach that provides an unbiased insight into molecular events that occur in the brain following seizures or status epilepticus. Data coming from such experiments have shed new light on molecular phenomena and metabolic pathways involved in determining the effect of seizures on brain function. Studies using animal models (of both seizures and status epilepticus) have confirmed previous results about the effects of seizures on the expression of genes coding for neurotrophic factors, growth factors, ion channels and receptors, a number of plasticity-related genes, and other genes mentioned earlier. In addition, several new groups of genes have been identified, including those involved in angiogenesis, cell cycle and proliferation, protein synthesis, and chemokine signaling. Advanced bioinformatics tools have been used for data mining in order to group these genes into meaningful functional classes, and have been useful for highlighting the most prominent metabolic pathways.

Future Goals

The work of several laboratories over few past decades has uncovered a large number of genes that are induced in the brain by seizures or status epilepticus. These genes belong to a variety of functional classes, and can have effects on many aspects of cell metabolism/function. Despite this knowledge, we still lack a unifying theory about the role of seizure-induced gene expression in the brain. Especially now, in this "genomic era," we are faced with many new genes with unknown roles in the brain. Before we can understand the outcome of changed expression of the whole assembly of genes, we have to address several questions for each gene. Given the phenotypic cell diversity in the brain, the expression of a given gene of interest in neuron versus microglia versus astroglia versus endothelium can have quite a different outcome from the point of view of brain function. Further, neurons and glia display great diversity, and can be divided into a large number of subclasses. In addition to cellular localization of the transcription, one should consider localization within the brain (e.g., hippocampus, cortex) or localization within brain structures (e.g., cortical layer, hippocampal division).

In the case of many novel seizure-induced genes, it is still a challenge to define the consequences of altered expression because of the lack of information about the function of protein products. Even if we know the localization of transcription, in many cases the only other available information is gene sequence. The information available about the function of a gene (or gene product) in other tissues or cell lines cannot always be easily translated to the brain. Therefore, much basic research is needed before we understand the net effect of global alterations of gene expression in the brain, or even attempt to determine if a given alteration in gene expression is beneficial of harmful.

See also: **Epileptogenesis**: Epileptogenesis and Plasticity; Gene Expression in Immature and Mature Hippocampus After Status Epilepticus; Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; Surrogate Markers for Epileptogenesis; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Post-Traumatic Epilepsy**: Posttraumatic Models.

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Seizure Initiation and Termination

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Introduction

A seizure is defined as an episode of excessive, synchronized neuronal activity associated with disturbance of sensation, loss of consciousness, convulsive movements, or combinations of these clinical signs. The precise combination is thought to reflect the brain regions that are synchronized in the discharge. Ever since electrical activity has been recorded in the brain, there has been reference to 'spontaneous' activity, the most marked form of which is seizure activity. Normally, brain activity does not appear to be highly synchronized. However, during a seizure, there is abnormal synchronization of brain circuits. Importantly, however, the occurrence of a seizure does not mean that the brain is abnormal. For example, seizures can be triggered in 'normal' brain by metabolic abnormalities such as hyponatremia and hypoglycemia, or by infection or inflammation. Every brain has a capacity to express a seizure.

The brain produces a variety of transient oscillations – synchronized activity in populations of neurons – that do not initiate seizures. This observation can be viewed in two ways. One is that there is a specific type of 'trigger' necessary to initiate a seizure. This trigger – such as an electrical shock – may itself produce excessive synchronization or may lower some threshold for seizure initiation. Alternatively, one could view the brain as having mechanisms that control the degree of synchronization; if these limiting factors fail, a seizure may begin. An example of such a process would be loss of inhibitory function in the cortex. Interestingly, if both excitation and inhibition are blocked, neurons tend to fire at a higher rate and in a more rhythmic pattern compared with activity during baseline conditions. This finding supports the idea that there are intrinsic mechanisms that determine neuronal excitability in brain networks, and that these intrinsic mechanisms could limit seizure initiation. Certainly, an active input (a trigger) could interact with loss of an

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SLEEP

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Brain Mechanisms Linking Epilepsy to Sleep

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Introduction

Even before proclaiming epilepsy independent of any malevolent deity, the Hippocratic school had already demystified sleep (God Hypnos in Greek mythology). The physicians of antiquity used sleep in their medical practice in various ways, and the relationship between sleep and epilepsy was recognized. Extensive clinical observations in the nineteenth century distinguished a large percentage of seizures (>20%) that occurred only at night (nocturnal), and a particular vulnerability to the occurrence of other types of epilepsy at awakening and sleep onset (i.e., transition times). Advances were made with the advent of electroencephalography (EEG) in the 1930s and with the widespread availability of polysomnography in the following decades, which led to the seminal series of studies that associated 'awakening seizures' to idiopathic generalized epilepsy (IGE) with tonic–clonic seizures. More recently, video-polysomnography and presurgical EEG monitoring have expanded and confirmed these associations, establishing the statistical likelihood of specific types of epilepsy to express their signs during particular sleep stages.

Aggravation of seizures by sleep would sound perhaps incompatible with the old ideas of sleep being a state of brain inhibition and restoration. Today, we know that sleep contains periods of intense neuronal activity, and that even when thalamic gates are closed to the outside world, intracortical dialogue is maintained. We distinguish three states of existence: awake, rapid eye movement sleep (REMS), and non-REM sleep (NREMS). Typically, as one goes to sleep, the low-voltage fast EEG pattern of wakefulness gradually gives way to slower frequencies, as NREMS goes from stage 1 (decrease in alpha) to stage 2 (spindles, K-complexes) to stage 3 and 4 (increasing amplitude and regularity of delta rhythm – see **Figure 1(a)**); stages 3–4 are therefore referred to as 'slow-wave sleep.' This pattern of change is the NREMS descending phase, which is followed by a more abrupt ascending phase (from stage 4 to stage 1) and an episode of REMS. Besides the characteristic saccadic eye movements, REMS is characterized by a high level of brain activity, the presence of low-voltage fast EEG activity (desynchronized EEG), the suppression of muscle tone, vivid dreaming, and the highest awakening threshold (therefore, 'paradoxical sleep'). In contrast, during NREMS, brain activity is reduced in most brain areas and thinking is usually nonvisual and ruminative. As displayed in the hypnogram in **Figure 1(b)**, every night's sleep progresses through several such ~90-min-long NREMS–REMS cycles, with NREMS predominating in

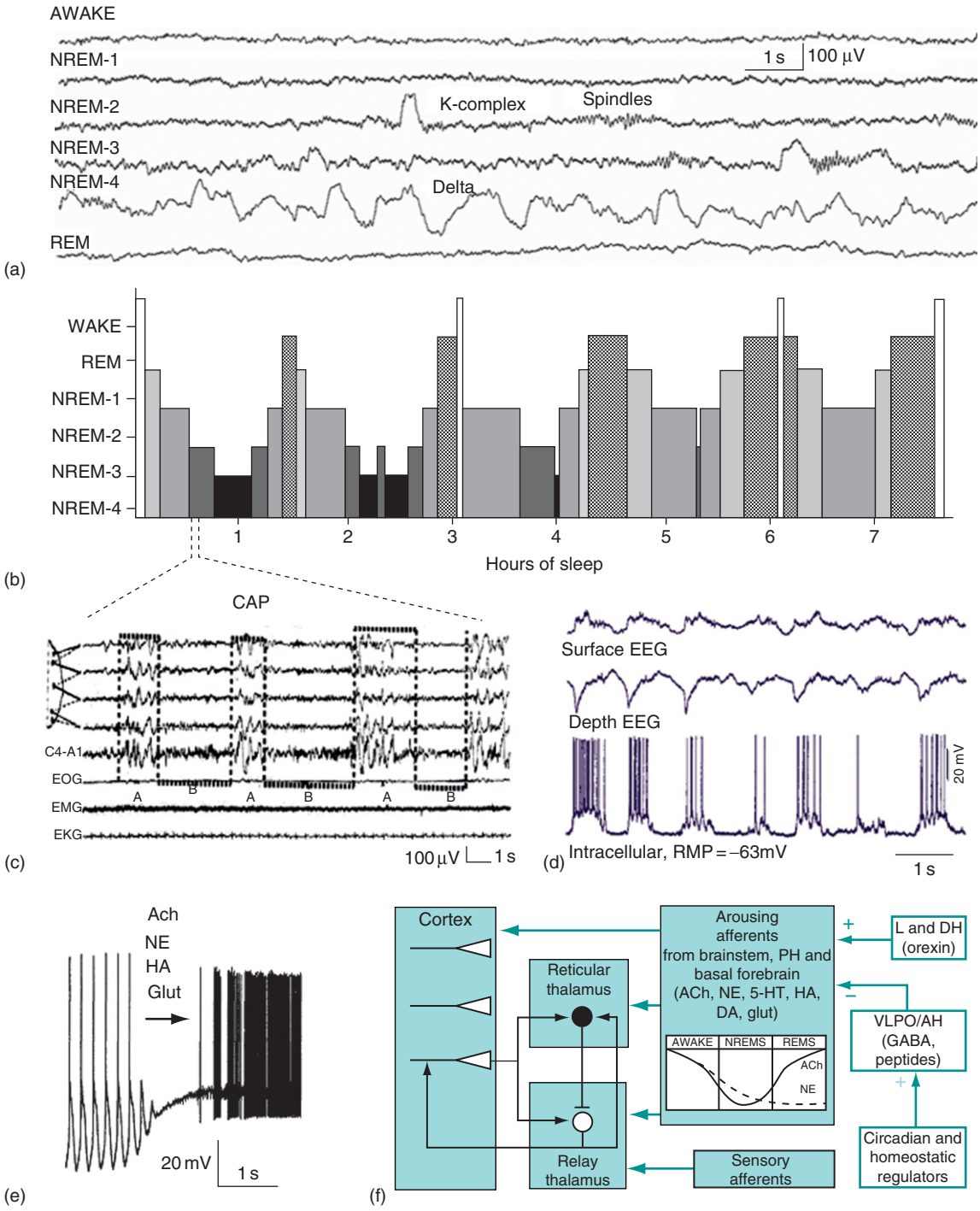


Figure 1 Mechanisms underlying EEG characteristics of sleep. (a) Characteristic EEG traces (all from CZ) from a human sleep. Note that compared with the wake condition the EEG of successive stages of non-REM sleep (NREMS-1 to NREMS-4) is characterized by an increase in power and decrease in frequency. Stage NREM-2 contains large negative or negative–positive waves called K-complexes, and spindles (a rhythm at 13–15 Hz). (b) Hypnogram of a night's sleep showing the time spent in each sleep stage. (c) During stages NREM-2–3 a CAP is observed, which clusters most of the prominent and globally synchronous EEG characteristics, like K-complexes and spindles, in periods ''A'' lasting a few seconds and alternating with rather quiet periods ''B''. Upper four traces bipolar parasaggital derivations from front to back. Next, monopolar recording of a central lead (C4), electrooculogram, electromyogram, and electrocardiogram. (d) experimental recordings from a sleeping cat brain. Note that EEG positive waves reflect negative field potentials in deeper lying cortical layers concomitant to depolarizing periods in a pyramidal neuron. The latter are termed 'up phases' and alternate with 'down phases' exhibiting hyperpolarization/disfacilitation. (e) Thalamic relay neurons appear to switch between two states of firing. In the hyperpolarized condition (left) the neurons tend to slowly oscillate and burst, while in the depolarized one (right) they tend to fire tonically and randomly. The first condition characterizes NREMS and precludes response to sensory input, while the second

the evening cycles (~75/15 min) and REMS increasing in the morning cycles (~60/30 min). NREMS in the morning cycles do not reach stage 4 (delta waves), and the longest part of a night's sleep is the stage 2 NREMS.

Although the importance of sleep is not questioned, and sleep deprivation (SD) can be life threatening, the exact role of sleep is still debated. It now appears that a crucial function of sleep is synaptic plasticity and homeostasis, and thus memory consolidation.

The link between sleep and epilepsy is reciprocal. Seizures have serious impact on sleep, and epileptic patients often have sleep deficits (even when seizure free). Antiepileptic drugs have highly variable effects on sleep organization, and antiepileptic chronic vagal nerve stimulation has been reported to improve alertness and sleep microarchitecture. Therapy for sleep disorders will undoubtedly benefit from a better understanding of the effect of seizures themselves, and of antiepileptic drugs, on sleep architecture. This article, however, will deal only with effects of sleep on epilepsy.

Understanding many types of epilepsy depends on understanding the mechanisms of sleep or SD, which influence them. This task is difficult and demands a systematic approach. One must examine how each of the physiological brain functions putatively involved in epileptogenesis (neuronal excitability and communication, synaptic plasticity and its homeostasis, network properties related to synchronization and/or rhythmicity, and the ability of the entire brain (and body) to express the particular seizure) is affected by the procession of sleep stages and their particular features. We are still far from hard answers, but clues from the clinical picture abound. After reviewing the clinical problem and the basic sleep mechanisms, this article will examine the recent advances showing how these mechanisms may affect epilepsy manifestations.

Background

The Clinical Problem and Questions from the Bedside

Nocturnal epilepsies are either idiopathic or hereditary and have variable course. Among types of partial epilepsy, seizures of frontal lobe epilepsy (nocturnal FLE) typically arise during NREMS – most often in stage 2 – and never during REMS. This is also the case with benign Rolandic epilepsy of childhood. In contrast, temporal lobe epilepsies (TLEs) do not show clear day–night preference. Patients with seizures arising from occipital or parietal lobes have rare sleep-onset seizures. Benign but not uncommon seizures, like those of Panayiotopoulos syndrome, tend also to occur during sleep. Seizures often present in children's sleep as continuous spike and wave discharges (SWD), or poly-SWD (as in Lennox–Gastaut syndrome), indicate predominantly nocturnal types of epilepsy. Of the epilepsies associated with awakening, most common are juvenile myoclonic epilepsy, absence seizures, and epilepsy with grand mal seizures on awakening. Typical absences appear particularly prone to present during drowsiness and transitions between sleep and arousal states. Awakening epilepsies are usually idiopathic or hereditary, with onset in childhood and usually a benign course. Epilepsies with random appearance (during the wake–sleep cycle) are more likely of symptomatic etiology, variable age onset, and relatively worse prognosis.

Although TLE patients have mostly daytime seizures, their EEG interictal epileptiform discharges (IEDs) are more often (and from more extensive neocortical areas) seen in NREMS stages 3 & 4 than in awake or REMS. In contrast, at NREMS 3 & 4, hippocampal spikes are suppressed and reach relative maximum in NREMS 2. This discordance between cortex and hippocampus suggests crucial differences in spike synchronization mechanisms between these two regions. Currently, we do not know which, if any, of the mechanisms involved in IEDs underlie sleep and/or epilepsy. What is apparent is that ictal and interictal events of a given seizure type may have different relationships to sleep.

IEDs increase after SD, a condition known to exacerbate most (perhaps all) seizure types, but more markedly IGE than partial epilepsies. Questions have arisen about whether the SD effect is due to the SD itself, to the fact that more sleep time is recorded after SD, or even to mechanisms underlying some stress associated with SD.

Obviously, questions from the bedside abound. Furthermore, some of them present intriguing paradoxes, warning

characterizes the wake and REMS states. Several synaptic transmitters may mediate this switch from the oscillatory non-responsive state to the tonic one (acetylcholine, norepinephrine, histamine, glutamate). (f) Simplified diagram of mechanisms underlying sleep initiation and maintenance focusing on the thalamocortical circuit pacing cortical rhythms. Filled and open neuronal somata denote inhibitory and excitatory neurons respectively. Among the subcortical modulatory influences, promoting the tonic rather than the oscillatory state of TC, the availability of acetylcholine in thalamocortical circuits synapses is high in both awake and REMS, while that of norepinephrine only in the awake condition. These arousing circuits are promoted (+) or inhibited (−) by other circuits indicated by their anatomical origin and neurotransmitter used (in parenthesis). AH, L&DH, PH respectively anterior, lateral and dorsal, and posterior hypothalamus; VLPO, ventrolateral preoptic nucleus; Ach, acetylcholine; DA, dopamine; HA, histamine; NE, norepinephrine; 5-HT, 5′-hydroxy-tryptamine; GABA, γ-amino-butyric acid; glut, glutamate. Part (c) adapted from Halász P, Terzano M, Parrino L, Bódizs R (2004) The nature of arousal in sleep. *Journal of Sleep Research* 13(1): 1–23. Part (d) modified from Amzica F (2002) Physiology of sleep and wakefulness as it relates to the physiology of epilepsy. *Journal of Clinical Neurophysiology* 19(6): 488–503. Part (e) adapted from Buzsaki G (2006) *Rhythms of the Brain*. Oxford University Press.

against attempts for simple explanations of an enormously complex, multifaceted, and dynamic relationship. From these observations, three fundamental questions arise: (a) What neurobiological differences make some partial epilepsies mostly nocturnal (i.e., NFLE) and some mostly diurnal (i.e., of occipital localization)?; (b) why does NREMS promote, and REMS antagonize epilepsy – i.e., what is peculiar about sleep stages with respect to epileptogenicity?; and (c) what is the neurobiological background of drowsiness and arousal state transitions that promote IGE seizure occurrence? In the following section, we will examine some of the fundamental mechanisms that differentiate neuronal behavior in the different wake–sleep states.

Sleep Mechanisms

During NREMS, there is an infra-slow oscillation (<0.02 Hz, 'cyclic alternating pattern,' CAP, **Figure 1(c)**) during which the synchronous EEG as well as motor and autonomic events appear to cluster together, thus creating successions of eventful phases 'A' and more quiet phases 'B'. Phases 'A' – and especially K-complexes – are considered sleep-promoting responses to endogenous or exogenous arousal stimuli.

A slow rhythm (<1 Hz) is nested within phase 'A,' which presents in individual neurons as sequences of hyperpolarizations ('down state' due to disfacilitation) followed by depolarizations ('up state,' when all activity patterns occur) (see **Figure 1(d)**). The transition from the down state to the up state is increasingly synchronized over wide brain areas as NREMS deepens (NREM 2 to NREM 4, **Figure 1(a)**), and often leads to large waves such as K-complexes or delta waves (**Figure 1(d)**). It is during the depolarizing 'up state' that spindles and higher-frequency rhythms like gamma may appear. Although the infra-slow and slow rhythms appear to be paced by cortical circuits, the pacemaker for the higher-frequency events is considered to be thalamic: thalamocortical (TC) neurons fire rhythmically in bursts when first hyperpolarized (**Figure 1(e)**), and impose this rhythm to cortical neurons responsible for generating EEG waves (**Figure 1(d)**). These neuronal events underlie the fundamental distinction between two states: a state of hypepolarized and bursting TC neurons that are not capable of accurately advancing sensory input to the cortex – which characterizes NREMS; and a state of depolarized TC neurons with tonic firing, with accurate response transmission properties – which characterizes the wake state (conscious processing of environmental input) and (in a different way) REMS (processing of 'internal' brain information).

The mechanisms responsible for the above two states of the TC circuits, which determine the wake–sleep cycle, are increasingly understood. Essential is the role of neurons of the reticular nucleus of thalamus, which are excited by both TC and cortical neurons, and which inhibit TCs as well as each other (**Figure 1(f)**). These neurons, like the TC neurons, have intrinsic currents promoting an escape from inhibition resulting in strong depolarization and burst discharge. The TC and cortical bursting reactivates reticular neurons, and their inhibitory feedback onto TC neurons maintains the rhythm at a frequency determined by the duration of the inhibitory periods imposed on the TC neurons.

Whether this thalamo-corticothalamic rhythmical reverberation will take place is determined by several subcortical inputs. The depolarized state of TC neurons, and therefore arousal, is initiated and maintained by a network of cholinergic neurons in the basal forebrain, histaminergic neurons in posterior hypothalamus, and glutamatergic neurons in the intralaminar thalamic nuclei. These TC neurons, as well as cortical neurons, are further modulated by brainstem afferents releasing norepinephrine (NE), serotonin (5-HT), and acetylcholine (ACh). Depolarization takes TC neurons from their inherent rhythmical bursting mode characteristic of NREM sleep to a tonic firing mode (**Figure 1(e)**), which is the mode compatible with their sensory transmission role and is mandatory for the awake state.

As we fall asleep, both cholinergic and aminergic neurons decrease their firing (**Figure 1(f)**), and so TC neurons return to their bursting mode. Initially, mutual excitation between TC and corticothalamic neurons elaborate the widely synchronous rhythm of spindles (NREMS stage 2). As TC neuron hyperpolarization becomes deeper, the even more synchronous and slower rhythm of delta waves appears (NREMS stage 4). To the extent that these rhythms persist, cortex cannot receive any information from the body or the external environment.

What, however, turns off the cholinergic and aminergic systems that support wakefulness? This role is attributed to active sleep mechanisms located in the ventrolateral preoptic (VLPO)/anterior hypothalamus (**Figure 1(f)**), and implemented through GABA and several peptide factors (including cytokines, growth hormone releasing hormone, and prolactin). Their inhibitory action on brainstem cholinergic and monoaminergic circuits is believed to be initiated by several influences, which constitute a circadian and a homeostatic regulator. The circadian circuit is controlled by pacemaker neurons in the suprachiasmatic nucleus, entrained to the day mainly by light. The homeostatic regulator increases its hypnogenic pressure as waking is extended; prolonged waking activates inducible nitric oxide synthase in the basal forebrain, which (through energy depletion) causes release of adenosine. Adenosine's hypnogenic action appears to be due to an inhibition of cholinergic neurons in basal forebrain and disinhibition of VLPO neurons. In sleep, metabolism is

slower and adenosine is cleared faster than it is produced. Interestingly, caffeine is a specific antagonist of adenosine receptors.

Apart from the waking condition, cholinergic neurons fire strongly also during REMS (**Figure 1(f)**) – and so in contrast to NREMS, TC are not allowed to oscillate and the sensory gate of the thalamus is open during REMS. However, the processing of environmental information is inhibited at higher or lower levels. Therefore, the TC circuits analyze only endogenous stimuli, to produce a dream content that has no time line or coherence due to inactivation of frontolateral cortex areas of working memory. Also, this content is highly emotional because of activation of limbic areas in REMS, and is not remembered because of lack of aminergic modulation. For REMS initiation, two types of 'switches' between REMS-on and REMS-off pontine circuits have been proposed, alternatively involving cholinergic/noradrenergic and GABAergic neurons. These regulate the activation of two glutamatergic projections to basal forebrain and ventromedial medulla, which respectively initiate the EEG features (desynchronization) and muscle paralysis characterizing REMS.

Methods

Electrophysiology has historically been the common thread in sleep and epilepsy research, supported by synaptic chemistry and pharmacology. Molecular biology has recently made some tremendous advances in both sleep and epilepsy research, but not yet so much on their interaction; computational modeling is increasingly used to theoretically explore this interaction. Electrophysiological experiments have provided clues from the level of whole brain EEG, to that of local field potentials, to the single cell membrane potential (**Figure 1(a), 1(d),** and **1(e)**). However, compared with the very long history of valid clinical observations, the experimental approaches to the mechanisms underlying sleep effects on epilepsy have been late and few. One inherent limitation has been the difficulty of applying reductionistic approaches (e.g., intracellular recordings, **Figure 1(d)** and **1(e)**), while carrying out long-term monitoring of a phenomenon as generalized in the brain – and as integrative of the whole body physiology – as sleep. The difficulty of combining good models of sleep and of epilepsy is further illustrated by the fact that most of the currently employed experimental models of epilepsy (e.g., genetic models, kindling) are in rodents, which unlike humans are nocturnal species. Most of the progress on cellular mechanisms has come from experiments in the cat (**Figure 1(d)**), which are increasingly uncommon. Polygraphic (EEG, EOG, EMG, ECG, etc.) and video recording in humans, leading to the construction of hypnograms (**Figure 1(a)–1(c)**) on which seizures time-course is noted, is currently the leading methodology, occasionally complemented with invasive recordings in epileptic patients. The recent ability to noninvasively image in 3D the metabolism (fMRI, PET) and electrophysiology (MEG) of the brain of sleeping epileptic patients raises new hopes for further insight.

Recent Advances on Brain Mechanisms Linking Sleep to Epilepsy

Several interesting advances have been made at the interface of basic mechanistic and clinical inquiry. We will examine them first in sequence of level of integration. Then we will examine the effects on epilepsy of sleep-related synchronous neuronal oscillations of infra-slow, slow, and fast frequency.

Genes, Channels, and Receptors

Strong genetic factors associated with awakening and nocturnal seizures have been increasingly substantiated. The well-known genetic predisposition of childhood absence epilepsy may result from a single nucleotide polymorphism in one of the low-voltage-activated calcium T-channel genes. Currents through these channels are instrumental for bursting in TC and cortical neurons (**Figure 1(e)**), and are therefore critical in the elaboration of normal sleep rhythms like sleep spindles, as well as of SWD of absences. The autosomal dominant nocturnal frontal lobe epilepsy (NFLE) is proposed to be partly due to mutations in the gene coding for the ligand-gated nicotinic receptors of acetylcholine, a major participant in arousing mechanisms (**Figure 1(f)**).

Neurotransmitters, Modulators, and Hormones

Unexceptionally, all neurotransmitter systems (from fast acting amino acids to amines and many neuroactive peptides) have experimentally suggested important roles in both sleep and epilepsy. For example, the cholinergic, serotonergic, and histaminergic components of arousing systems have antiepileptic actions in several seizure models, while the norepinephrine system is changed in kindling and absence models.

The neuromodulator, adenosine, has been proposed both as an endogenous hypnogen (the homeostatic regulator – see **Figure 1(f)**) and an endogenous antiepileptic agent. The two effects are most likely independent, since adenosine's hypnogenic effect appears to be exerted locally (in posterior hypothalamus) but its antiepileptic effect is generalized. Under normal conditions, there is a tonic effect of $[\text{adenosine}]_o$ (suggested by the observation that caffeine is excitatory), which is kept in check by an active uptake of adenosine (uptake blockers increase $[\text{adenosine}]_o$ and depress neurons); the baseline level of

adenosine is in a range of 25–250 nmol, which is anticonvulsant. During epileptic seizures, $[adenosine]_o$ rises rapidly to micromolar levels, a 6–31-fold increase sufficient to terminate ongoing seizure activity. Application of adenosine uptake antagonists can completely block epileptiform discharges in human cortex slices. Furthermore, adenosine receptor density appears to increase after seizures, and remains increased for prolonged periods (in contrast to the more transient GABA receptor changes). Finally, adenosine is neuroprotective, antagonizing excititoxic cell damage. It therefore makes sense to look for antiepileptic medications, based on facilitating this endogenous antiepileptic system, which appears to be rather specifically turned on with seizures. The restriction of adenosine's hypnogenic role to hypothalamus makes the sleep-related side effects of such medication less likely to be problematic. Melatonin, a hormone of the pineal gland, is released at night and affects the sleep–wake cycle. It also has antiepileptic effects in several experimental models in vivo and in vitro, but its levels and therapeutic potential in humans remain undetermined. The hypothalamic peptide, *orexin* (or hypocretin) has a crucial role in waking (**Figure 1(f)**). Its intraventricular application causes behavioral seizures, and its levels increase during experimental epileptogenesis. In slices, it modulates the balance of GABAergic and glumatergic neurotransmission in a way that suggests that endogenous orexin may play an epileptogenic role. The endogenous hypnogen, *delta sleep-inducing peptide*, potentiates GABA and blocks NMDA effects, and has demonstrated antiepileptic action. Finally, pituitary–adrenal secretory activity has been implicated in nocturnal epileptogenesis; this activity, and inhibitory control over cortical stimulus processing, is lower in the early part of sleep.

Regional Brain Energy Consumption and Epilepsy

In most brain regions, relatively lower blood flow and metabolism (as well as neuronal hyperpolarization) are characteristic of inactivation periods in NREMS (especially in association with K-complexes and delta waves). Could energy shortages affect neuronal responsiveness to endogenous epileptogenic activations? At a single cell level, significantly reduced blood flow may affect clearance of extracellular $[K^+]_o$ as well as extracellular space volume. Given that high $[K^+]_o$ and small extracellular space can result from neuronal bursting, and in turn tend to increase excitability through depolarization and ephaptic transmission (respectively), brain metabolism does indeed appear to be an important factor in controlling epileptogenicity. To be sure, not all brain areas change their metabolic activities in the same way during different sleep stages. It may be significant, however, that thalamus appears to be hypoperfused during NREMS, but activated during the phasic periods of REMS. Most relevant to partial epilepsy, normal human amygdala, hippocampal, and parahippocampal metabolic activity increases in REMS and decreases in NREMS, while metabolic activity in lateral prefrontal and temporal cortex decrease during both REMS and NREMS. Cases of unilateral temporal lobe epilepsy have been associated with interictal hypoperfusion in the frontal lobe.

Epileptogenic Sleep-Related Infra-Slow Modulations of Excitability

The appearance of generalized epilepsy with 3-Hz SWD seems to depend on the relationship between two long-term processes, with respective periods of 90–100 min and 24 h, and their interactive modulation. In this relationship, the onset of sleep and onset of arousal are strong boundary conditions; SWDs are promoted by transitional states (between wake and NREMS and between NREMS and REMS), and are inhibited by REMS and alertness. Negative DC shifts have been observed during transitions from wake to sleep. Assuming they have a neuronal origin, such negative DC shifts are usually thought to reflect concomitant periods of mild but widespread depolarization of pyramidal cell apical dendrites and associated increased pyramidal neuron excitability, which have been shown to be a requirement for SWD.

Although the 90-min cycle determines the NREMS–REMS recurrence during a night, the CAP (**Figure 1(c)**) modulates gross cortical excitability during NREMS, with a periodicity of about 20–40 s (~0.02 Hz) organizing EEG and autonomic arousal events in its phase 'A.' Phase A of CAP triggers and modulates the distribution of epileptic events and IEDs of either focal or generalized nature (**Figure 2**). It is noteworthy that IEDs are also correlated to the slow oscillation in stage 2 NREMS, when there are yet no delta waves. Similar observations of very slowly oscillating network excitability were found in hippocampus of rats strains highly susceptible to epilepsy. The phase-locking of epileptic events to the infra-slow cortical oscillation provides a putative mechanism for the as yet enigmatic aggravation of epileptic activity during sleep. The 3-Hz SWD of IGE are proposed to occur during 'phasic arousals without awakening' – i.e., CAP phase A. It remains unclear whether the epilepsy-promoting mechanism is related to the increased neuronal excitability (or to the synchronization) caused by some global event (neuronal, glial, or vascular) associated with the particular phase of CAP.

Epileptogenic Sleep-Related Slow Oscillations

NREMS is characterized by a slow (<1 Hz) oscillation between depolarizing ('up state') and hyperpolarizing ('down state') phases in individual cortical neurons (**Figure 1(d)**).

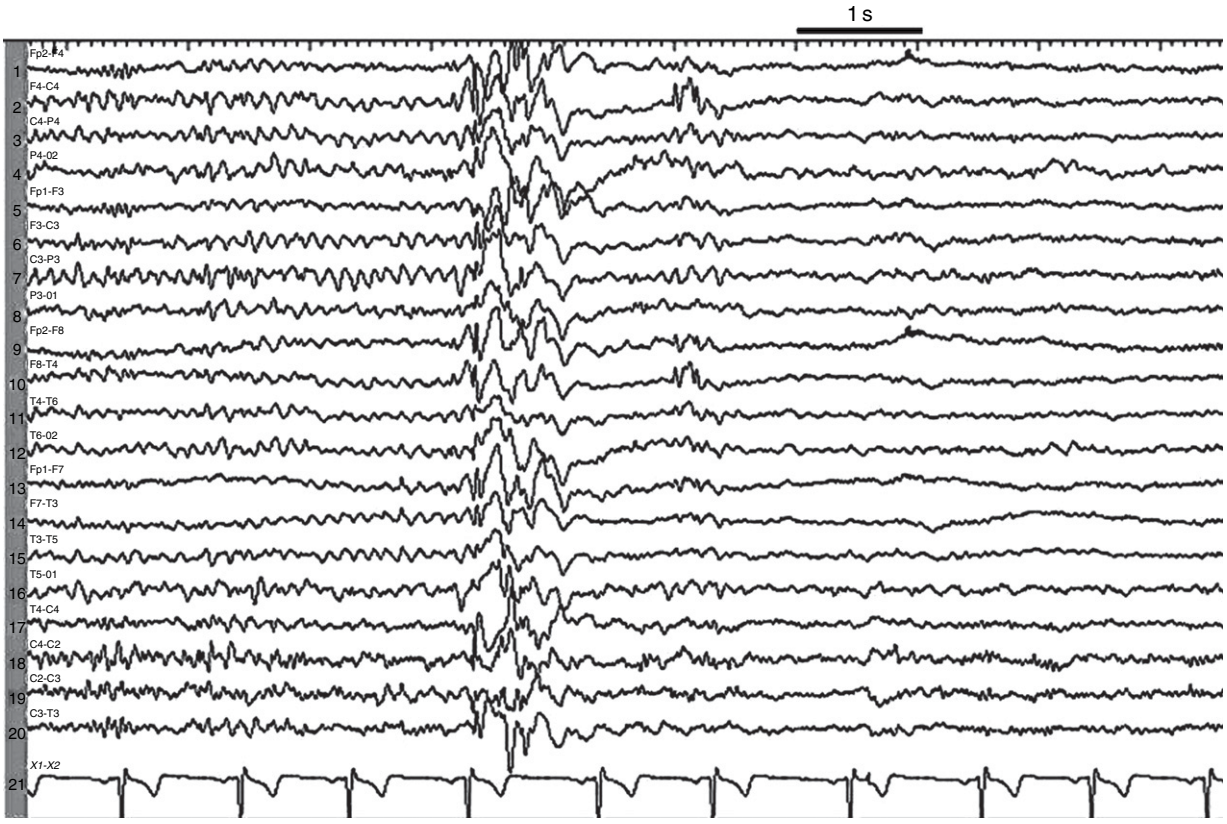


Figure 2 EEG of a 26-year-old man with IGE (JME). Myoclonic seizures on awakening since age 20 years, two GTCS at 25 and 26 years following volleys of myoclonic jerks on awakening. Generalized polyspike-wave discharge during stage 2 of slow sleep separating a phase A from B of a CAP. A second abortive generalized discharge occurs one second later (within phase B). Data courtesy of Dr. Michael Koutroumanides, Department of Clinical Neurophysiology and Epilepsies at Guy's & St. Thomas' NHS Foundation Trust, London, UK.

This neuronal effect is reflected in similar frequency EEG oscillations – to the extent that this rhythm becomes synchronized in large populations of neurons. Under conditions of high synchronization, the EEG corresponding to the 'up states' includes high power representation of almost the entire spectrum of frequencies, as well as stage-specific EEG waves, like K-complexes and spindles. At the single cell level, the 'up states' are periods of increased excitability (although specific cell types in particular brain areas, during particular phases of NREMS, may not participate in this 'increased excitability'). Can this bistability contribute to epileptogenesis during NREMS? And if so, how?

Sleep stages have been shown to influence human single neuron activity in seizure-generating areas. Most illustrative of the epileptogenic potential of NREMS is the demonstrated change of firing properties of cortical neurons, a change which in NREMS challenges the boundaries between the four categories of cortical neurons described from in vitro studies (regular spiking, fast spiking, fast rhythmic bursting, and intrinsically bursting neurons). NREMS favors the appearance of neuronal bursting (**Figure 1(d)** and **1(e)**). Sensory responses appear stronger in 'down states' than in 'up states.' However, of particular importance to nocturnal epileptogenesis may be the transition to the 'up state,' which is facilitated by TC bursting. The slow oscillation has been shown to constitute a nonstationarity of the sensory evoked potentials, which reach a maximum during the negative-to-positive slope of K-complexes and delta waves at the transition from 'down' to 'up' state.

No less relevant to epileptogenesis is the fact that the hyperpolarizations of the 'down state' periods do not reflect synaptic inhibition but rather disfacilitation (paucity of excitatory inputs) and summation of Ca^{2+}- and Na^{+}-dependent outward K^{+} current. There is an increased membrane resistance, maximal at the end of the down state, which may (a) favor distal synaptic input over proximal input (reducing the larger current loss of distal inputs through their longer cellular paths), and so nonspecific over specific TC input, and longer-over shorter-range corticocortical input; (b) favor, nondiscriminately, any input that will be large enough to overcome this hyperpolarization. Both these biases would presumably favor generalization and spread of epileptiform activity.

The mechanisms underlying disfacilitation are not known, but they may include adenosine-mediated depression of presynaptic release, since the adenosine levels maximize before sleep onset and increase further to micromolar levels during epileptic activation.

The characteristic K-complex of stage 2 NREMS (**Figure 1(a)**) is considered a solitary forerunner of delta rhythm waves, representing a transition from a stable low firing rate of cortical activity to an unstable high firing rate. Epileptic discharges – both generalized and focal – tend to cluster around K-complexes. But does this clustering constitute evidence of an influence of K-complex-related mechanism on epileptogenic activity? Similar clustering has been reported around delta waves. Thus, the clustering may simply reflect a general increase of excitability during the activated phase 'A' of CAP (explained earlier) rather than K-complex-specific mechanisms. Within the framework of such a generalization, one must be specific about the particular aspects and the types of epilepsy in question. Lighter stages of NREMS seem to best promote seizures, while deeper stages of NREMS seem to best activate IED. In patients with primary generalized epilepsy, the occurrence of spikes, polyspikes, and SWD is enhanced during the stage 2 of NREMS in conjunction with K-complexes, which is termed 'dyshormia' by Niedermeyer. The latter is defined as faulty or deviant arousal, during which the epileptic K-complex is found localized mid-anterior-frontally rather than at the vertex. This shift suggests an involvement of the supplementary motor area – which has been shown by MEG to be exceptionally active in sleep.

SWD of absence seizures have been hypothesized to emerge from the same TC mechanisms (**Figure 1(e)** and **1(f)**) that elaborate sleep spindles, under conditions of hypersynchronous cortical hyperexcitability. It is worth noting that in the experiments supporting this hypothesis, which demonstrated the development of SWD as transformation of spindles after i.m. penicillin in the awake restrained cat, SWD were not associated with paroxysmal depolarization shifts (PDSs) or other signs of abnormal neuronal discharges; SWD were associated with rhythmical hypersynchronous EPSP–IPSP sequences. The same is true for absence discharge in the rodent. Experiments studying local SWD produced by cortical deafferentation or topical application of large doses of convulsants, in animals sleeping or under excitatory anesthetics, have yielded insights at the membrane/neuronal/circuit level into the putative mechanisms relating sleep to epilepsy with SWD. However, some of these findings (i.e., PDSs reported during spikes of SWD) may model SWD associated with the particular animal model rather than SWD characterizing absence seizures of IGE. SWDs are best characterized, in behaviorally validated models, by neuronal discharges that are very synchronous but not abnormal; most importantly they are nonconvulsive.

In any event, spindles representing a series of depolarizations of lower (type I) or higher (type II) firing capacity (riding on top of a DC negativity) constitute a state of relatively higher cortical excitability, consistent with the correlation of IED with spindle activity – a correlation reported to be even higher than the correlation with slow-wave activity and with longer spindle duration just prior to NFLE seizures. Spindles have been associated with processes of neuronal plasticity and memory consolidation. Sleep mechanisms, similar to those involved in learning, may be employed during both epileptogenesis (establishing new connections) and the expression of seizures (spread of excitability over paths 'facilitated' by the discharges of previous seizures). A recently advanced hypothesis suggests that spontaneous brain activity – especially the bursting activity during the 'up state' of NREMS – may not only serve synaptic homeostasis, but also promote epilepsy.

It may be concluded that the slow (<1 Hz) oscillation of NREMS, and in particular spindles, K-complexes and delta waves, share some features that may contribute to the aggravation of epileptic phenomena. These effects may be related to the dynamic bistability of neuronal membrane potentials and neuronal readiness for bursting and widespread synchronization.

Epileptogenic fast oscillations during sleep

Along with IEDs, epileptic patients show fast oscillations (fast ripples, FRs, 200–500 Hz), whose current generators may more accurately locate epileptic foci than IEDs. FRs, as well as ripples (80–200 Hz), are more frequent during NREMS than during awake and REMS states. Although ripples are considered excitatory, and they are stronger in the 'up state' of NREMS than in awake or REMS, only spontaneous FR – and not ripples – are considered epileptogenic; the two appear to have different generators. FRs are considered to reflect synchronized population spikes within an interconnected network composed primarily of pyramidal cells. Facilitators of this synchronization may be the succession of up and down states of NREMS, which decrease temporal variability between neuronal discharges. As a useful contrast, FRs accompanying sensory evoked potentials in healthy humans are specifically depressed during NREMS.

Conclusions and Challenges

Sleep is recognized as a major precipitator of epileptic activity, but the intervening mechanisms remain enigmatic. Several sleep factors, mechanisms, or conditions that aggravate seizures (in NREM) are found:

- increased number of intrinsically bursting neurons;

- depolarization during the 'up state';
- disfacilitation and increased membrane resistance at the end of the 'down state';
- 'A' phase of CAP and its constituents, such as K-complexes;
- fast ripples;
- synaptic plasticity and associated homeostasis;
- ephaptic transmission and other mechanisms promoting synchronization;
- local blood flow not meeting the metabolic demand related to neuronal bursting.

Relatively few sleep mechanisms that 'antagonize' epilepsy have been recognized:

- hyperpolarization of motoneurons (sleep paralysis in REMS);
- desynchronization and effective inhibition (in REMS);
- enhanced release of neuromodulators like hypnogenic adenosine (at sleep starting time).

Although the findings described above mostly indicate correlations rather than causal relationships, they have opened new ways of understanding the apparently opposing roles of REMS and NREMS stages in epilepsy. However, the fundamental questions are still with us. Why are some epilepsies (i.e., NFLE) promoted by sleep (NREMS) and others (i.e., IGE) by drowsiness? What exactly is neurobiologically peculiar about drowsiness? Do some IEDs – e.g., those of TLE – spread more effectively during NREM simply because the ongoing activity of the brain is more synchronized? Why, then, does NREM not facilitate the spread of all types of IEDs? Typically, investigations aimed at answering these questions raise more questions than they answer, and paradoxes still abound. For example, deep NREMS is shown by transcranial magnetic stimulation studies to be a more excitable state compared with light NREMS; however, both evoked potentials and IEDs are stronger in the latter. The excitability of the entorhino-hippocampal circuits is higher in NREMS than in awake and REMS. Why, then, does NREMS aggravate preferentially FLE and not MTLE?

New directions of research are certainly needed, and relevant new ideas have emerged. Some of them are rather radical – e.g., that epilepsy may be affected not by sleep per se but by factors (endocrine, temperature) simply covarying in time with sleep. Other directions focus on reexamining some of our older assumptions and generalizations. For example, awakening (and to a lesser extent nocturnal) epilepsies are usually of hereditary or idiopathic etiology, whereas diffuse epilepsies are generally symptomatic. Does this difference imply that the links between sleep and epilepsy are mostly constitutional, and so possibly attributable to a single mechanism underlying both sleep and epilepsy, rather than to a causal interaction between the respective mechanisms? What is long overdue is a study of the effect of sleep on epileptogenesis rather than on the expression of seizures. Of particular interest is a study of the development of intractable seizures, whose relationship to sleep is still elusive.

In the earlier paragraphs, I have suggested that research should move from studying the effects of different sleep stages on epilepsy to examining the effects of interactions between brain rhythms. This change of emphasis would involve a shift from a focus on the degree of synchronization (high in NREMS, low in REMS) towards a focus on the role of phase relationships between nested oscillations in the EEG, and on the levels of various global neuromodulators. Understanding the processes of membrane and circuit bistability characterizing specific moments of the sleep–wake cycle, and of neuronal plasticity during sleep, may prove to have important implications for understanding the mechanisms underlying sleep effects on epilepsy.

Acknowledgements

The author thanks Dr. Michael Koutroumanides, Department of Clinical Neurophysiology and Epilepsies at Guy's & St. Thomas' NHS Foundation Trust, London, UK, for critical suggestions on the draft and the Hellenic Ministry of Development for support (PENED).

See also: **Absence Seizures**: Oscillations in Thalamocortical Networks and the Putative 'Initiation Site' of Typical Absence Seizures; Thalamic Neurons and Networks Related to Absence Epilepsy; **Non-Synaptic Mechanisms**: Non-Synaptic Mechanisms of Neuronal Synchronization: A Possible Role in Fast Oscillations; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; **Sleep**: Epileptic Components of NREM and Antiepileptogenic Components of REM Sleep; Seizure Initiation: The Transition from Sleep-Related Oscillations to Epileptiform Discharge.

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Epileptic Components of NREM and Antiepileptogenic Components of REM Sleep

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Introduction

The two main sleep states, non-rapid-eye-movement (NREM) sleep and rapid-eye-movement (REM) sleep, have different physiological components and contrasting effects on generalized ictal and interictal discharges (IIDs). Epileptiform discharges are likely to propagate during NREM sleep – i.e., during periods of synchronized electroencephalographic (EEG) background activity, during transients such as k-complexes and sleep spindles, and during transitional 'drowsy' EEG arousal periods. In contrast, REM sleep, with its asynchronous cellular discharge patterns and skeletal motor paralysis, is resistant to propagation of epileptic EEG potentials and to clinical motor accompaniment even though focal IID may persist at this time. The contrasting effects of NREM and REM on IIDs and clinically evident seizures are, to some extent, nonspecific with respect to epileptic syndrome (as defined by seizure type, etiology, and clinical course). Among the clinical syndromes in which epileptiform activity occurs prominently during sleep are childhood disorders with Continuous Spike-and-Wave activity during Slow-sleep (CSWS), such as Landau–Kleffner syndrome (LKS), Electrical Status Epilepticus during Sleep (ESES), and to a lesser degree West syndrome.

Increased severity of the epileptic syndrome, which reaches a peak in symptomatic generalized epilepsies such as West syndrome, is more likely to be associated with dispersion of ictal and IID events across the sleep-wake cycle, dissociation of physiological sleep components, medical refractoriness, and poor prognosis. However, there can be severe neurobehavioral consequences of uncontrolled seizures in sleep without overt neuropathy, especially in childhood. The cognitive, perceptual, and emotional consequences typically outlast the seizure disorder, and may be avoided with effective early intervention. Understanding the mechanisms responsible for these consequences should help to direct the development of early interventions and should have implications for developmental brain–behavior interactions.

The clinical observations raise three questions which this manuscript addresses: (1) Which components of NREM account for the propagation of ictal (seizure) and IIDs? (2) Which components of REM sleep suppress them? (3) How might the physiological mechanisms of seizure-prone sleep states overlap with those underlying other neurological consequences of LKS and ESES, including some autistic behaviors?

Background and Methods

To address these questions, many laboratories have established animal models that parallel the different clinical syndromes with respect to the timing of EEG and motor seizure manifestations. For example, systemic penicillin

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Epileptic Components of NREM and Antiepileptogenic Components of REM Sleep

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Introduction

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Background and Methods

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and electroconvulsive shock (ECS) provide models of idiopathic primary generalized epilepsy (PGE), and amygdala kindling is a model of the localization-related seizure disorder, temporal lobe epilepsy (TLE). Our studies employed cats studied at different ages, ranging from preadolescence to adulthood. The sleep-waking state distribution of seizures was quantified before and after several manipulations, as detailed later.

Subjects

Experiments employed cats ranging in age from 2.5 months to over 12 months of age. This time frame roughly corresponds in humans to about 3–4 years of age through adulthood. There are several reasons for selecting cats as subjects: (1) The mechanisms for nearly all the sleep components studied here were initially described in cats, and certain 'dissociative' manipulations, such as the lesion creating REM sleep without atonia, were developed in cats; (2) The brain size of cats, even at the youngest age studied, is large enough and the calvarium strong enough to perform chronic studies of seizure activity at multiple brain sites using a cranial implant that remains stable to adulthood; (3) Brain development is slow enough in cats to capture sleep and seizure development throughout the developmental course in the same animals, thus permitting longitudinal and cross-sectional studies of age effects. These opportunities are either limited or unavailable in rodents.

Animal Models

Feline penicillin epilepsy: Injection of large dosages of penicillin (300 000–400 000 IU/kg, i.m.) into cats induces seizure manifestations of idiopathic PGE, particularly juvenile myoclonic epilepsy (JME). The effect is unique to cats in that it can not be duplicated in any other species. JME is the most common form of convulsive epilepsy on awakening in humans. Spike-wave and multi-spike-wave complexes with myoclonic jerks occur contemporaneously with sleep EEG transients such as sleep spindles and k-complexes during slow-wave-sleep (SWS) (feline term for human NREM sleep) and decline during stable REM sleep. Nearly all generalized tonic-clonic convulsions (GTCs) occur during drowsiness after awakening. Feline penicillin epilepsy is an acute model; all effects subside with metabolism of the penicillin, and multiple trials can be conducted in the same animals without concern for residual effects of penicillin on sleep or epilepsy.

Electroconvulsive shock (ECS): High voltage electrical shock, applied through a thalamic lateral geniculate nuleus (LGN) electrode (paired to a reference electrode in the skull) induces a primary generalized epilepsy with tonic-clonic convulsions that occur predominantly in SWS but not in REM. ECS is generally thought of as an acute rather than a chronic seizure model, can be done in any species, and is often performed through corneal electrodes using a variety of amperages depending upon the species and age. At a high enough current, stimulation any nucleus or brain structure will induce ECS. In rat pups <4 days old, we have used 100 mA interaurally. In cats, we established thresholds aimed at obtaining the lowest possible amperage to achieve a generalized tonic-clonic convulsion (GTC) in each sleep or waking state before and after the REM sleep dissociative manipulation (see results).

Amygdala kindling: Amygdala kindling provides a model of the localization-related seizure disorder, TLE. TLE is one of the most common forms of convulsive sleep epilepsies in humans. As in humans, the spread of EEG seizure discharge from one site to another, called seizure discharge propagation, and spontaneous GTCs occur most often in SWS and in the transition into REM. Focal discharge persists and is highly localized in REM unless the cat wakes up. Also as in humans, several subconvulsive seizure types may be evoked or occur spontaneously at different times in the sleep-wake cycle.

Repetitive electrical stimulation of amygdala (once per workday, between 1 and 3 mA, for 4–37 days) in 2.5–6.5 month old kittens creates a unique spontaneous convulsive sleep epilepsy that can persist to adulthood. The younger the animal, the higher the current needed for initial afterdischarge (AD), the faster they kindle, (defined as the number of ADs required to achieve the first stimulus induced GTC), and the more likely they are to develop spontaneous seizures shortly after initial kindling. [Adults very rarely develop spontaneous seizures as a result of this kindling paradigm, and when they do, it usually results from multiple evoked convulsions after initial kindling.] The objective of our work in amygdale-kindled kittens has been to study the basis for onset of spontaneous seizures, their timing in the sleep-wake cycle, and their developmental course. We also wanted to determine whether sleep disorders and other behavioral correlates of epilepsy resemble age-related findings in human TLE.

All three of these epilepsy models resemble human counterparts in that generalized seizures occur during NREM sleep or transitional arousal periods. These seizure-prone states are characterized by background EEG synchronization, often with phasic events which include sleep EEG transients such as sleep spindles, k-complexes and ponto-geniculo-occipital (PGO) waves, and may involve state or stage shifts. In contrast, EEG seizure discharge generalization is reduced during states characterized by tonic EEG desynchronization, such as alert waking and particularly REM sleep. REM sleep is the most antiepileptic state in the sleep-wake cycle for generalized EEG and motor seizure activity, even though phasic events and localized EEG discharge persist in REM.

Results

We have performed experimental manipulations of discrete physiological characteristics or components (described below) in these epilepsy models, and recently described continuous electrographic seizures in SWS. This electrical seizure activity and the neurobehavioral consequences of sleep-related seizures may provide insights into to seizure phenomena in human ESES and LKS.

Seizure Initiation Associated with Activation of Transient Epileptiform Events

Phasic events of sleep are thought to reflect aborted arousals related to brainstem processing of intrinsic stimulation and can be provoked by extrinsic stimulation such as a loud sound. We have hypothesized that epileptogenesis is associated with abnormal (epileptiform) phasic events, which may sometimes simply disrupt sleep by awakening the animal but at other times precipitate seizures. **Figure 1** shows that exogenous stimulation, in this case photic stimulation (PS) ($20\ s^{-1}$), can not only evoke epileptiform phasic events during SWS, but can also precipitate GTCs in spontaneously epileptic kindled kittens.

The most vulnerable periods for extrinsic stimulus-evoked GTCs are those in which spontaneous seizures most frequently occur, notably in SWS and the transition into REM. The least vulnerable time for evoked seizure activity is REM sleep. Spontaneous phasic activity and highly localized multifocal EEG seizure discharge persist during REM and may be evoked by photic stimulation, but there is no clinical accompaniment. These findings suggest that phasic activity can provoke epileptiform discharges in any sleep state, but that the extent of epileptic EEG discharge spread and the clinical motor accompaniment depend upon tonic EEG and motor sleep components.

Dissociating the Tonic (Sustained) Components of REM and NREM Sleep

The roles of two tonic sleep components (synchronous vs. asynchronous background EEG and presence vs. absence of tone) were evaluated before and after the experimental manipulations depicted in **Figure 2**. The top tracing (1) shows normal REM sleep with tonic EEG

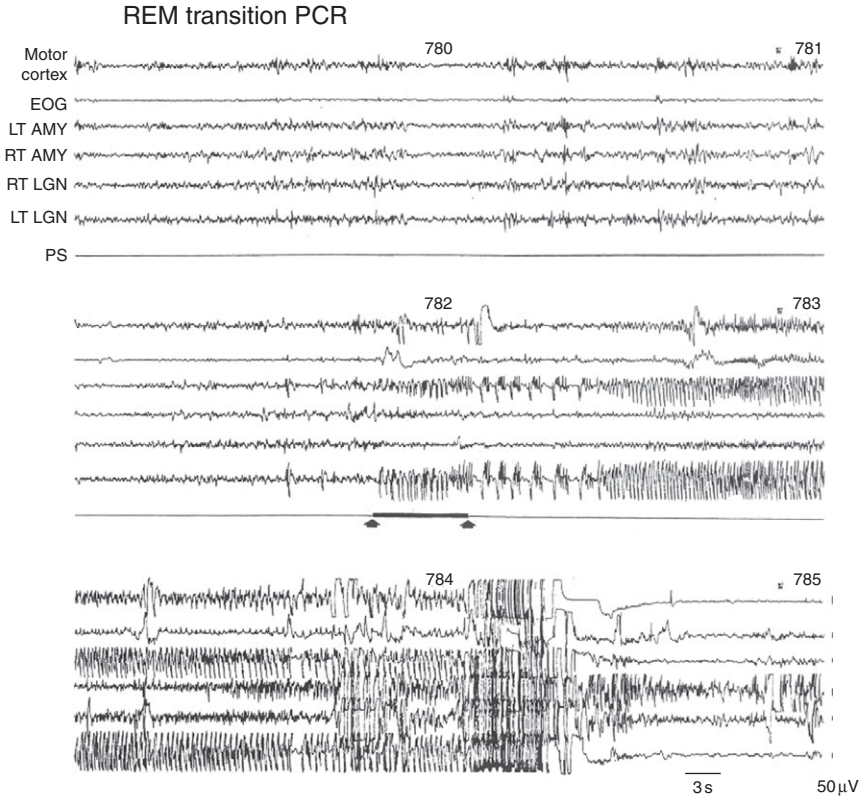


Figure 1 Continuous polygraphic recording showing a REM transition photoconvulsive response (PCR) elicited by $20\ s^{-1}$ PS. Arrows indicate PS onset and offset. PS initially evoked epileptiform PGO spikes in kindled amygdala and ipsilateral LGN. Epileptiform k-complexes in the kindled amygdala and ipsilateral LGN outlasted PS and were followed by EEG seizure discharge propagation with clinical accompaniment. Paper speed is 10 mm. AMY, amygdala; LGN, lateral geniculate nucleus of thalamus; EOG, electrooculogram; EMG, electromyogram. Adapted from Shouse *et al*. (1995) Paroxysmal arousals in amygdala-kindled kittens: microarousals, could they be subclinical seizures? *Epilepsia* 36: 290–300.

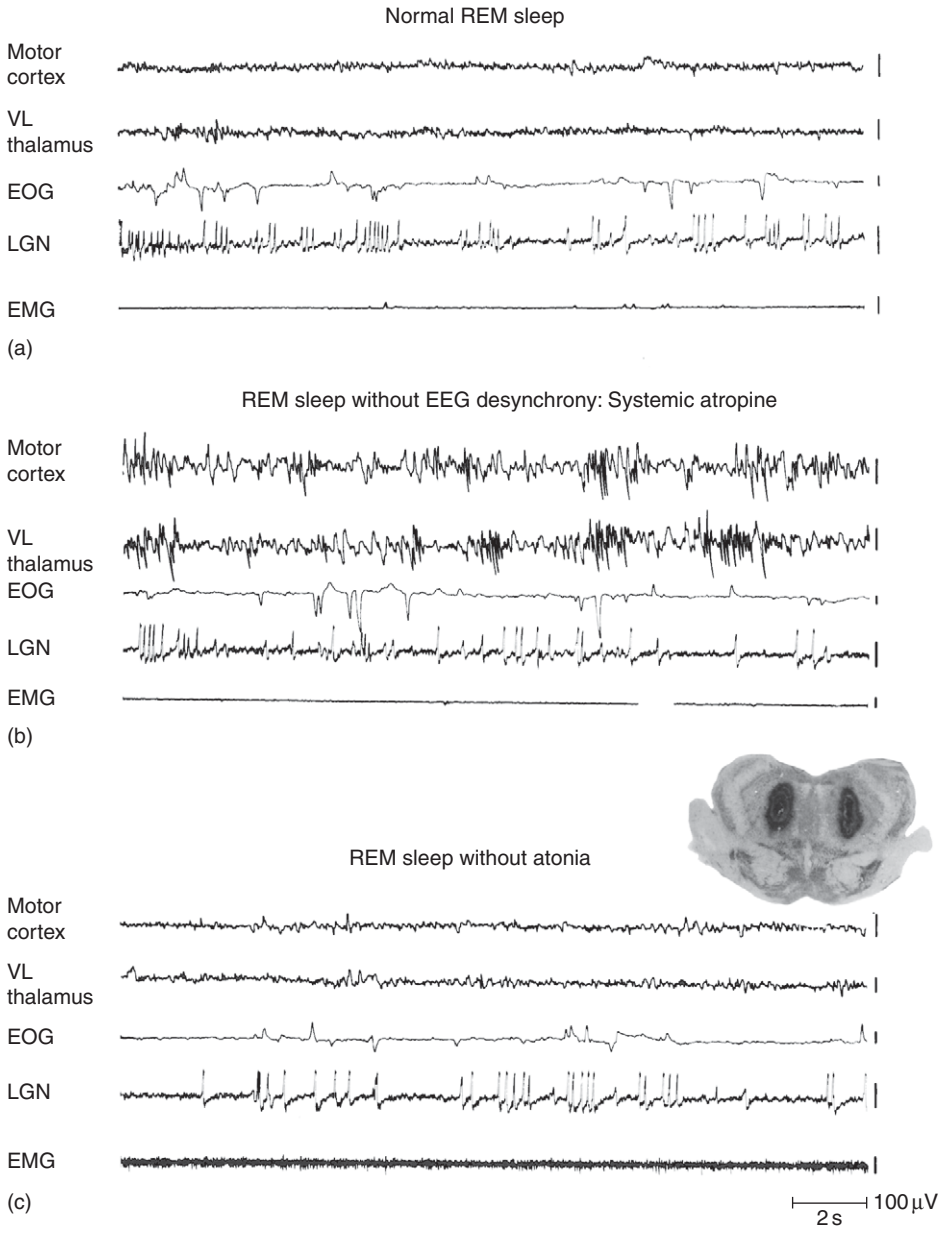


Figure 2 REM sleep before (a) and after experimental techniques designed to selectively alter either EEG synchronization or atonia. (b) Systemic atropine induces REM sleep without EEG desynchronization. (c) A bilateral medial-lateral pontine lesion induces REM sleep without atonia. Abbreviations as in **Figure 2** except for ventral lateral (VL) nucleus of thalamus. Adapted from Shouse *et al*. (1989) Mechanisms of seizure suppression during REM sleep in cats. *Brain Research* 505: 271–282.

desynchronization (motor cortex and ventral lateral thalamus channels), rapid eye movements (EOG channel), PGO waves (lateral geniculate nucleus or LGN channel), and atonia (EMG channel). The middle tracing (2) shows a model of REM sleep without EEG desynchronization induced by systemic administration of atropine. The anticholinergic agent presumably acts by blocking activity in the pedunculopontine tegmentum and nucleus basalis, which are thought to be the critical generators of EEG desynchronization in waking and REM. The result of this treatment is a pervasive NREM-like EEG with sleep spindles. The other phasic and tonic components of REM are also intact, including rapid eye movements, PGO waves, and atonia (note electrical silence in EMG channel). The third tracing (3) shows REM sleep without atonia, created by lesions of the medial-lateral pontine tegmentum. This lesion is thought to destroy cholinoceptive and glutaminergic cells that silence lower motor neurons and create sleep paralysis during REM sleep. REM sleep components are intact except the cat has tone (EMG channel).

Figure 3 illustrates the effect of these dissociative sleep manipulations on penicillin epilepsy. The top tracing

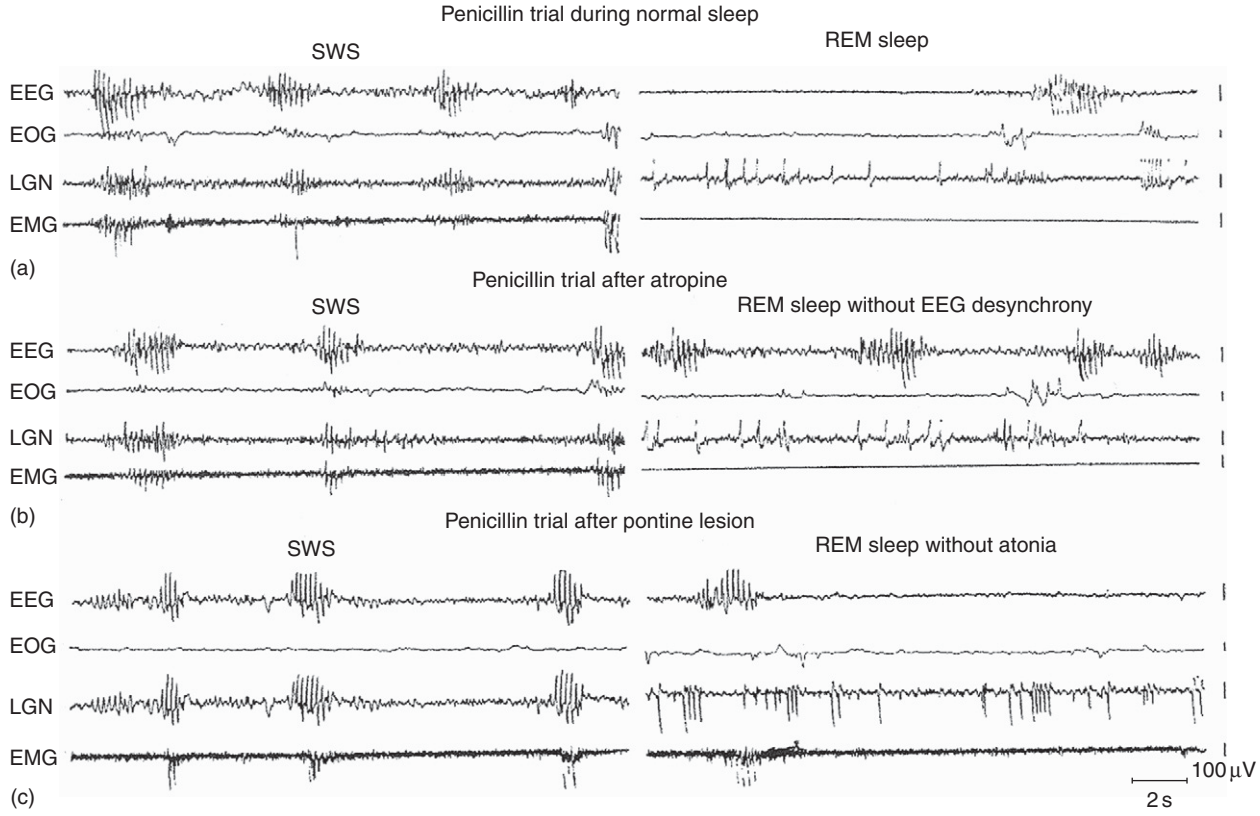


Figure 3 Penicillin epilepsy during SWS (left) and REM sleep (right) before (a) and after dissociation techniques. (b) Atropine creates a SWS-like EEG synchrony in REM and selectively increases incidence of EEG seizure susceptibility in REM without clinical motor accompaniment. (c) Pontine lesions which cause REM sleep without atonia selectively increase clinical motor accompaniment. Abbreviations as in **Figure 2**; the top channel is a motor cortex EEG. Adapted From Shouse *et al*. (1989) Mechanisms of seizure suppression during REM sleep in cats. *Brain Research* 505: 271–282.

Table 1 Parallels and dissimilarities of age-related amygdala kindling in kittens to electrical status epilepticus (eses) and/or Landau–Kleffner syndrome (LKS)

	Parallels	*Dissimilarities*
Age dependency	Critical period for onset	GTCs can last to adulthood in kittens
Spontaneous seizure-related symptomatology	Seizure types (GTCs, partial motor, atypical absence, complex-partial); IID types and cortical 'foci'; Foci change over time	ESES, not CSWS in kittens; Clinical seizures start first kittens and ESES versus LKS and ASD; Fronto-central foci in ESES versus Centro-temporal foci in LKS and kittens
Sleep patterns	NREM onset seizures; ↑ IIDs and spread in NREM and ↓↓ IIDs and spread in REM	Occasional ESES symptoms in kittens; Sleep deficits in kittens
Developmental/ neurobehavioral deficits	↑ Sensitivity and IIDs to sound; Agnosia; Social isolation; Mood change, Stereotypies, Hyperactive, atonic/dystonic aggressive/placid	Type/extent of residual deficits depend on early onset, site and duration of CSWS in humans versus GTCs in kittens
Prognosis and AED response	Good for seizures; ESES self-limited; Residual neuro-behavioral symptoms; Clonidine good for autistic symptoms	Same AEDs untested
Encephalopathy; pathophysiology	Localization-related with subtle/minor cell loss/pathology at foci	?

GTCs, generalized tonic-clonic convulsions; ↑, increased; ↓, decreased; CSWS, continuous spike-and-wave during slow sleep; ASD, autistic spectrum disorders.

(a) shows spike-and-wave complexes riding upon sleep spindles in SWS and accompanied by myoclonic twitching (note related discharge in EMG channel). During REM sleep, spike-and-wave activity is rare and occurs without clinical motor accompaniment. The middle tracing (b) shows that atropine does not affect the rate of EEG and motor seizure activity in SWS. However, the REM sleep cortical EEG is synchronized, and EEG seizure discharge

rate cannot be distinguished from SWS (shown) or waking (not shown). Unlike waking and SWS, there is still no clinical motor accompaniment in REM sleep, presumably because lower motor neuron inhibition remains intact. The bottom tracing (c) shows seizure activity during REM sleep without atonia. Spike-wave activity is as infrequent as during normal REM, but, when a spike-and-wave complex does occur, there is clinical motor accompaniment, presumably because the animal can now move. Similar results were obtained after these dissociative sleep manipulations in 26 cats undergoing penicillin epilepsy ($n = 10$), ECS ($n = 10$) or amygdala kindling ($n = 6$).

Amygdala Kindled Kittens ESES and Landau–Kleffner Syndrome

Recent 24 h video recordings of group housed kittens $\leq$5 months old, during the initial stages of kindling, revealed a far more extensive and complex clinical picture than is seen after kindling older pre-adolescent and adult cats. These young animals display several similarities to ESES and LKS (and occasionally to ASD), as summarized in Table 1.

Amygdala-kindled kittens, like ESES/LKS patients at ~4–7 years of age, display a critical, age-dependent period for development of multiple foci (including, but not limited to, fronto-central and centro-temporal areas), and multiple seizure types (partial, atonic, atypical absence, tonic-clonic, subclinical). IIDs generalize during SWS or NREM sleep, when most clinically evident seizures occur. Sleep disorders common to both the animal model and to the human syndromes include delayed sleep onset, increased wakefulness after sleep onset, and abbreviated sleep time (mostly in children with autistic symptoms). Common behavioral correlates include increased sensitivity to sensory stimuli, extreme fluctuations in temperament (docile or depressed to aggressive) and social isolation. Other behavioral characteristics shown by some kindled kittens better resemble Kluver-Bucy syndrome, notably visual agnosia, hypersexuality and docility. Anti-epileptic drugs can arrest the incidence and progression of seizures and the associated behavioral complications. Unlike human TLE with hippocampal sclerosis, the seizures and their sequelae tend to remit spontaneously (by or during adolescence), although residual language and behavioral symptoms usually persist. Histological changes are nominal in ESES and LKS, and minimal gliosis and cell loss are also a feature of kindled kittens, except for those animals that remain symptomatic to adulthood due to uncontrolled seizures and/or continued evoked seizure trials.

The findings in kindled kittens and LKS suggest an age-dependent pathophysiological process rather than a static or progressive course more often associated with structural anomalies. Kindling is believed to have 'trans-synaptic' effects, in that cells distal to the kindled focus undergo transient or permanent changes in membrane properties, synaptic reorganization, and transmitter release as well as variable degrees of cell loss. These changes are hypothesized to explain the extra-focal alterations in seizure susceptibility, as well as the many other behavioral sequelae of kindling which range from sleep disorders to changes in agonist/antagonist social behaviors. These trans-synaptic effects seen in adults are not always detected in the young.

Some of the concepts currently discussed to explain age-related differences in kindled TLE without hippocampal sclerosis in young rodents may be helpful in explaining the clinical course of amygdala kindling in kittens and perhaps ESES/LKS as well. Young rats (7–14 days old) kindle much more rapidly than adults, and the electrical seizures are far more likely to become multifocal. Different limbic sites kindle with equal speed in young rats, whereas adults usually exhibit preferential kindling sites (e.g., piriform cortex kindles faster than amygdala). Immature rats also do not display the 24 h seizure-resistant or refractory period that occurs between evoked seizure trials in adults,

Table 2 Spontaneous seizure parameters after once daily amygdala microinfusion of either the α-2 agonist idazoxan or the α-2 antagonist clonidine

Type of drug infused	*No. and percent of animals per group with Spontaneous Seizures*	*Spontaneous seizure types, no. of animals with each and mean GTC duration (s) ± S.E.M.*	*Mean No. and duration ± S.E.M. and range (min/max) of post-kindling GTCs to onset of spontaneous GTCs or to end of protocol*
Idazoxan ($n = 7$)	7 (100%)	Focal or generalized slow spike-and-wave with staring ($n = 2$) Generalized subcortical fast trains with staring ($n = 1$) GTC ($n = 7$)[a]; 66.7 ± 3.8 s (53–74.5)	5.86 ± 0.3[a] (5–7); 65.8 ± 3 s (60.3–71.6)
Clonidine ($n = 7$)	0 (0%)	0 (0%)	24.71 ± 0.9 (20–26); 67.85 ± 5 s (54.5–93.0)

[a]Simple *F*-test (df = 1,12), $p < 0.01$ from clonidine.

Number (No.) of spontaneously epileptic animals, spontaneous seizure types, and evoked seizures were noted prior to the onset of spontaneous seizures or to the end of the protocol. Adapted from Shouse *et al.* (2007) The α-2 adrenoreceptor agonist clonidine suppresses evoked and spontaneous seizures, whereas the α-2 adrenoreceptor antagonist idazoxan promotes seizures in amygdala kindled kittens. *Brain Research* 1137(1): 58–68.

nor do they exhibit 'kindling antagonism' in which alternating once daily stimulations between two anatomical sites blocks seizure development at one of the sites. Also unlike adults, immature rats are resistant to structural damage resulting from multiple seizures and/or other cerebral insults, do not exhibit synaptic reorganization of mossy fibers, and do not display increased inhibitory transmission in dentate granule cells.

Moshe and others have concluded that inadequate inhibitory mechanisms, potentially involving GABAergic and noradrenergic transmitter systems, may be responsible for increased vulnerability to multifocal epilepsy in the young. Early in development, GABA has excitatory (depolarizing) rather than inhibitory (hyperpolarizing) effects on neurons and thus might enhance rather than discourage seizures and other disruptive behaviors. Norepinephrine (NE) has well-established inhibitory effects on seizure activity in adults and may not be fully operational in immature brains. For example, NE secretion is believed responsible for establishing and maintaining both the interictal refractory periods and the inter-site 'kindling antagonism' seen in mature animals. Increased NE secretion is also considered critical to selective sensory processing during wakefulness, which is deficient in ESES and LKS. Alpha-2 adrenoceptor agonists (e.g., clonidine) can effectively suppress seizures in kittens (**Table 2**) as well as several behavioral symptoms of autism. It remains unclear how the development of neural networks affects or is affected by seizures (and other behavioral anomalies), but maturation clearly alters the dynamics. Early detection and intervention may halt or attenuate the progression of symptoms.

Future Goals

We propose two major goals for future research. First, there is an important need for establishing better models of continuous spike-wave during slow sleep (CSWS), the EEG seizure signature of ESES and LKS. One approach to this goal is to examine the effects of kindling cortical sites involved in the clinical syndromes. We have seen ESES in amygdala-kindled kittens, but not continuous spike-wave activity per se. Second, we believe that it is critical to develop a more sophisticated behavioral inventory, to help determine the efficacies of new antiepileptic drugs, and to assess whether early intervention in the electrical component of seizure disorders can prevent the neurological/clinical sequelae.

Acknowledgements

This work is sponsored by Department of Veterans Affairs.

See also: **Cognitive Dysfunction and Other Comorbidities**: Epilepsy and Autism in Tuberous Sclerosis; The Relationship of Epilepsy to Other Conditions/Disorders; **Models**: Characteristics and Mechanisms of Kindling; **Neurotransmitter Function**: Impact of Norepinephrine on Epileptogenesis; **Pediatric Epilepsy**: Developmental Aspects of Seizures; **Sleep**: Brain Mechanisms Linking Epilepsy to Sleep; Seizure Initiation: The Transition from Sleep-Related Oscillations to Epileptiform Discharge.

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Seizure Initiation: The Transition from Sleep-Related Oscillations to Epileptiform Discharge

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Introduction

This article is devoted to examining the potential initiating mechanisms of seizures, with particular emphasis on the transition from sleep-related oscillations to seizure activity. Toward this goal, we consider the example of nocturnal seizures, which develop almost exclusively from slow sleep oscillations, and then extend our analysis to other spike-wave (SW) paroxysms. Although the contribution of glial cells during epileptic events has been previously acknowledged, few studies have addressed the interaction between neurons and glia. Here, we consider the contribution of glial cells to the triggering of the seizures, to the oscillatory process associated with this initiation process, and to the generalization/propagation of the paroxysms through the brain. Special emphasis is on the modulation of the neuronal activity by the ionic concentrations of the extracellular space.

SW seizures occur predominantly during resting sleep, as has been reported in children with absence epilepsy and in the Lennox–Gastaut syndrome. The vast majority of seizures occurring during sleep are dominated, in their electrographic aspect, by recurrent SW complexes. The electroencephalographic (EEG) SW complexes recur with a frequency of about 2–4 Hz; the precise frequency is determined by various pathologic, structural, and intrinsic factors. In this article we consider the cellular mechanisms generating SW complexes regardless of the etiology of the related seizures; we believe that, in spite of the clinical variety of epileptic seizures displaying this EEG feature, the underlying cellular mechanisms of SW complexes are similar across seizure types.

Background

Physiological Sleep Slow-Wave Oscillations

A continuous range of slow waves and oscillations, generally <15 Hz and of relatively high amplitude, dominates the electrographic pattern of sleep. Current studies of the cellular substrates of these rhythms have indicated that most of the sleep oscillations are generated within the corticothalamic network, under the control of ascending modulatory projections from the brainstem core and basal forebrain (see Steriade in Further Reading).

Although sleep oscillations emerge in the cortex as coalesced events, two rhythms with distinct mechanisms and generated in various substructures of the corticothalamic network can be distinguished: spindles (7–14 Hz) and delta (<4 Hz) waves. The latter frequency band contains several phenomena with distinct generating mechanisms. Among them, a remarkable oscillation, termed 'slow' (less than but close to 1 Hz), assumes a particular role in combining other sleep rhythms and in constituting the germ for paroxysmal development during nocturnal seizures.

Neuronal Mechanisms of the Slow Oscillation

In spite of its ubiquitous presence during sleep, the slow (<1 Hz) oscillation was only recently described. The main rhythm is within the frequency range of 0.5–0.9 Hz, although slower and slightly higher frequencies are not uncommon as a function of the sleep stage, with a clear tendency toward acceleration as sleep deepens. The first description of this slow oscillation came from anesthetized cats and was made at the intracellular level, and subsequent studies described slow oscillations under various anesthetics and during natural sleep in chronically implanted cats as well as in the sleep of humans. One cycle of the slow oscillation consists of an excitatory phase and an inhibitory phase (**Figure 1**). During the excitatory phase, neurons rapidly depolarize to reach a plateau level that is crowned by occasional action potentials; the depolarization lasts for 0.5–1 s and is generated by EPSPs, IPSPs, and intrinsic currents (mostly NMDA and Na^+ persistent).

An important issue concerning the slow oscillation remains the understanding of cellular and/or ionic mechanisms responsible for the switching between the depolarizing and the hyperpolarizing phase. It is not yet understood why the cortical network oscillates, or what elements are responsible for setting the pace of this oscillation. Investigators have demonstrated that the end of the depolarizing phase is accompanied by a progressive disfacilitation during which the excitatory drive within cortical networks diminishes; they have postulated that the progressive diminution of network activity would promote a gradual disconnection between neurons – resulting in cell hyperpolarization. An active inhibition eliciting the hyperpolarizing phase of the slow oscillation

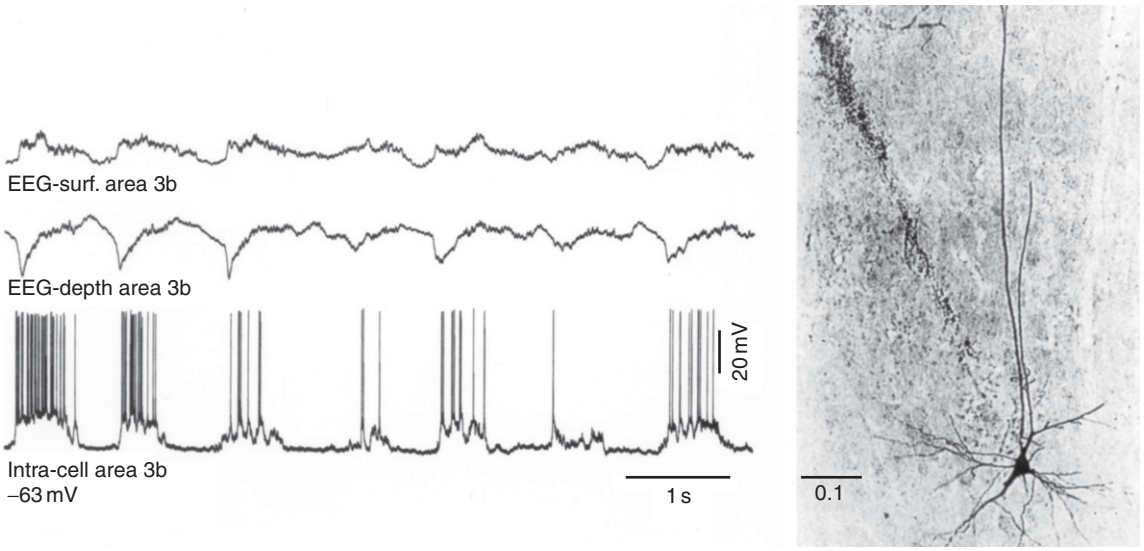


Figure 1 Pyramidal cell from the somatosensory cortex (area 3b) during the slow oscillation. The left panel shows an intracellular recording and simultaneously recorded EEG in the vicinity (~1 mm) of the cell. The EEG was recorded by means of coaxial electrodes located on the surface and at a depth of ~0.6 mm. In this and following figures, polarity of EEG and evoked field potentials is as for intracellular recordings (positivity up). The cell oscillated at 0.9 Hz with depolarizing phases corresponding to depth-EEG negative (surface-positive) potentials. The right panel shows the corresponding cell stained with Neurobiotin (calibration bar in mm). Modified from Contreras D and Steriade M (1995) Cellular basis of EEG slow rhythms: A study of dynamic corticothalamic relationships. *Journal of Neuroscience* 15: 604–622, with permission from the Society for Neuroscience.

has been ruled out. The disfacilitation hypothesis is supported by measurements of the extracellular calcium concentration, showing that the depolarizing phase of the slow oscillation is associated with a progressive diminution of the extracellular calcium (probably due to postsynaptic uptake of calcium), with the effect of a parallel reduction of synaptic efficacy. Another synergistic mechanism involves spatial buffering of potassium by glial cells (see the later sections).

Several lines of evidence support the idea that the slow oscillation is generated within cortical networks. First, the slow oscillation survives in the cortex after extensive thalamic lesions and in preparations with high brainstem transections. Second, the slow oscillation is absent from the thalamus of decorticated animals, while it is expressed by thalamic reticular and thalamocortical cells in intact animals. Third, the slow oscillation has been replicated in vitro, in cortical slices. The mechanisms underlying this oscillatory behavior are partly elucidated at the neuronal level. Studies have shown that slow oscillations are expressed within various cortical areas and by virtually all neuronal types, and they display wide-range and short-range cortical synchronization. There is a large current sink in cortical layer III during the depolarizing phase, and the coherence of the rhythm is impaired after inactivation of synaptic activity or after cortical transections, indicating that intracortical linkages are instrumental to the propagation of the slow oscillation.

The slow oscillation assumes an operational role in its relation with other brain activities during sleep. Its cortical coherence ensures periodic and synchronous inputs to subcortical structures, mainly to the thalamus, where it simultaneously triggers spindle sequences. Once a spindle is generated in the thalamus, it is transmitted to the cortex, thus having a direct impact on the EEG. In this way, the slow oscillation is reflected in the gross brain activity of the sleeping brain. The slow oscillation is expressed in the local cortical field potentials that reflect the membrane currents generated by neurons (and possibly glial cells). Since the depolarizing phase of the slow oscillation reflects the excitation of all cortical neurons, the corresponding extracellular field potential consists of a negative deflection. The shape of this negative potential is similar to that of the K-complex, an EEG wave described many decades ago. The fact that the depolarizing (excitatory) phase of the slow oscillation is conveyed by corticothalamic axons to the thalamus also explains the periodic triggering of spindle sequences in the thalamus and, at the EEG level, the association between K-complexes and spindles. Spindles are triggered in the thalamus mostly during the early stages of slow wave sleep.

Glial Mechanisms of the Slow Oscillation

More insight into the cellular mechanisms responsible for the pacing of slow oscillations, and the switch between depolarizing and hyperpolarizing phases, has come from experiments examining the possible dialog between neurons and glial cells – and particularly exploring the possibility that glia may not only passively reflect, but directly

influence, the state of neuronal networks. Dual simultaneous intracellular recordings from neurons and adjacent glial cells were performed during sleeplike EEG patterns produced by ketamine–xylazine anesthesia. The behavior of simultaneously recorded neurons and glia is illustrated in Figure 2. During spontaneously occurring slow oscillations, the onset of the glial depolarization did, in the vast majority of the cases, follow the onset of the neuronal depolarization, with an average time lag of about 90 ms – considerably longer than the lag seen between pairs of neurons (around 10 ms). Presumably, this glial depolarization reflects, with virtually no delay, the potassium uptake into glia. As potassium is also continuously recaptured by neurons through Na^+/K^+-ATP pumps, the actual onset of the glial depolarization may thus mark the moment at which the increase of extracellular potassium concentration exceeds the capacity of the neuronal pumps. This behavior suggests that glial cells are essential for maintaining extracellular potassium within controlled values.

Toward the end of the depolarizing phase, the glial membrane begins to repolarize before the membranes of neurons (Figure 2). This observation excludes two mechanisms that could have been responsible for the onset of the hyperpolarizing phase of the slow oscillation: an active GABA inhibition at the end of the depolarization and a slow calcium-dependent potassium current. In the first case, the release of GABA would depolarize glial cells either through direct action on $GABA_A$ receptors or indirectly through the potassium output resulting from the neuronal activation of $GABA_B$ receptors. The alternative implication of calcium-dependent potassium currents

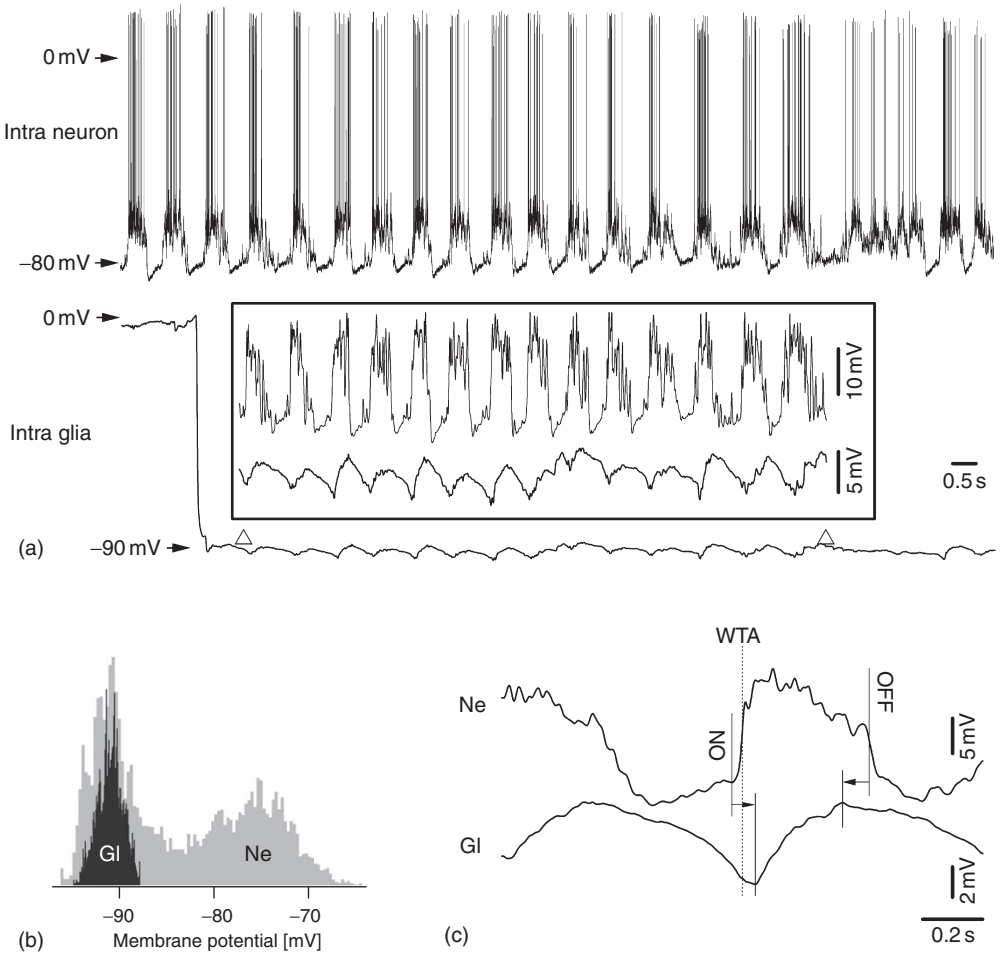


Figure 2 Simultaneous recording of a neuron-glia pair in the cortex of the cat during slow oscillatory patterns. (a) Relationship between neuronal and glial depolarizations on the one hand, and between neuronal hyperpolarization and glial repolarizations, on the other hand (note enlargement within the box). The glial impalement occurred during this period of the recording and is depicted as a brisk drop of the potential from the extracellular (0 mV) to the intracellular (−90 mV) level. (b) Histogram of membrane potential variations in the neuron (in gray) and glial cell (in black). (c) Typical time relationship between the onset and offset moments of the two phases of a slow oscillatory cycle. Note that the onset of the depolarizing phase starts in the neuron, but that the end of this period is anticipated in the glia. Modified from Amzica F and Massimini M (2002) Glial and neuronal interactions during slow wave and paroxysmal activities in the neocortex. *Cerebral Cortex* 12(10): 1101–1113, with permission from Oxford University Press.

should be discarded because the extracellular potassium starts to drop before the onset of the neuronal hyperpolarizing phase.

In addition, the glial membrane potential returns to control value at the end of each of the recurring oscillatory cycles, which has led us to postulate that glial cells might control the pace of the oscillation through changes in the concentration of the extracellular potassium. The overall synchronization of the slow oscillation in the cortex appears to be assisted by glial cells, which are imbedded in a gap junction-based network, through the phenomenon of spatial buffering. Spatial buffering evens local increases of extracellular potassium by transferring it from the extracellular milieu into local glial cells, then through gap junctions and along the potassium concentration gradient to more distant sites with lower concentrations of potassium, where it is again released into the extracellular space. This pathway is preferred to the direct one through the extracellular space, because the tortuosity of the extracellular space makes diffusion difficult. During slow oscillations, the relationship between intracellular glial potentials and extracellular potassium shows little accumulation of potassium in the extracellular space (<1.1 mM per cycle), so there is a minimal imbalance between the extra- and intracellular potassium concentrations. Given that, under physiological conditions such as sleep, glial cells normally deal with low amounts of potassium – but have a high uptake capacity – it is likely that, during the slow oscillation, spatial buffering generally occurs at a relatively local spatial scale.

The local spatial buffering during slow oscillations might play two roles: (i) it contributes to the steady depolarization of neurons during the depolarizing phase of the slow oscillation in a Nernst-related manner and (ii) it modulates the neuronal excitability. The latter mechanism could favor the synaptic interaction within cortical networks at the onset of the depolarizing phase of the slow oscillation, but it could equally induce a gradual disfacilitation as the extracellular potassium concentration reaches higher values toward the top of the glial depolarization. The amplitude of the potassium variations during the slow sleep oscillation reaches 1–2 mM above resting level at its peak; when added to the physiological values of resting concentration (~3 mM), this increased extracellular potassium may assist cortical neurons in oscillating between hyper- and hypoexcitability. This hypothesis was confirmed after testing the excitability of the neurons during the various phases of the slow oscillation cycle with cortical electric stimuli. Neuronal excitability is maximal toward the beginning of the depolarizing cycle and almost zero toward the end of the depolarizing phase of the slow oscillation; it then increases during the hyperpolarizing phase, in phase opposition with the extracellular potassium concentration.

Methods

The results illustrated in this paper were obtained in experiments performed on cats maintained under ketamine–xylazine anesthesia (10–15 and 2–3 mg kg^{-1}, respectively, i.m.). This anesthesia produces the best EEG sleeplike pattern of available anesthetics. Intracellular recordings from both cortical neurons and glia were obtained under stable recording conditions that included muscular paralysis (accompanied by artificial ventilation), hip suspension, drainage of *cisterna magna*, and bilateral pneumothorax; the calvarium was filled with a solution of 4% agar. The animal was kept within stable physiological parameters: body temperature between 37 and 38 °C, heart rate 90–110 beats min^{-1}, end-tidal CO_2 between 3.5% and 3.8%. The intracellular recording electrodes were made of glass capillaries filled with a 3 M potassium acetate solution (resistance, 25–45 MΩ). Field potentials were recorded by means of tungsten microelectrodes (3–8 MΩ resistance) inserted at different depths into various cortical areas. Epileptic seizurelike activity occurred spontaneously, directly from the sleeplike patterns. Extracellular ionic concentrations were measured with micropipettes filled with potassium or calcium ionophores.

Results – Transition from Sleep to Seizures

To understand the mechanisms underlying the occurrence of nocturnal SW seizures, one must consider the precipitating factors acting before and throughout the paroxysmal episode, and the (minimal) critical structures involved in the genesis of SW discharges. The occurrence of seizures may rely on a multitude of factors, ranging from genetic malformations of K^+ channels to imbalances of ionic concentrations or abnormal recruitment of cellular aggregates. In most cases, it is likely that seizures result from a combination of factors.

Consistent with this view, it is noteworthy that nocturnal seizures occur during a state in which coherent oscillations are already present. During the slow sleep oscillation, the neuronal membrane potential varies between hyperpolarized and depolarized values. The hyperpolarization of the neuronal membrane often deinactivates some excitatory currents of the low threshold type, which in turn will have a greater impact on the synchronization of the network. It has been proposed that the slow oscillation acts as a precursor of nocturnal seizures of the Lennox–Gastaut type (**Figure 3**). The slowly oscillating pattern accelerates during a transition period, evolving toward an oscillation at around 1.5–2 Hz; the amplitude of the phasic depolarizing segments increases, while their duration diminishes. Thus, the

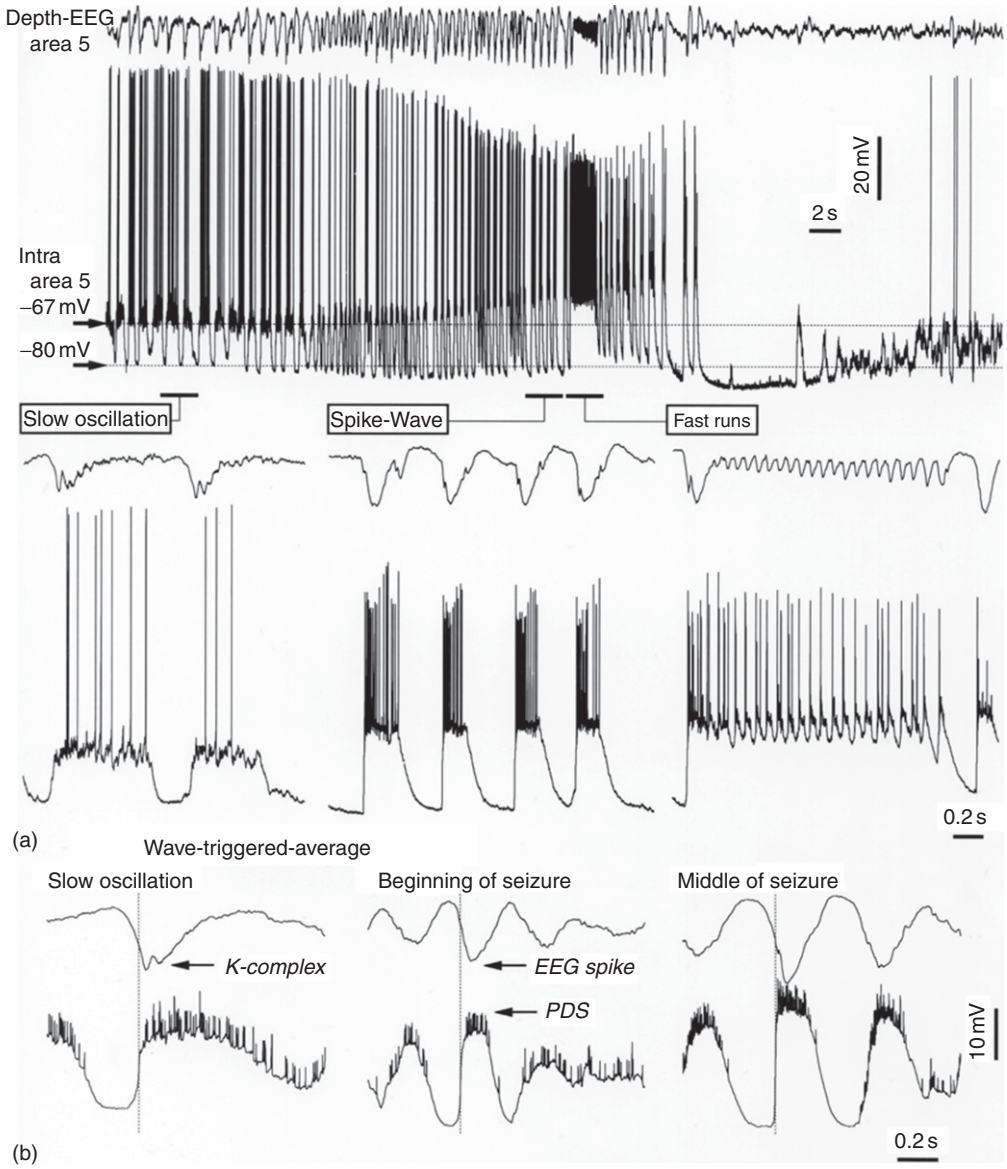


Figure 3 Spontaneously occurring seizure, developing without discontinuity from slow sleep-like oscillations. Intracellular recording from regular-spiking area 5 neuron together with depth-EEG from the vicinity in area 5. (a) Smooth transition from slow oscillation to complex seizure consisting of SW complexes at ~2 Hz and fast runs at ~15 Hz. The seizure lasted for ~25 s. Epochs of slow oscillation preceding the seizure, SW complexes, and fast runs are indicated and expanded below. Note postictal depression (hyperpolarization) in the intracellularly recorded neuron (~6 s), associated with suppression of EEG slow oscillation (compare to left part of trace). (b) Wave-triggered-average during the slow oscillation, at the beginning of seizure and during the middle part of seizure. The averaged activity was triggered by the steepest part of the depolarizing component in cortical neuron (dotted lines), during the three epochs. The depth-negative field component of the slow oscillation (associated with cell's depolarization) is a K-complex. During the seizure, the depolarizing component reaches the level of a paroxysmal depolarizing shift (PDS), associated with an EEG spike. Modified from Steriade M, Amzica F, Neckelmann D, and Timofeev I (1998) Spike-wave complexes and fast components of cortically generated seizures. II. Extra- and intracellular patterns. *Journal of Neurophysiology* 80: 1456–1479, with permission from The American Physiological Society.

normal depolarizing events of the slow oscillation are transformed into paroxysmal depolarizing shifts (PDSs), and K-complexes (at the EEG level) evolve into EEG paroxysmal 'spikes.'

The smooth transition from the physiological to the pathological pattern suggests that the slow and the paroxysmal oscillations have common mechanisms. The PDS is a giant EPSP that also contains IPSPs. Occasionally, the PDSs and their EEG counterparts, the 'spikes,' are superimposed by faster oscillations (at about 10–15 Hz) that produce corresponding indentations on the EEG 'spike,' leading to a polyspike pattern (**Figure 3**). This pattern of discharge is a typical signature for those EEG events occurring in the Lennox–Gastaut syndrome, and their

frequency (1.5–2 Hz) appears to be a resonating phenomenon within the cortical network. This conclusion resulted from a series of experiments where the cortical network was stimulated with trains of electrical shocks at various frequencies; in these experiments, responses to stimulation were maximal (in amplitude and reliability) at a stimulation rate of about 2 Hz.

The wave component of the SW complex is not due to active inhibition because, as demonstrated with multiple intracellular recordings, virtually all neurons (local inhibitory interneurons included) are silent during this phase of the oscillation. In addition, the firing rate tends to diminish toward the end of the PDS due to the inactivation of action potentials. The activity of local-circuit inhibitory cells tends to weaken toward the end of the PDS, and undergoes the general disfacilitation affecting all neurons belonging to the cortical network. The somatic input resistance is lowest at the beginning of the PDS, and continues to increase up to the next PDS, assuming its largest value during the hyperpolarization. This observation would not be consistent with the possibility that GABAergic currents induce the arrest of firing. The disfacilitation could be promoted, as in the case of the slow oscillation, by reduced synaptic efficacy consequent upon the depletion of extracellular calcium ions. Such reduced synaptic efficacy is consistent with the relative refractoriness of cortically elicited PDSs.

Among the ions with a significant impact on the neuronal excitability, potassium fluctuations have been investigated in depth. These studies have shown that the more synchronized the network, and the higher the amplitude of its oscillations, the more potassium is expelled into the extracellular space during action potentials and other currents. During SW seizures, the extracellular potassium concentration increases from resting values (3–3.5 mM) to pathological levels of 9–12 mM. An increase in the extracellular potassium has proved to favor initiation of SW seizures. As indicated above, the extracellular potassium is regulated by glial cells uptake followed by spatial buffering through gap junctions connecting the glial syncytium. While this latter phenomenon 'evens out' the extracellular potassium, it may also contribute to the general depolarization of the neuronal membrane potentials by positively shifting the Nernst equilibrium potential.

The synchronization of neuronal activity, the striking feature of sleep slow activities, creates a favorable environment for seizure genesis because synchronous activities generate local increases of extracellular potassium. The more synchronous the activities, the more potassium will be released. The spatial buffering performed by glial cells will transport the excess of potassium to more distant locations, setting the neurons of those remote sites in a hyperexcitable state. In addition, those distant neurons will receive synaptic bombardment from the neurons involved in generation of the synchronous discharge, thus contributing to the propagation of the oscillations. The protective mechanism against this spread of excitability seems to rely on the ability of glial cells to regulate potassium through uptake; impaired glial uptake of K^+ has been shown to cause epileptiform activity in the hippocampus. Circumscribed foci of epileptic activity can be generated at locations with impaired glial functionality, with spread of this hyperexcitability to remote sites through the same process of spatial buffering.

Besides the steady, very slow variations of potassium, which are reflected by similar changes in the membrane potentials of glial cells, the rhythmic ictal discharges are also accompanied by variations of the extracellular potassium concentration and corresponding phasic potentials in glial cells (**Figure 4**), indicating that the potassium uptake and spatial buffering also undergoes oscillatory influences at the frequency of the intraseizure oscillations (1.5–2 Hz). Further, at this frequency, an additional glial-dependent modulation may occur.

The implication of glial cells in the genesis of SW seizures may also be viewed in the light of recent studies that have expanded our understanding of neuron–glia communication pathways. On the one hand, glial cells are now known to be capable of neurotransmitter release, particularly glutamate, which is released from astrocytes via a calcium-dependent vesicular release mechanism. In agreement with the evolution of the neuronal excitability within each cycle of a SW discharge, the depolarization of glial cells subsequent to potassium uptake may provoke calcium-dependent neurotransmitter release. On the other hand, glial membranes possess receptors for a variety of transmitters, among which are those involved in behavioral state control, such as glutamate (non-NMDA), $GABA_A$, acetylcholine, and norepinephrine. Glutamate, the ubiquitous transmitter in the central nervous system, depolarizes glia through receptors similar to those on neurons and triggers slow calcium-dependent oscillations (as seen in brain slices containing cortical and hippocampal astrocytes). Calcium waves may travel through gap junctions between astrocytes and may unidirectionally cross glia–neuron gap junctions. Such waves may be crucial for the spread of activities from an epileptic focus, a possibility that is especially important since it has been repeatedly shown that during SW seizures the extracellular calcium is depleted at a level that is incompatible with synaptic transmission.

The participation of glial cells during SW seizures has several consequences. First, in vivo intracellular measurements have shown a persistent increase of glial membrane capacitance, developing in parallel with the progression of the seizure. In contrast, the capacitance of neurons did show stable values. This specific behavior of glia is directly proportional to the strength of the seizure and inversely proportional to the distance of the recorded glial cell from the presumed seizure focus. The glial capacitance

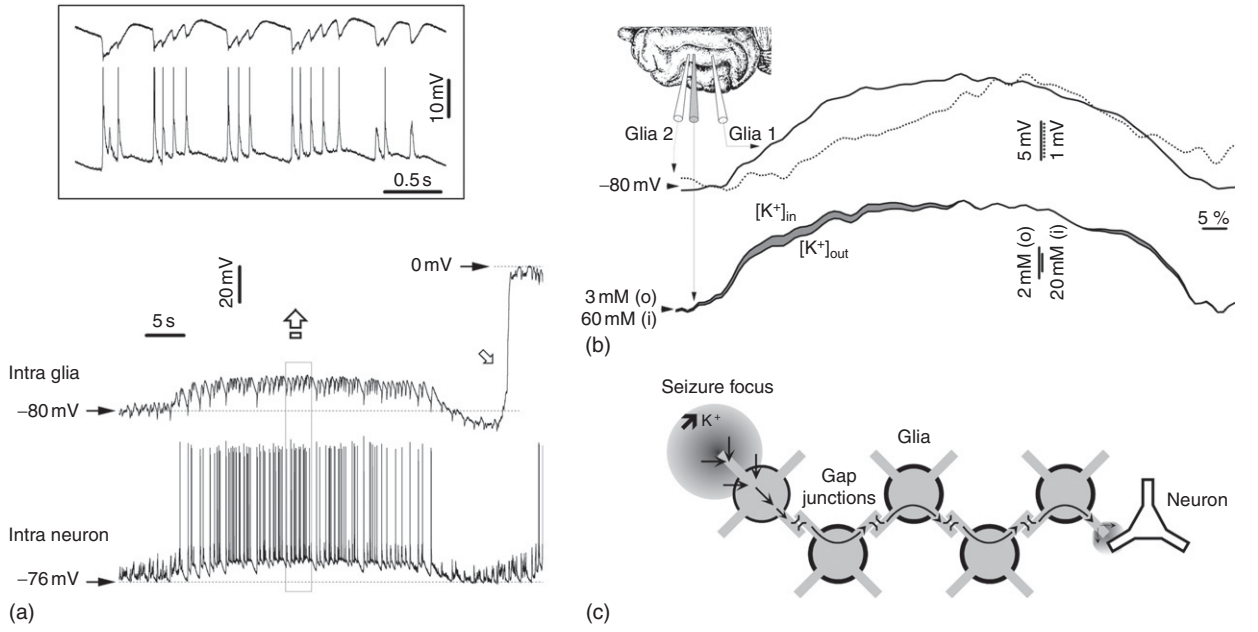


Figure 4 (a) Neuron-glia interaction during SW seizures. Continuous recording containing a double neuron-glia impalement in cortical association area 7. The two electrodes are separated by <1 mm. At the end of the period depicted the microelectrode recording the glial cell was withdrawn from the cell (*oblique open arrowhead*) to show the polarization of the glial membrane. The epochs within the square is expanded above. (b) Propagation of K^+ waves during SW seizures. Dual intraglial recording together with the extracellular potassium concentration $[K^+]_{out}$. The disposition of the recording electrodes in the suprasylvian gyrus is shown in the *inset*. The traces represent the average of 20 normalized seizure envelopes. The *upper* superimposition contains the intracellular seizures in the pair of glial cells expanded at their maximum amplitude (see different voltage calibrations – continuous line for cell 1 and dotted line for cell 2, also corresponding to the envelope traces). From the higher amplitude of the signal, it may be inferred that *cell 1* was closer to the presumed seizure focus. The *lower* panel displays the intra- and extracellular K^+ concentrations superimposed and expanded at their maximum amplitude. The $[K^+]_{in}$ was calculated from the Nernst equilibrium potential in relation with the $[K^+]_{out}$ and the intracellular trace that was recorded closely to the K^+ microelectrode (2). Toward the beginning of the seizure, the estimated $[K^+]_{in}$ rose faster than the $[K^+]_{out}$ (*gray area* between the two traces). (c) Schematic functioning of the spatial buffering during SW seizures. Important increases in the $[K^+]_{out}$ may not be buffered at short distances, in which case the internalized K^+ may travel through the glial syncytium and is externalized at a location with lower $[K^+]_{out}$ values, where it would modulate the activity of nearby neurons. Panel (a) is modified from Amzica F and Steriade M (2000) Neuronal and glial membrane potentials during sleep and paroxysmal oscillations in the neocortex. *The Journal of Neuroscience* 20: 6648–6665; panels (b) and (c) are modified from Amzica F, Massimini M, and Manfridi A (2002) Spatial buffering during slow and paroxysmal sleep oscillations in cortical networks of glial cells in vivo. *Journal of Neuroscience* 22: 1042–1053, with permission from the Society for Neuroscience.

modifications may be attributed to swelling and/or to the modulation of gap junction conductances. The latter process may be due to the fact that the steady glial depolarization observed during SW seizures would induce intracellular alkalinization, which results in increased permeability of gap junctions. The swelling of glial cells during SW seizures could result from the net uptake of K^+ to raise the intracellular osmotic pressure, and is consistent with previous reports about the shrinkage of the extracellular space during periods of increased extracellular potassium. As a direct consequence, glial swelling and shrinkage of the extracellular space would facilitate intercellular ephaptic transmission. We have recently measured such phenomena, reflected as negative intraglial potentials that develop during SW seizures. Ephaptic transmission may become an important factor in the propagation of SW seizures, especially in association with impaired action potential generation due to the high amplitude of the PDSs, and with sustained depletion of extracellular calcium concentration that directly affects synaptic transmission.

It appears, therefore, that the tight homeostasis of the extracellular potassium plays a pivotal role in keeping the normal brain oscillations from evolving into paroxysmal events. Glial cells normally fulfill this function. However, once the potassium regulation is compromised, these same cells may participate in the propagation/generalization of interictal activities. Several studies have drawn the attention to the fact that glial dysfunction is intimately associated with the genesis of epileptic behavior. Among other factors, the opening of the blood–brain barrier and the ensuing extravasation of blood proteins entrain a cascade of events that begin with astrocytic activation, down-regulation of inward-rectifying potassium (Kir 4.1) channels in astrocytes, and impaired interstitial potassium buffering – ultimately leading to the genesis of epileptogenic foci.

Conclusion and Directions for Future Research

Such mechanisms are probably at work, regardless of the vigilance state of the epileptic patient. However, it is clear that the presence of an already widely synchronized state – such as sleep – requires a lesser dysfunction of the buffering properties of glia in order to initiate a seizure. This widespread synchrony during sleep may explain why SW paroxysms are so much more common during sleep than during alert/desynchronized states.

Several aspects still require clarification: We still do not know many of the factors that precipitate the transition from a quiet slow-wave sleep state into an epileptic episode. It is conceivable that all normal sleepers develop some form of epileptiform sleep discharge, without really becoming epileptic. Those suffering from an epileptic syndrome might simply be those whose paroxysmal discharge 'overflows' into clinical symptoms. We also do not know whether the generalization of epileptic seizures might, despite its often devastating expression, have beneficial effects, in that this generalization distributes over large cerebral territories the pressure that would otherwise harm more severely local neurons (at the point of seizure origin). Our understanding of the involvement of the blood–brain barrier in the genesis and spread of seizures is still minimal; it would be interesting to know to what extent blood flow maintains protection or harms the parenchyma – i.e., what the consequence of an open blood–brain barrier is.

See also: **Glia**/**Astrocytes**: Role of Astrocyte Dysfunction in Epilepsy; **Non-Synaptic Mechanisms**: Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Modulation of Neuronal Excitability by Changes in Extracellular Ion Composition; **Sleep**: Brain Mechanisms Linking Epilepsy to Sleep; Epileptic Components of NREM and Antiepileptogenic Components of REM Sleep.

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TEMPORAL LOBE EPILEPSY

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Altered $GABA_A$ Receptor Subunit Composition in Temporal Lobe Epilepsy

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Introduction

γ-Amino butyric acid (GABA) is the principal inhibitory neurotransmitter in the mammalian brain. It acts through two classes of receptors: the $GABA_A$ receptors that are ligand-operated ion channels, and the G-protein-coupled metabotropic $GABA_B$ receptors. GABAergic neurons are ubiquitously distributed and encompass a fundamental role in processing and integration of all brain functions. A great number of differently assembled $GABA_A$ receptors that are differentially distributed throughout the brain have been observed. It has been suggested that dysfunction of the GABAergic system may have a crucial role in the propagation of acute seizures and in the manifestation of epilepsy syndromes. Indeed, blockade of the fast inhibitory $GABA_A$ receptors by bicuculline, pentylenetetrazole, or picrotoxin causes severe motor seizures in experimental animals, and mutant mice lacking the synthetic enzyme for GABA production (glutamate decarboxylase – GAD) or expressing mutations of certain $GABA_A$ receptor subunits are prone to spontaneous epileptic seizures.

$GABA_A$ receptors are central to GABAergic transmission. Their roles are seen not only at the synaptic level, but also in volume transmission, synchronization of neurons, and generation of network oscillations. The multiple molecular forms in which $GABA_A$ receptors are assembled in the membrane (discussed in the following sections) indicate highly diverse physiological properties of these receptors. There is growing evidence that mutations in certain $GABA_A$ receptor subunits can lead to altered brain functions, including epilepsies. Further, it has been proposed that an initial severe seizure event (status epilepticus) may alter the expression profile of individual $GABA_A$ receptor subunits, causing different assembly and thus altered function of $GABA_A$ receptors in certain brain areas. These changes could influence (support or inhibit) epileptogenesis. Acute or recurrent seizures may also influence the sensitivity of $GABA_A$ receptors to anticonvulsant drugs (such as barbiturates or benzodiazepines) known to act through the $GABA_A$ receptor.

Changes in the expression of individual $GABA_A$ receptor subunits in subfields of the hippocampus may reflect altered receptor function in this brain area – a region thought to be involved often in epileptogenesis or drug resistance. The cellular sites at which such changes take place may be critical for their roles in these processes.

Background

The $GABA_A$ receptor is a member of a superfamily of ligand-gated ion channels that also includes the nicotinic

acetylcholine receptor, the 5-hydroxytryptamine type 3 receptor, and the glycine receptor. $GABA_A$ receptors are composed of five subunits forming a chloride channel. So far, a total of six α, four β, three γ, one δ, one ε, one π, one θ, and three ρ subunits of $GABA_A$ receptors have been identified in the mammalian nervous system. This subunit heterogeneity is further increased by alternative exon splicing of the premRNA, which generates two forms of the γ2 subunit (γ2S and γ2L), from one gene, that are differently distributed in the brain. Splice variants have also been detected for other subunits, and subunit homologues have been identified in nonmammalian species.

The majority of native $GABA_A$ receptors, however, appear to be composed of two α, two β, and one γ subunit. A δ subunit, or less frequently, an ε or π subunit, can replace the γ subunit in the $GABA_A$ receptor, whereas the θ subunit may be able to replace a β subunit. A given $GABA_A$ receptor complex may consist of various α and β subunits; for example, a functioning receptor may contain two α1 subunits, two β2 and one γ2; or one α1, one α2, two β2 and one γ2 subunit; or any pair of existing α subunits. Also, the β subunits within a given $GABA_A$ receptor may originate from the same or from different genes. The most abundant subunits in the rodent brain are α1 to α5, β1 to β3, γ2, and δ.

The binding site of GABA to the $GABA_A$ receptor is located at the interface of an α and a β subunit. Binding of benzodiazepines occurs at the interface of an α and a γ subunit. Benzodiazepines bind with high affinity to receptors containing α1, and with somewhat lower affinity to those containing an α2, α3, or an α5 subunit (but not to α4 or α6 containing receptors), and only the γ2 (no other γ subunit or δ) subunit can mediate the benzodiazepine-induced increase in affinity to GABA. Barbiturates exert their action after binding at the chloride channel pore, and seem to bind also to α4, α6, and/or δ subunit containing receptors. $GABA_A$ receptors containing only α and β subunits have been described in hippocampal pyramidal cells. They do not respond to benzodiazepines but are highly sensitive to Zn^{2+} ions.

The subunit composition of $GABA_A$ receptors also critically determines their physiological and pharmacological functions. GABA exerts its action by opening a chloride channel. Benzodiazepines and barbiturates can increase the affinity for GABA, thus increasing the frequency or duration of channel opening, respectively. The direction of the chloride flux is dependent on the chloride gradient at the cell membrane. In the mature brain, the chloride transporters (such as the potassium-dependent chloride transporter KCC2) extrude chloride from the cell, allowing in an *inward* chloride flux and a hyperpolarization after channel opening. In the immature brain, the expression level of KCC2 is low; instead, a sodium–potassium-dependent cotransporter NKCC1 is expressed that transports chloride into the neuron. The resulting high intracellular chloride concentration drives an outward chloride flux and depolarization of the cell upon stimulation of $GABA_A$ receptors. Altered chloride transporter expression or function has been associated with genetic forms of epilepsy. It has also been proposed that expression of KCC2 may be altered in certain neurons and lead to impaired inhibition.

In the adult mammalian CNS, synaptic transmission through GABA synapses is characterized by fast and precisely timed inhibitory activity in the form of inhibitory postsynaptic currents that mediate phasic inhibition. A second form of inhibitory transmission, resulting from GABA diffusion out of the synaptic cleft and activating $GABA_A$ receptors located outside the synapse, has been characterized recently and is referred to as tonic inhibition. Tonic conductance has been found in a large variety of principal neurons and interneurons, including those found in the cerebellum, cortex, hippocampus, thalamus, and spinal cord. Depending on the brain region, certain subunits such as α4, α5, and δ preferentially constitute such extrasynaptic receptors and mediate tonic inhibition.

Methods

Using histochemical methods, we investigated changes in the expression of $GABA_A$ receptor subunits after systemic application of kainic acid (KA; 10 mg kg^{-1}, i.p.) in rats. In this model, an initial status epilepticus leads, after a 'latent phase,' to epilepsy characterized by recurrent spontaneous seizure activity. In order to differentiate changes during the acute KA-induced status epilepticus from changes during epileptogenesis (i.e., during the latent phase) and in chronic epilepsy, we investigated $GABA_A$ receptor subunit expression at 24 h, 8 days, and 1–3 months after the initial kainate-induced status epilepticus. Expression was studied at the mRNA and protein level using in situ hybridization and immunohistochemistry, respectively. For in situ hybridization, we used 20 μm thick cryotome-cut sections from snap frozen brains. We incubated them with synthetic oligo-DNA probes labeled by reaction with ^{35}S-dATP in presence of terminal deoxynucleotidyl transferase. For immunohistochemistry, we used 40 μm thick sections from 4% paraformaldehyde fixed brains and processed them by the indirect peroxidase method. Antibodies specific for individual $GABA_A$ receptor subunits were supplied by Prof. Werner Sieghart, Brain Research Center, Medical University Vienna. For semiquantitative evaluation of changes in the expression of individual subunits, we performed densitometry on film autoradiographs and on immunolabeled brain sections.

Studies in human brain tissue were done in collaboration with Prof. Thomas Czech, Department of Neurosurgery, Medical University of Vienna, who supplied us with specimens collected from TLE patients during routine surgery (after approval of the Institutional board of the

Medical University of Vienna). The specimens were either snap-frozen or immersion fixed in 4% paraformaldehyde. For in situ hybridization, the method described earlier was used, applying probes complementary to the respective human RNA sequences. For immunohistochemistry in the human specimens, we performed the slightly more sensitive avidin–biotin method (ABC-kit by Boehringer, Ingelheim, Germany) after treating the sections with target retrieval solution; the same antibodies used for investigating rat brains were also applied for the human tissue.

Results

Studies in Animal Models of Epilepsy

In the studies using the KA rat model of temporal lobe epilepsy (TLE), we observed marked losses in $GABA_A$ receptors that were obviously related to neuronal cell losses (mainly in sectors CA1 and CA3 of the Ammon's horn, hilar interneurons, and the subiculum). This result is consistent with losses in $GABA_A$ receptor binding sites in the whole hippocampus. These prominent neurodegeneration-related changes suggest marked alterations in $GABA_A$ mediated transmission. In addition, there were also significant changes in $GABA_A$ receptor subunit expression in the molecular layer of the dentate gyrus – a structure that remains relatively intact in this animal model. Most conspicuous in this region was a rapid decrease in subunit δ (Figure 1). Indeed, downregulation of subunit δ was a consistent observation in most animal models, including the kindling model, recurrent seizures induced by electrical stimulation, and the mouse model of pilocarpine-induced status epilepticus and epileptogenesis. Interestingly, expression of the α4 subunit (a frequent partner of the δ subunit in the $GABA_A$ receptor complex) is not reduced but increased in the KA model of the rat (Figure 1) and in the pilocarpine model of the mouse. We also observed a clear-cut reduction in the expression of subunit α5 in the dentate gyrus, as well as in the Ammon's horn – although these latter changes were obscured by the severe neurodegeneration in this area after kainate-induced status epilepticus. Houser *et al.* reported less severe damage of the pyramidal cell layer in the mouse pilocarpine model, and were able to demonstrate a reduction in the expression of subunit α5. They also discussed the possibility that these observed decreases in δ subunit expression (and possibly in α5 subunit expression) are associated with reduced tonic inhibition in the epileptic mice.

In contrast to receptors containing the δ subunit, receptors containing the γ2 subunit are primarily associated with synaptic or phasic inhibition. In addition, the γ2 subunit mediates the sensitivity of the receptor to benzodiazepines, and the γ2 subunit is importantly involved in the targeting of the $GABA_A$ receptor complex to the cell membrane. In our initial experiments, we observed a marked and rapid – but transient – reduction in γ2 mRNA levels in the dentate gyrus (6 h) after kainate injection. In the pyramidal layer, a persistent reduction (presumably related to cell damage) was seen. Using immunohistochemistry, we observed conspicuous labeling for subunit γ2-immunoreactivity of dendrites of pyramidal cells and interneurons, lasting up to 1 month. We interpreted these data as a

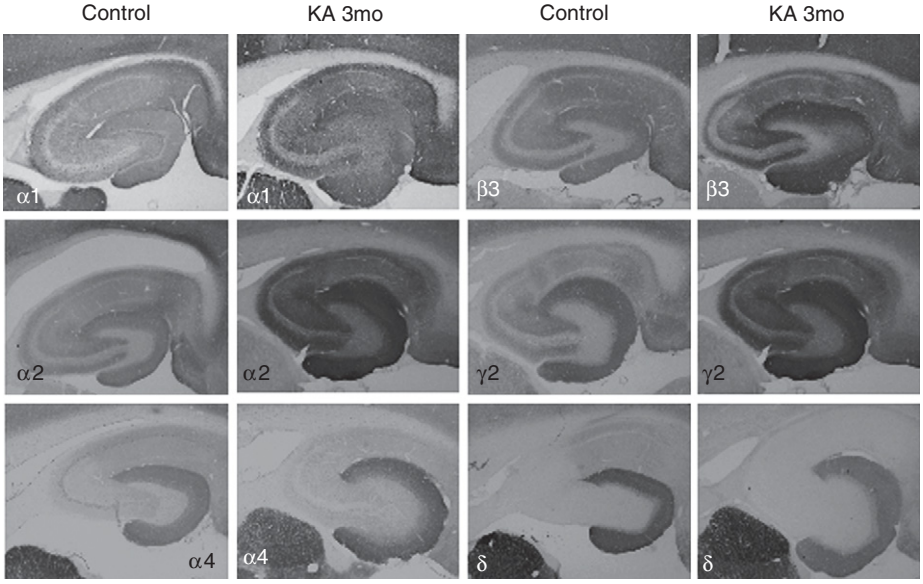


Figure 1 Changes in immunoreactivites of $GABA_A$ receptor subunits in rats with spontaneous seizures, 3 months after status epilepticus induced by injection of kainic acid. Note the decrease in δ subunit immunoreactivity, contrasted by increases in subunits α2, α4, β3, and γ2. Apparent increase in subunit α1 immunoreactivity in the epileptic rats is mainly present in interneurons.

reflection of overexpression of this subunit. Such an increase in γ2-immunoreactivity can also be seen in the dentate molecular layer up to 4 months after KA injection (Figure 1).

Recent studies by Kapur *et al.* and Wasterlain *et al.*, have demonstrated rapid internalization of γ2 subunit protein following status epilepticus. Using immunofluorescence, they demonstrated as early as 1 h after seizure induction, a translocation of subunit γ2-immunoreactivity from the cell membrane to the cytoplasm of the respective neurons. Functionally, this translocation may have considerable consequences. First, it may result in receptors devoid of γ2 subunit. Such receptors (if active at all in vivo) would have strongly altered physiological and pharmacological functions. As mentioned earlier, receptors consisting only of α and β subunits have high sensitivity to the ion Zn^{2+} that can be released from mossy fibers; this sensitivity to mossy fiber-released zinc could result in impaired GABA-mediated inhibition. Receptors lacking the γ2 subunit are also resistant to treatment with benzodiazepines. And, since the γ2 subunit is important for anchoring the $GABA_A$ receptor complex in the cell membrane, internalization of the subunit γ2 may also limit the survival of the whole receptor in the cell membrane. This translocation phenomenon does not affect all $GABA_A$ receptors and/or is not permanent, since even 3 month after kainate-induced status epilepticus we observed high levels of γ2-immunoreactivty in granule cells and pyramidal cells (Figure 1). Consistent with these findings, Houser *et al.* also observed an increase in γ2-immunoreactivty in the chronic phase after pilocarpine-induced status epilepticus in the mouse.

In the normal molecular layer of the dentate gyrus of the rat, the α2 subunit is thought to be the most prevalent. The α1 subunit appears to be preferentially expressed in interneurons of the hippocampus. Expression levels of subunit α4 appear to be low in the rat hippocampus (compared with the thalamus for example), but more abundant in the dentate molecular layer than in the strata oriens and radiatum of the hippocampus proper. In contrast to the human hippocampus, there is no α3 subunit expressed in rat dentate granule cells, and little in interneurons. Subunit α5 is more extensively expressed in pyramidal cells than in granule cells of the rat. Again, this pattern is somewhat different from the expression pattern in humans, where equally high levels of subunit can be seen in dendrites of granule cells and pyramidal cells. Expression patterns of individual $GABA_A$ receptor subunits are also slightly different in the mouse.

As noted earlier, subunits α4 and α5 are presumably related to tonic inhibition. The subunit α4, expressed only slightly in normal brain, is slightly upregulated and subunit α5 downregulated in the dentate gyrus, notable at the later intervals after kainate injection. At this time, in situ hybridization and immunohistochemistry also show increased expression of subunits α1 and α2 in rats treated with KA or after electrically induced status epilepticus.

All three β subunits are expressed in principal cell layers of the rat hippocampus. Expression of subunit β3 appears to be the strongest, that of β1 weakest. Subunit β2 is even more abundant in hippocampal interneurons (coexistent with subunit α1 and γ2, less with δ). In most TLE models, notably in the KA model, there is some upregulation of all three β subunits (notably of β2 and β3).

Taken together, these data indicate increased expression of $GABA_A$ receptors in the dentate gyrus following kainate-induced status epilepticus; however, there is a clear shift from receptors associated with tonic inhibition to receptors associated with phasic inhibition. Increased concentrations of $GABA_A$ receptors in the dentate hilus were reported more than 20 years ago (using ligands for either GABA or benzodiazepine-binding sites) – even in the face of neurodegeneration related receptor losses in Ammon's horn). Why subunits related to phasic inhibition are preferentially lost remains to be determined. One possibility is that GABA produced at high concentrations in granule cells may be released extrasynaptically onto granule cell dendrites, and may then contribute to downregulation of extrasynaptic receptors.

Studies in the Human TLE Hippocampus

There are only a few studies on the expression of $GABA_A$ receptor subunits in specimens removed by surgery in TLE patients. Difficulties experienced in such studies include the availability of valid antibodies (specifically detecting the human subunits) and difficulties in comparing fresh (surgically removed) tissue with post mortem control tissue (in which mRNA and proteins may be degraded to varying extents). The first extensive study was published by Loup *et al.*, who showed upregulation of subunits α1, α2, β2/3, and γ2 subunits in granule cells and granule cell dendrites, downregulation of these subunits in the subgranular zone, and loss of α1 subunit immunoreactivity in interneurons of the hippocampus proper. Using in situ hybridization and immunohistochemistry, we also observed increases in all three β subunits, notably β1 and β3, in the molecular layer, the hippocampus proper and subiculum. In the subiculum (which is highly preserved in human TLE), we observed increases in subunits α3 and γ2, but losses in subunits α1, α3, and γ2 in Ammon's horn (Figure 2).

Taken together, these data indicate that in human TLE there are marked changes in the expression and assembly of $GABA_A$ receptors, suggesting remodeling of GABAergic transmission. Subfields of the hippocampus that are less vulnerable to seizure-induced neurodegeneration, such as the subiculum and dentate granule cell layer, display upregulation of various subunits, indicating

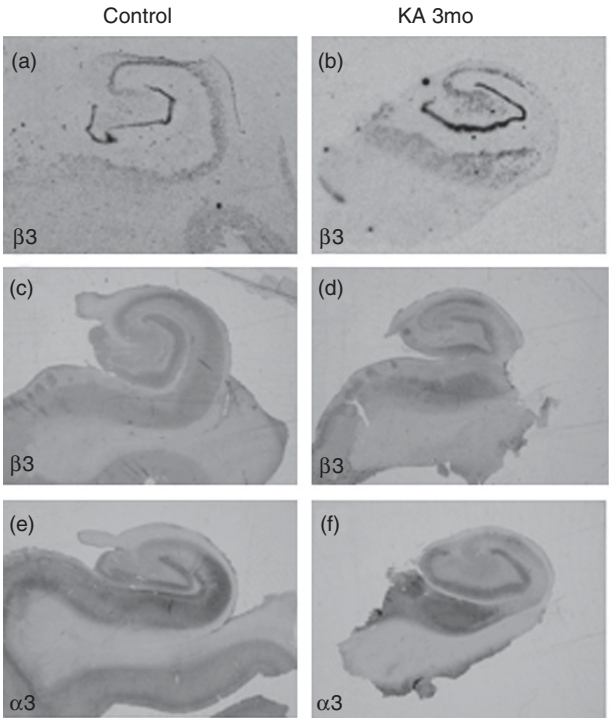


Figure 2 Changes in immunoreactivites of $GABA_A$ receptor subunits in TLE patients. Note the increased expression of subunit β3 mRNA (b vs. a) and immunoreactivity (d vs. c) in the dentate molecular layer and in the subiculum and the increase in α3 immunoreactivity in the subiculum (f vs. e).

facilitation of GABAergic transmission. Striking and difficult to explain is the especially pronounced expression of β subunits – which could result in imbalanced assembly of $GABA_A$ receptors (such as receptors containing three β and two α and no γ subunit, or three β, one α and one γ2 or δ subunit). Studies with recombinant receptors indicate that they would not bind GABA with high affinity or capacity – and if they lacked γ2, they may not be affected by benzodiazepines but would be sensitive to Zn^{2+}. Due to the difficult comparison with postmortem controls, we have so far been unable to obtain evidence for downregulation of subunits δ or α5 in the human tissue, which would (in analogy to the rat) be indicative of reduced extrasynaptic tonic inhibition in the epileptic brain. Appropriate human controls are essential since, as mentioned earlier, the distribution (and thus the function) of certain $GABA_A$ receptor subunits is different in the human hippocampus compared to that in the rat.

Future Goals

It will be crucial to continue work in the human brain, since the physiological properties of single $GABA_A$ receptor may vary much depending on the circuitry in which they are integrated and less critically on their actual subunit composition. Detailed knowledge about the pathology of altered receptor expression will be important for developing appropriate drugs targeting individual $GABA_A$ receptor subtypes (e.g., acting through the benzodiazepine binding site). A limitation of our studies is that although we have learned something about changes in the $GABA_A$ receptor subunit distribution, we have yet to determine the details about changes in actual receptor composition. We are currently performing neurochemical studies, using subtractive purification methods in microdissected hippocampal subfields from KA rats, to approach this question.

Acknowledgements

The work was supported by the Austrian Science Fund and by the EC contract number LSH-CT-2006–037315 (EPICURE) FP6 – Thematic priority LIFESCIHEALTH.

See also: **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; Pathophysiology of Status Epilepticus; Synchrony Through GABAergic Inhibition; **Interneurons**: Plasticity and Reorganization of GABA Neurons in Epilepsy; **Neurotransmitter Function**: Synaptic Activities and Transmitter Function: Receptor Trafficking.

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Functional Genomic Analysis in Temporal Lobe Epilepsy

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Introduction

Milestone progress in large-scale genetic characterization, such as the completion of the Human Genome Project, has opened free online access to virtually all genetic data about the human organism. The transcriptional regulation and interaction of genes raises the challenging issue of functional genomics – and the identification of potential drug targets in human diseases. Not surprisingly, the high-throughput analysis of biological systems by the use of genome-wide expression profiling has also become a fundamental key technology in epilepsy research. Different kinds of array designs have been developed, including DNA microarrays and RNA oligonucleotide microarrays. In addition, recent advances have opened new avenues for establishing protein or antibody microarrays, thus bridging the gap toward post-translational approaches – i.e., proteomics.

Functional genomics deals with the presumption that physiological and pathogenic conditions are caused by, and cause, specific transcriptional alterations. To carry out such studies, access to living tissue without RNA degradation is pivotal. And is frequently a limiting step in the experimental setup of functional genomics studies. This issue is of special importance in the study of many brain disorders, such as epilepsy. For obvious reasons, the availability of human brain tissue is restricted to autopsy samples or specimens obtained by neurosurgical treatment strategies (in pharmacoresistant focal epilepsies). Alternatively, animal models provide useful tools to elucidate those processes that occur before chronic seizure activity appears – i.e., in the period termed 'epileptogenesis.' The present article will offer an overview of the main trends in microarray technologies and their application in epilepsy research. Pros and cons will be highlighted by focusing on studies in temporal lobe epilepsy (TLE), a major cause of focal epilepsies in children and young adults.

The Microarray Experiment

Expression profiling based on microarrays has evolved as a valuable approach to determine transcript patterns in anatomically heterogeneous structures such as the brain, and to monitor genomic responses in complex diseases. A growing number of customized cDNA microarrays and oligonucleotide arrays, also designated as 'chips,' has dramatically accelerated the throughput of gene expression analyses. The core idea of large-scale 'chip' approaches is a 'nonhypothesis'-driven attempt to find gene transcripts that otherwise would not have been expected to play a physiological or pathogenic role. Frequent strategies for chip analysis include comparisons between identified cell populations, brain regions, or groups of neuropathologically characterized individuals.

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Background

Expression Profiling in Temporal Lobe Epilepsy

Epilepsies are among the most frequent CNS disorders, affecting ~1.5% of the population worldwide. In TLE, the seizure origin typically involves the hippocampal formation, a structure located in the mesial temporal lobe. Many TLE patients develop pharmacoresistance (i.e., seizures can not be controlled by antiepileptic drugs), and therefore have to undergo surgical treatment strategies.

Hippocampal biopsies obtained during surgery from pharmacoresistant patients reveal two major neuropathological entities. The most frequent finding, in approximately two-third of the patients, is Ammon's horn sclerosis (AHS). Histopathologically, AHS hippocampi exhibit substantial neuronal cell loss in the areas CA1, CA3 and CA4, whereas the CA2 subfield and dentate gyrus granule cells (DG) appear less vulnerable to seizure-induced neurodegeneration. Those areas strongly affected by neuronal cell loss also exhibit pronounced fibrillary astrogliosis and, thereby, sclerosis. Further alterations in AHS hippocampi include dispersion and focal bilamination of the dentate granule cell layer, persistence of Cajal–Retzius-like interneurons, and mossy fiber sprouting. In approximately one-third of pharmacoresistant patients, TLE is associated with focal lesions in the temporal lobe. These lesions include highly differentiated malformations as well as benign glial and glioneuronal neoplasms, such as oligodendrogliomas, pilocytic astrocytomas, pleomorphic xanthoastrocytomas (PXA), gangliogliomas, dysembryoplastic neuroepithelial tumors (DNTs) – referred to as long-term epilepsy associated tumors (LEAT). Within this broad spectrum, two major groups – represented by gangliogliomas and focal cortical dysplasias – are of major interest because of their rather frequent occurrence in the subpopulation of lesion-associated TLE.

Microarray Technology

Different expression array technologies have been developed. While cDNA nylon arrays rely on radioactively labeled probes and semiquantitative phosphorimaging analysis, large-scale oligonucleotide and glass microscope slide DNA arrays are hybridized with fluorescent probes and analyzed by specific detection systems. In the following paragraphs, we will focus on a commercial oligonucleotide microarray procedure (based on the Affymetrix platform) that has been successfully used in our laboratory. For example, modern gene chips for rat RNA provide comprehensive coverage of the transcribed rat genome on a single array with 30 000 transcripts and variants from over 28 000 well-substantiated rat genes. Microarray solutions are supplemented by protocols and reagents for straight-forwarded sample cryopreservation, mRNA isolation, probe labeling, hybridization, high-resolution scanning, and data mining procedures.

Methods

Overview

The Affymetrix microarrays combine more than a million oligonucleotide probes on each 'chip.' The principle of the procedure is to load the chip with the whole transcriptome of a tissue- or cell-type sample. Biotin-labeled RNA fragments are hybridized to the probe array and stained with streptavidin phycoerythrin conjugates. The signal intensity fluorescent images produced during chip hybridizations are read using the scanning systems and digitalized into a cell file (cel) data format using GeneChip operating software of the manufacturer. The signal intensity is proportional to the bound target at each location on the probe array. To obtain biotin-labeled RNA fragments, preparatory procedures on RNA samples include the isolation and cleanup of total-RNA, the reverse transcription (RT) of the mRNA into cDNA, and then in vitro transcription into cRNA using biotin-conjugated nucleotides that can be targeted by the streptavidin phycoerythrin conjugate after hybridization. The cRNA is fragmented before hybridization, as each transcript is represented by several probes on the chip. Hybridization to the probes is designed to either match or mismatch. The signal proportions of matching and mismatching probes are then used to calculate if, and to what extent, a transcript may be present or not.

Experimental Setup

Microarray technology is not a simple genetic 'cash cow.' As in almost any scientific experiment, the careful selection of appropriate samples and controls (or matched pairs of samples) is required for valid and reliable findings. The statistical menace of false-positive findings within a plethora of transcriptional data is one of the biggest obstacles in applications of this technology. To diminish the false-positive discovery rate as much as possible, the a priori inclusion of sufficient replicates is mandatory.

For obvious reasons, access to living brain tissue is frequently restricted. But even if access is possible, there are additional challenges. Relevant transcriptional alterations in distinct cell types may be obscured because of tissue heterogeneity within the sample. Other sources of heterogeneity are inevitable, including (in humans) interindividual genetic background. Another limitation, especially of human surgical specimens, is the strong mRNA degradation effects because of local hypoxic

periods or mechanical stress during tissue resection. Nonetheless, microarray analyses have been successfully established in human brain (e.g., hippocampal) specimens.

Data Mining

Microarray technology allows the experimenter to profile gene expression of thousands of genes at the same time. However, the number of relevant candidate genes that can be further characterized in subsequent validation studies is rather small. This discrepancy between large-scale analysis and the need to focus on distinct candidates makes it necessary to 'filter' the genes of interest. As already indicated earlier, the statistical menace of false-positive findings within a plethora of transcriptional data is a major obstacle. For example, when dealing with 10 000 transcripts and using a *t*-test between two groups with a cut-off of $p < 0.05$, about 500 genes would be expected to pass this filter just by chance. The false-positive discovery rate can be diminished by increasing the stringency of the filter – i.e., applying lower cut-off values. However, this strategy increases the rate of discarded false-negative genes. This higher discard rate can be unfortunate, since even somewhat less reliable genes – i.e., those low abundant transcripts, or high abundant genes with minor but stable expression alterations – may be of scientific interest (exert relevant biological effects).

To find the discriminating genes between two or more conditions, a sequential filtering of the data can be performed. For example, genes of interest can be required to meet the following criteria: (1) perfect match/mismatch ratio to be called present or marginally present, calculated by the analysis software tool based on probe hybridization signals the should match (i.e., perfect match (PM)) and those that should not match (i.e., mismatch probes (MM)); (2) valuable abundance (higher than the median gene expression) in at least one of two conditions; (3) a fold-change of at least two; and (4) interindividual homogeneity measured, e.g., by a two-sided *t*-test with a cut-off of $p < 0.05$ and multiple-testing correction.

Chip to chip variability of signal intensity because of sample processing variability must be adjusted by various normalization strategies. Per gene normalization can be performed with control replicates as reference samples. However, one must keep in mind that mathematical normalization strategies cannot compensate for intrinsic biological variability between individual specimens, a factor in virtually all expression profiling studies. Introduction of certain reference or housekeeping genes enables the investigator to carry out a systematic comparison between different sets of experiments, including complex estimations such as cluster analysis. However, the identification of reliable housekeeping genes may considerably change with experimental paradigms and always needs careful attention.

Complex, sporadic brain disorders such as TLE represent a major challenge for functional genomics, since a variety of molecular pathways and genes are involved and differentially regulated during the long medical history of the disease. Large-scale expression arrays have proven to be efficient tools for monitoring gene expression and open new avenues for the identification of pathogenesis-related molecules and mechanisms of different diseases. Nonetheless, it will be an important future challenge to carry out such analyses in homogeneous patient samples, standardized for distinct pathogenetic aspects (e.g., type of seizures), onset of the disease, and resistance to specific modes of pharmacotherapy.

Validation of Expression Data

The study of gene expression using microarray technology has gained considerable acceptance in the research community. However, gene expression data based on chips must be independently quantified. The most common method is quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), a method that enables the investigation of mRNA levels even from single cells. Furthermore, it allows absolute or relative quantitation of a target transcript. For relative mRNA quantitation, a suitable reference gene is essential as an internal control. Ideally, it should show a constant and high level of expression, independent of experimental conditions. After normalization of the target signals to the reference signal, comparisons between different tissue types, across various developmental stages, and between control and disease tissue can be performed. In our laboratory, validation of the microarray-based gene expression profiling is performed by a quantitative real-time RT-PCR (TaqMan®) assay using the SYBR Green PCR detection system in a three-step RT-PCR amplification cycle. Duplicate reactions are carried out for each gene. The SYBR Green fluorescence signal is measured in each PCR cycle. The comparison of transcription levels is finally calculated by the ΔΔCt-method.

Recent Results

Microarray Analyses in Human Specimens

Hippocampal biopsy specimens from patients with pharmacoresistant TLE have been used for functional genomics. However, these samples only reflect the chronic stage of the disease; this situation may explain why such studies are rarely compared with expression analyses in animal models. When dealing with human specimens, the first issue is to obtain a sufficient yield of mRNA – a potential problem since there is a need for high amounts of starting material. Complex tissue composition (such as is the case for the brain), with expression alterations in only one cell type, may obscure biologically relevant

differences. Laser capture microdissection can be used to obtain pure cell populations, but aggravates the difficulty of obtaining sufficient mRNA amounts. Nonetheless, by introducing amplification steps into the sample preparation protocols, it may be possible to obtain sufficient mRNA for microarray procedures. Use of snap-frozen tissue sections, labeled with standard histochemical stains, is recommended for RNA extraction. This strategy has been further refined and applied to studies using laser microdissection of tissue from immunohistochemically labeled cells after RT of mRNA into cDNA (because DNA is more resistant against degradation during immunohistochemistry than is mRNA). Additional applications, using DNA isolated from microdissected tissue, include analysis of loss of heterozygosity (LOH) or screening for somatic mutations or mosaicisms in distinct cell types. Certainly, tissue heterogeneity on the cellular level must be taken into account in expression studies, but the difficulty may be solved using a two-step approach, with cell-type specific analyses after initial microarray experiments.

Another problem is the need for appropriate control CNS tissue for expression analysis in TLE. For obvious reasons, appropriate age-matched nonepileptic control specimens – e.g., resected hippocampi without any histopathological abnormalities – are virtually impossible to obtain. Cases of nonepileptic tumor patients with resection of a noninfiltrating tumor mass in the close vicinity of the hippocampus rarely occur, so are not feasible for systematic experimental design with large case numbers.

We have used a two-step strategy to study gene expression in complex human CNS disease by combining a cDNA-array-based expression profiling procedure with subsequent real-time quantitative RT-PCR analysis of gene expression levels in histologically specified and laser-microdissected cells. In this setting, epileptic human hippocampi were compared with control biopsy samples from nonepileptic tumor patients, and alternatively compared with hippocampi from epilepsy patients without histopathological alterations of the hippocampal formation but perihippocampal focal lesions. We found about 3.55% of all represented genes to be dysregulated, with the highest signal increase in AHS for ataxin-3. Real-time RT-PCR validation experiments confirmed initial microarray results of an upregulation of ataxin-3 and GFAP, and a reduction in calmodulin expression in AHS hippocampal slices. RNA in situ hybridization approaches confirmed the cellular expression pattern of ataxin-3 and correlated well with the results of the quantitative RT-PCR experiments. Ataxin-3 in situ hybridization showed hybridization signals in DG and hippocampal pyramidal neurons, but not in glial cells. These data indicate that hippocampal biopsy specimens of pharmacoresistant TLE patients are suitable for large-scale expression analyses.

Arion and colleagues performed a microarray study of anterolateral temporal cortical specimens of patients with chronic TLE to compare 'spiking' (abnormal) and 'nonspiking' (normal) brain areas. Gene expression profiling revealed a downregulation of multiple GABA system-related genes as well as an upregulation of oligodendrocyte and lipid metabolism transcripts in the spiking samples. Expression level alterations were also found in transcripts related to signaling transduction systems such as the classical MAPK cascade, suggesting that spiking behavior is substantially correlated with distinct transcriptional fingerprints.

In another cDNA microarray experiment, entorhinal cortex was subjected to expression profiling and compared with corresponding lateral temporal neocortex of the same individuals as control tissue. To discard expression alterations due to physiological differences between brain areas, the experimenters required that the initially found genes lacked regional differences in nonepileptic autopsy samples. Six transcipts fulfilled this criterion: serotonin receptor HTR2A, neuropeptide Y receptor type 1, the FHL2 protein associated with the KCNE1 potassium channel subunit and with presenilin-2, and three immune system-related proteins (C3, HLA-DR-gamma, and CD99). The authors suggested that local dysregulation of the neurotransmission and complement systems in the EC is a frequent event in human MTLE.

Animal Models of TLE

An important aspect of TLE pathogenesis is that the disease usually develops in children or young adults. The process of epilepsy development has been termed 'epileptogenesis,' and the underlying process is suggested in the dictum 'seizures beget seizures.' Underlying pathophysiological as well as pathomorphological mechanisms – i.e., cellular hyperexcitability and axonal fiber sprouting – are likely to reflect distinct alterations in gene expression. However, in the latent period of epileptogenesis, human specimens are not available for functional genome analysis. In this time window, molecular mechanisms of epileptogenesis can only be elucidated using animal models of TLE. The complexity of histopathological alterations in the affected structure of the mesial temporal lobe, the hippocampus, suggests expression alterations in various genetic and molecular signaling pathways specifically involved in key aspects of human epileptogenesis, particularly those expression changes related to synaptic and axonal reorganization resulting in hyperexcitability. There is growing interest in the question of whether onset of recurrent chronic epileptic seizures may be prevented by pharmacological treatment during the latent stage of epileptogenesis. Pathogenic or course-modifying gene expression patterns may provide candidate structures for the design of innovative therapeutic targets.

Currently used TLE animal models can be separated into two major groups: induction of status epilepticus (SE)

by application of an excitotoxic compound (kainic acid, pilocarpine) followed by development of a spontaneous epilepsy condition; and electrically stimulated chronic recurrent seizures referred to as kindling-induced TLE. In the kainic acid and pilocarpine models, segmental neuronal cell loss and astrogliosis of hippocampi intriguingly reflect human AHS. Further, neuropathological findings in lesion-associated TLE are consistent with hippocampi of animals exhibiting chronic seizures after subconvulsive electrical kindling of the amygdala or tractus perforans.

Beside electrical and excitotoxic compound elicitation of SE, other models without induction of SE have also been investigated. One such model focuses on mimicking prolonged febrile seizures in young children. Since many patients with AHS have a history of prolonged febrile seizures during early childhood, an etiological role for these seizures in the development of TLE has been suggested. Although neuronal damage induced by febrile seizures is rather mild, recent findings support an impact of experimental prolonged febrile seizures on structural and molecular changes resulting in hippocampal hyperexcitability, including reduced seizure threshold to convulsive agents.

A common important feature of all experimental TLE models is the possibility to monitor expression dynamics from the 'beginning' of epileptogenesis (i.e., from the time of the epileptogenic insult). In a recent study, we have used oligonucleotide microarrays to carry out expression profiling in vulnerable hippocampal CA1 neurons versus resistant DG at distinct stages of epileptogenesis in the pilocarpine TLE model. An episode of SE, induced by application of pilocarpine to rats, results in chronic TLE after a seizure-free latency interval. We showed that on day 3 following SE, mechanisms of cellular stress and injury as well as inflammatory responses predominated, followed by alterations of the associated transcription factors. The latter changes were consistent with cytoskeletal changes and synaptic reorganization processes seen at 14 days after SE. Finally, the stage of chronic recurrent seizure activity was characterized particularly by expression changes of genes involved in neurotransmission.

The observation of shared features of nervous system development and epileptogenesis has led Elliott and colleagues to hypothesize common molecular mechanisms in both states. And indeed, DNA microarray analysis in the dentate gyrus identified similar patterns of regulation during development and epileptogenesis. Common spatial and temporal gene expression patterns were found (i.e., several genes were either up- or downregulated during both processes). Functionally, many genes were implicated in cell morphology and axon outgrowth or cellular proliferation and fate determination. The relatively high density of plasticity-related genes supported the concept that nervous system development and epileptogenesis may share common mechanisms. Another study focused on expression changes after kainic acid stimulation (activation of non-*N*-methyl-D-aspartate (NMDA) glutamate receptors) resulting in seizures, neurodegeneration, and remodeling processes. The gene profile revealed 36 novel genes that mainly have roles in transcription, and to a lesser extent have roles in cell death, extracellular matrix remodeling, cell cycle progression, neuroprotection, angiogenesis, and synaptic signaling.

Based on the observation that kainate acid-induced SE in immature rats causes neither cell death nor recurrent spontaneous seizures, another microarray analysis was performed to elucidate the mechanisms of these distinct responses with respect to transcriptional changes in neuropeptides following SE. Intriguingly, neuropeptides were differentially regulated by seizures in both immature (P15) and mature (P30) hippocampi. However, the changes were more pronounced at P30 compared with the changes at P15, not only for neuropeptides with convulsive properties but also for those with neuroprotective properties. The authors suggested that distinct neuropeptides might play a neuroprotective role in immature rats by preventing neuronal loss after seizure in adults.

Pitkänen and colleagues have analyzed epileptogenesis-related expression profiles in hippocampal and temporal lobe specimens after electrically induced TLE. Many of the differentially expressed genes were associated with signal transduction, synaptic and axonal plasticity, and cellular metabolism. Recently, first evidence has been found for specific gene expression signatures in response to pharmacotreatment in experimental TLE. Levetiracetam (LEV) treatment was shown to induce substantial effects on gene expression. Several novel genes, as well as many epilepsy-related genes, such as neuropeptide Y, thyrotropin-releasing hormone (TRH), and glial fibrillary acidic protein (GFAP), were identified from the gene chip experiments and confirmed to be upregulated by kindling and partially normalized by LEV treatment. Moreover, some expression changes correlated with the kindled phenotype. Such studies are promising steps towards a better comprehension of the genetic background of susceptibility for pharmacotreatment and pharmacoresistance.

Comparison of Experimental and Human Data

The studies described earlier have addressed transcriptional alterations based on large-scale microarray analyses in different animal models of TLE, with different emphases on distinct spatial or temporal alterations. While the animal models allow for better technical processing and the possibility of analyzing epileptogenesis, it is unclear whether these model findings can be transferred to human conditions. To address this question, comparisons of genomic responses between human and experimental TLE were carried out, with a focus on relationships involving 3409 rat genes and 4126 human

genes. Correlation coefficients of the corresponding genes were in the range of 0.4, but rose to 0.6 when the interspecies comparison concentrated on the expression ratios between DG and CA1 regions of each species. The highest congruency was detected between the chronic epileptic stage in experimental epilepsy and chronic human AHS. In contrast, markedly less codifferentiated gene ratios were observed between human AHS and samples from rats at acute or intermediate stages after SE. Remarkable similarity in gene expression profiles were associated with key cellular functions, such as cell–matrix interaction, neuronal growth, and signaling. Thus, corresponding microarray experiments suggest that changes observed in experimental TLE show a valuable overlap with findings in human disease. Nonetheless, further studies are needed to address the question of whether these genes contribute to the pathogenesis of human TLE.

Future Directions

Recent expression profiling studies in hippocampi of pharmacoresistant TLE patients have yielded results that reflect significant variability between individual specimens. It will be important to carry out such analyses in homogeneous patient samples, standardized for distinct pathogenic factors – e.g., type of seizures, onset of the disease, and resistance to specific modes of pharmacotherapy. Highly structured dynamic expression databases have been developed to cope with the wealth of expression profiling data. These platforms support the integration of expression and clinical data, as well as the automatic exchange and comparison of data from different groups. An additional challenge will be the correlation of RNA expression and proteomics data. Future efforts will need to focus on functional relevance beyond transcription profiling. Novel molecular biological tools, such as small interference RNA technology, may allow the development of high-throughput strategies to functionally validate gene expression profiles.

See also: **Epileptogenesis**: Patterns of Gene Expression during Epileptogenesis: Micro-Array Studies in Rats; **GABA**: $GABA_A$ Receptor Plasticity in Epileptogenesis; **Plasticity**: Proteomic Approaches to the Analysis of Protein Alterations at the Synapse in Kindling; **Pyramidal Cells**: Receptor Subtype Localization in CA3 Pyramidal Cells in Epileptic Brain; **Seizures**: Seizure-Induced Gene Expression; **Temporal Lobe Epilepsy**: Altered GABA Receptor Subunit Composition in Temporal Lobe Epilepsy.

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Genetic Determinants of Temporal Lobe Epilepsy

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Introduction

Temporal lobe epilepsy (TLE) is one of the most common, and poorly controlled, forms of seizure disorder. It has been extensively characterized – clinically, electrophysiologically, and histopathologically. Indeed, most of our animal models of chronic seizure disorders have been developed to mimic TLE. At both the clinical and basic research levels, a strong underlying assumption has been that TLE is the result of some brain insult that is eventually manifest in a

genes. Correlation coefficients of the corresponding genes were in the range of 0.4, but rose to 0.6 when the interspecies comparison concentrated on the expression ratios between DG and CA1 regions of each species. The highest congruency was detected between the chronic epileptic stage in experimental epilepsy and chronic human AHS. In contrast, markedly less codifferentiated gene ratios were observed between human AHS and samples from rats at acute or intermediate stages after SE. Remarkable similarity in gene expression profiles were associated with key cellular functions, such as cell–matrix interaction, neuronal growth, and signaling. Thus, corresponding microarray experiments suggest that changes observed in experimental TLE show a valuable overlap with findings in human disease. Nonetheless, further studies are needed to address the question of whether these genes contribute to the pathogenesis of human TLE.

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Introduction

Temporal lobe epilepsy (TLE) is one of the most common, and poorly controlled, forms of seizure disorder. It has been extensively characterized – clinically, electrophysiologically, and histopathologically. Indeed, most of our animal models of chronic seizure disorders have been developed to mimic TLE. At both the clinical and basic research levels, a strong underlying assumption has been that TLE is the result of some brain insult that is eventually manifest in a

characteristic neuropathologic profile. For example, investigators have focused on the epileptogenic nature of traumatic brain injury (TBI), with a particular interest in subsequent TLE. This assumption of TLE as an 'acquired' epilepsy currently appears to be under critical scrutiny. For example, there is a serious debate about the role of early (infant and childhood) complex febrile seizures – or prolonged status epilepticus – in initiating the epileptogenic events that later result in TLE.

The histopathologic features generally associated with TLE include hippocampal cell loss, particularly among CA1 pyramidal cells and/or CA3 and hilar neurons (mesial temporal sclerosis), selective loss of subtypes of GABAergic interneurons, and mossy fiber sprouting. The electrophysiological features may be somewhat varied, but 'by definition' of a partial epilepsy syndrome, these features include seizure initiation in a distinct brain region (the temporal lobe – and usually mesial temporal lobe structures) and secondary spread to the rest of the brain. Behaviorally, the seizures often involve alteration in consciousness and behavioral automatisms. However, it is becoming increasingly clear that the phenomenology covered by this TLE umbrella is quite varied – and both clinicians and researchers are beginning to consider the idea that 'TLE' may in fact reflect a number of distinct disorders, with different underlying etiologies, pathologies, and electrophysiological and behavioral characteristics. For example, a significant number of TLE hippocampi – resected because the seizures were refractory to conventional treatments – show little neuropathologic abnormality.

The possibility that TLE may cover a number of different disorders raises the possibility (likelihood) that distinct therapeutic approaches may be appropriate in different cases. It is, therefore, important to understand the origins and process of these 'syndromes.' Given the growing awareness of genetic determinants of many seizure syndromes, and the knowledge that genetic variation can give rise to different levels of seizure sensitivity, it is worthwhile exploring the possibility that at least some forms of TLE involve important genetic predisposition factors. Identification of these factors may facilitate diagnosis and treatment.

Background

Genetic Determinants of Seizure-sensitivity and of Epilepsy in Animal Models

A large number of studies have now identified genes, the absence or overexpression of which modify excitability. The manipulated genes include those that code for such disparate substances as channel and receptor subunits, modulatory peptides such as NPY and galanin, and ion and neurotransmitter transporters. In addition, experimental observations have led to the identification of a number genes, the mutations of which give rise to human epilepsies. In most cases, these human 'epilepsy genes' are not completely penetrant – that is, their mutations are quite variable in producing an epileptic phenotype. In this respect, then, they too are 'modulatory' of brain excitability. In animals studies (particularly in mice), spontaneous gene mutations have been described that produce epileptic genotypes, and in some cases these genes have human homologs. Interestingly – and perhaps predictably given the widespread expression of these critical genes – most of these epileptic mice display generalized epileptic phenotypes, reminiscent of absence-like epilepsies in human patients.

At a more subtle level, a number of laboratories have determined – in both rats and mice – the importance of background strain for seizure susceptibility (i.e., sensitivity to epileptogenic agents), seizure intensity, seizure-related brain pathologies, and other morbidities (including seizure-related mortality). It is, therefore, quite clear that genetic influences – both subtle and obvious – can be involved in manifestations of seizure and can influence seizure sensitivity.

Genetic Associations with TLE

Given that the same genome is present in all cells of the brain, it seems intuitively attractive that genetic features would have a generalized effect on brain excitability. This expectation has been confirmed by the identification of human and mouse 'epilepsy genes' associated with generalized seizure phenomenology. However, it is also clear that different genes have regionally – and cell type-specific – distinct expression patterns. Thus, there is a reasonable basis for expecting that mutation/dysfunction in some genes might give rise to local changes in excitability and focal epilepsies – a defining feature of TLE. Recent studies of TLE have even identified a particular gene – LGI1 (leucine-rich, glioma-inactivated 1 gene) – mutations of which appear to give rise to an autosomal dominant partial epilepsy in humans (lateral TLE with auditory features). In separate studies, the LGI1 gene product was shown to associate with the Kv1.1 potassium channel α subunit, a particularly interesting finding given that Kv1.1 dysfunctional mutation – in both mouse and man – has sometimes been associated with a seizure phenotype. At least in mouse, the 'epilepsy' seen in Kv1.1 knockout mice has many of the characteristics of TLE, as commonly described in both animal models of TLE (e.g., pilocarine and kainate injections, amygdala kindling, etc.) and in tissue from TLE patients.

A different kind of genetic disorder is associated with abnormalities of brain development, and gives rise to dysplastic lesions closely associated with epileptic disorders. Depending on the location of the dysplasia, these genetic abnormalities may give rise to a TLE-like

syndrome. For example, one of the common histopathological features seen in tissue from TLE patients is granule cell dispersion in the dentate region of the hippocampus. This disorganzational feature – initially believed to be a product of seizure activity per se – is now known to be a product of aberrant brain developmental patterns. Granule cell dispersion is reproduced by several genetic mutations, the best-studied of which is probably the mutation/deletion of the reelin (RELN) gene. The reeler mouse – a spontaneous mutant with a dysfunctional RELN gene – has been known for many years; a human homolog has been identified in a rare epileptic syndrome (autosomal dominant lissencepahly with cerebellar hypoplasia). In mice, the p35 mutation also produces granule cell dispersion, along with an epileptic phenotype which has features of limbic epilepsy (i.e., TLE). While many areas of the brain appear somewhat disorganized in the p35 (and RELN) knockout, the seizure phenotype again appears to be localization-specific and TLE-like. Disorganization of the hippocampal dentate region, with reorganization of afferent and intrinsic connections, has been hypothesized as the basis for this temporal lobe-specific epilepsy manifestation.

Methods

In our laboratory, we have taken advantage of available 'knockout' mice to study models in which a gene mutation/deletion gives rise to what appears to be a limbic-like seizure disorder. In behavioral observations of these mice – the Kv1.1 and p35 knockouts – spontaneous seizures can be described according to the Racine scale for rating amygdala-kindled limbic seizures. Video-EEG monitoring has been used to relate clinical/behavioral phenomena with electrical abnormalities (ictal episodes as well as interictal abnormalities).

It is noteworthy that even in inbred mice, this gene deletion does not appear to produce an 'equivalent' seizure phenotype in 100% of the animals; seizure onset timing, frequency, and severity can differ considerably from animal to animal. Therefore, in addition to monitoring spontaneous seizures, we have tested animals for seizure threshold, primarily using flurothyl to provoke seizure activity. Animals can be compared with age-matched, normal background strain controls.

Animals have also been sacrificed to generate slices of hippocampus for in vitro studies of cellular electrophysiological characteristics, as well as for histopathological features. In hippocampal slices, extracellular and intracellular recording techniques are used to study the normal and reorganizational characteristics of hippocampus, in an attempt to determine what features give rise to the epileptic abnormality. In parallel, histological and immunocytochemical approaches – for gliosis, cell degeneration, changes in intracellular structure, and mossy fiber sprouting – have been employed to relate structural features to electrophysiological dysfunction.

Recent Results

The Kv1.1 knockout mouse has a spontaneous seizure phenotype that varies across homozygous −/− mice (Figure 1). Seizure activity appears developmentally within the second postnatal week, a time that corresponds approximately to the period in which Kv1.1 should begin to appear in hippocampal and cortical neurons. Knockout mice are unusually 'jumpy' and sensitive to external stimuli. In adult knockout mice, seizures often take the form of initial immobility, with subsequent facial automatisms (whisker twitching, licking, etc.) and Straub tail. In some cases, these initial stages progress to forelimb clonus and then generalized tonic–clonic motor seizures. This progression is similar to the 'clinical' stages described initially by Racine for limbic kindled seizures, and used widely to describe limbic (i.e., temporal lobe) epilepsy in animal models (e.g., pilocarpine, kainate). At the EEG level, tonic–clonic seizure activity is consistently related to obvious ictal activity in cortical recordings; immobility/freezing is also associated with ongoing EEG abnormality.

Seizure threshold testing has confirmed that with exogenous seizure-inducing stimuli, these knockout mice are more seizure-prone than $Kv1.1^{+/+}$ littermate controls. Interestingly, while the Kv1.1 heterozygous mouse does not display spontaneous seizures or EEG abnormalities, it does show a seizure threshold that is intermediate between the wildtype and knockout mouse.

Histopathological examination of Kv1.1 animals that have a history of seizure episodes reveals neuropathology that mimics the changes normally associated with human TLE – widespread gliosis, cell loss particularly in the hilus of hippocampus, and mossy fiber sprouting. Critically, however, these TLE-like abnormalities are not seen in young animals (i.e., at a time before the mice have begun to exhibit seizures). Interestingly, however, reactive gliosis is seen at early timepoints, particularly in those regions that should have (but do not in the knockout) high expression of Kv1.1 – suggesting a clinically subthreshold, localization specific discharge within the hippocampal dentate and CA3 regions. Electrophysiological data from $Kv1.1^{-/-}$ hippocampal slices have shown that this tissue is seizure-sensitive, and that the major cellular abnormality involves axonal conduction (and perhaps synaptic release).

These results are consistent with histological and electron microscopic data that indicate a normal Kv1.1 localization that is particularly high in the axons of hippocampus (perforant input from entorhinal cortex, mossy fiber projections from granule cells to CA3, and CA3 axon collaterals). The Kv1.1-containing channels are heteromers

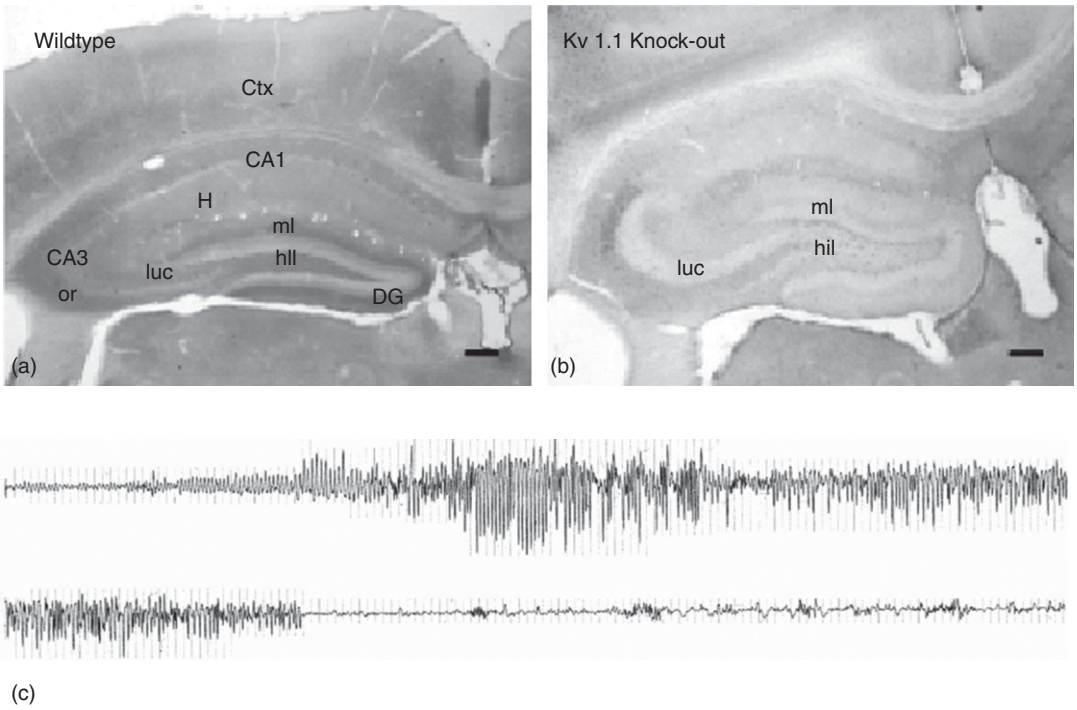


Figure 1 (a) Immunocytochemistry for the Kv1.1 α subunit in wildtype mouse hippocampus. Note particularly high density in the dentate molecular layer (ml), hilus (hil), and CA3 oriens (or) and lucidum (luc). (b) In the knockout mouse, Kv1.1 immunoreactivity is absent. (c) Spontaneous seizure episode recorded from the skull surface (1 s calibration bar).

that generally involve another Kv1 channel subtype (e.g., Kv1.2 and/or Kv1.4) and beta subunits, with Kv1.1-containing channels specifically localized to the extraparanodal region of axons and some terminals. At least in hippocampus, Kv1.1 is not found in the membranes of principal cell somata, but is found in somata of subtypes of interneurons. Immunocytochemistry has confirmed that in Kv1.1 knockouts, the Kv1.1 subunit is completely absent; possible compensatory changes in other subunits are subtle.

It appears that the loss of Kv1.1 from hippocampal axons is sufficient to give rise to seizure activity in these animals. In these locations, in combination with Kv1.4 subunits, the Kv1.1 subunit normally contributes to a channel that mediates 'A-current.' In the knockout, this fast, easily inactivated potassium current is lost, and action potentials become broader (with the danger that long depolarization of axon membrane will trigger aberrant repetitive action potential discharge and/or mediate the release of abnormal amounts of neurotransmitter). Compensatory changes in related Kv1 subunits do not appear to 'normalize' this dysfunction – although such changes may help account for the fact that seizure expression is so variable across knockout animals. Typical TLE-like histopathology does occur in these animals, but not until after they have experienced multiple and/or prolonged seizures. Thus, it appears that these structural changes are a result – not a cause – of the seizures.

In p35 knockouts, the seizure phenomenology – the typical Racine scale description of seizure activity – is similar to that described for the Kv1.1 knockout. However, the relationship between the aberrant structure and the seizure activity is quite different. In these animals, granule cell dispersion, as well as abnormalities in GABAergic interneuron subpopulations, is seen even in immature animals well before seizures can be observed or recorded. In this animal, therefore, the genetic determinant of tissue hyperexcitability is expressed not through a direct modulation of cellular electrical properties, but rather as a consequence of the abnormal structural features (or structural reorganization) that result from abnormal brain development. For example, both in vitro and in vivo studies have shown characteristic mossy fiber reorganization associated with hyperexcitability in tissue from p35 knockouts. Granule cells are monosynaptically connected to each other via excitatory axon collaterals which make contacts not only on the dendritic sites in the inner molecular layer of the dentate, but also on the basal dendrites and displaced granule cell somata in the hilus. This positive feedback from one granule cell to another appears sufficient to trigger hyperexcitable discharge within the hippocampus.

Future Goals

A major challenge in working on localization-specific epilepsies in mouse models is to establish a clear and convincing description of the site of seizure initiation

(and, if possible, the pathways of spread). Since the brain is so small and seizure activity spreads so quickly, it is difficult to establish an initiation site – and requires implantation of microelectrodes into multiple suspect regions. While current data suggest a temporal lobe (limbic) seizure phenotype, we have still not established the temporal lobe – and hippocampus in particular – as the site of origin of seizure activity. Thus, a firm determination of seizure type as 'partial' and 'temporal lobe' must still be established for both Kv1.1 and p35 knockouts.

In trying to elucidate the mechanisms underlying the seizure activity in these mutant animals, it is clearly not enough to identify the aberrant gene or gene product. Understanding the bases of epileptiform activities requires research ranging from the level of molecules to the intact brain. Of key interest, in both the models discussed in this article, is an understanding of how the Kv1.1 alpha subunit protein and the p35 protein normally interact with related gene products. For example, it is clear that appropriate subcellular targeting and channel function for Kv1.1 is determined in large measure by other associated Kv1 subunits. The nature of this interaction in the normal brain, and the changes in subunit interaction (compensatory?) in the knockout brain should be explored. Similarly, it is clear that p35/cdk5 and reelin interact in normal brain to provide guidance for neuronal migration. While the pathways are apparently independent, they appear to interact to produce granule cell dispersion.

The electrophysiological bases for epileptic activity in these models remain incompletely characterized. Our past studies have identified a set of potential epileptogenic mechanisms in hippocampal tissue, but this description needs to be expanded. One obvious area of neglect is the role played by inhibitory (GABAergic) systems in these epileptic tissues, and the possible aberrant features of interneuron populations in these knockouts. Another issue of potential importance is the likelihood that extra-hippocampal regions also express hyperexcitable properties – a possibility still to be explored.

Finally, in order to relate the observations in animal models to the features of human epileptic disorders, it is important to identify common variables – molecular, histological, electrophysiological – in tissue from human epileptic brain. If, as hypothesized, the salient epileptogenic underpinnings of these genetic mutations are preferentially localized to temporal lobe, then evidence for their involvement in epileptiform activities should be obtainable from human epileptic temporal lobe resected for medically intractable epilepsy.

See also: **Development/Malformations**: Epilepsy-Associated Reelin Dysfunction Induces Granule Cell Dispersion in the Dentate Gyrus; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Interneurons**: Plasticity and Reorganization of GABA Neurons in Epilepsy; **Ion Channels**: Channelopathies in Epilepsy; Intrinsic Properties of Neocortical Neurons Relevant to Seizure Discharges: The Lesson of Epileptogenic Channelopathies; **Models**: Model Characterization in Relationship to Human Disorders; **Temporal Lobe Epilepsy**: Morphological Abnormalities Associated with Genetic Epilepsies.

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Morphological Abnormalities Associated with Genetic Epilepsies

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Introduction

In recent years, numerous gene mutations associated with human epilepsy have been identified; these mutations, together with information obtained from spontaneous mutations and targeted deletion of genes in mice, have provided insights into genetic links to seizure disorders. Many of these genes appear to be related to altered functional excitability (e.g., mutations in channel- and receptor-related genes). Other genes are associated with molecules critical to normal brain development, and mutations in such genes cause severe structural abnormalities. In both types of genes, mutations are frequently associated with not only characteristic anatomical neuropathologies but also developmental disabilities and seizures/epilepsy. Moreover, in mutations of both types of genes, it is often unclear how the altered/deleted gene product causes a seizure phenotype.

Within this context of genetically-determined epilepsies, there is a growing need to understand the relationship between aberrant brain structure and functional abnormalities. In particular, it is important to understand what structural abnormalities lead to epilepsy, and why. Also critical, particularly from the perspective of developing more effective treatments, is the question of how/if functional abnormalities (e.g., channelopathies) give rise to structural abnormalities – and whether that outcome has further consequences for epileptogenicity.

One approach to investigate these issues is the use of animal models. Many such models have been studied, particularly in mice and rats, in which abnormal brain structures have been induced by experimental manipulations (e.g., freeze, undercut, γ-irradiation). Alternatively, in animal models one can attempt to mimic specific gene mutations found to cause epilepsy in the human brain. Among the most commonly studied of these mutations are those that affect voltage-gated channels or that cause various forms of cortical dysplasia. Two such models – the $p35^{-/-}$ (knockout) mouse and the $Kv1.1^{-/-}$ mouse – provide useful illustrations of how structure and function may interact and contribute to epileptogenicity. In the $p35^{-/-}$ mouse, a genetically-induced aberrant cortical structure is primarily – and presumably—the causal factor leading to spontaneous seizures/epilepsy in these animals. In contrast, deletion of the *Kcna1* gene causes hyperexcitability and epilepsy, initially in the absence of any obvious neuropathology; however, neuropathological features do develop as a consequence of repeated seizure activity, and may exacerbate (or at least contribute to the maintenance of) seizure activity.

Background

$p35^{-/-}$ Mouse

The *p35* gene plays a key role in brain development. In wildtype mice, the p35 protein participates in the signaling pathway involved in corticogenesis. It acts as a neuron-specific activator of cyclin-dependent kinase 5 (Cdk5), which in turn regulates neocortical lamination. The brains of mice lacking p35 or Cdk5 show aberrant cortical lamination patterns, including callosal axonal guidance defects. During corticogenesis, the signals that guide the normal 'inside-out' lamination pattern are disrupted (i.e., later born waves of migrating neurons fail to move past pre-existing neurons), thus resulting in a dysplastic neocortex. In addition to the neocortical lamination defects, there are also dysplastic changes in the hippocampus, involving particularly displaced pyramidal neurons and dentate granule cells. The dysplasia also involves disorganized dendritic and axonal arbors and aberrant connectivity among neurons. As these $p35^{-/-}$animals mature, they also exhibit abnormal functional properties, including spontaneous limbic-like seizures. The patterns of pathology – both seizures and dysplasia features – are somewhat different from animal to animal, and the severity depends (at least to some extent) on the genetic background of the mouse.

Recent studies of the pathways involved in neuronal migration have suggested close relationships/interactions between the p35/Cdk5 signaling and other signaling systems that regulate neocortical lamination. Of particular interest for our studies, the reelin pathway has been shown to play a key role in neuronal migration both for neocortical development and for dentate granule cell layering in hippocampus. In the mutant 'reeler' mouse, reelin deficiency leads to aberrant migration patterns similar to those seen in the $p35^{-/-}$ mouse. Although previous studies revealed no seizure phenotype in reeler, recent studies have shown these mice to be seizure-prone. Further, 4 investigations show changes in reelin expression in epileptic hippocampi from patients with temporal lobe epilepsy (TLE) and in experimentally-induced epileptic animals. These studies suggest that in the epileptic hippocampus, abnormalities in either the p35/Cdk5 or

reelin signaling pathways may contribute to granule cell pathologies (e.g., cell dispersion – see below), and subsequently to the aberrant circuitry that underlies seizure activity in TLE.

Kv1.1$^{-/-}$ Mouse

The Kv1.1$^{-/-}$ mouse is one of dozens of single gene mutation models that are collectively referred to as examples of 'channelopathies.' They are particularly interesting as models, since many of the single gene mutation epilepsies in human appear to be channelopathies. The Kv1.1$^{-/-}$ mouse (deletion of the Kv1.1α subunit) is somewhat different from most of the other single gene mutation mice, inasmuch as it exhibits a tonic-clonic seizure phenotype, with features similar to those of temporal lobe epilepsy. In contrast, many of the other mouse channelopathy models present with an absence-like seizure phenotype. The Kv1.1$^{-/-}$ mouse also exhibits enhanced seizure propensity (i.e., lower thresholds to seizure inducing agents) as well as a gene 'dose' effect (Kv1.1+/− mice are more seizure-prone than wildtype). Potassium channels play an important role in hyperpolarizing neuronal membranes, so it is perhaps not surprising that loss of the Kv1.1α subunit results in brain hyperexcitability. What is surprising is the fact that Kv1.1-containing channels are heteromers, containing four primary subunits and auxiliary subunits (in addition to Kv1.1, Kv1.2, and Kv1.4α subunits, Kvβ1 and/or Kvβ2 auxiliary subunits, also participate in these channels). When Kv1.1 homotetramers are studied in expression systems, the channel mediates a delayed rectifier current. However, when Kv1.1 combines with, e.g., Kv1.4 – as it almost always does in the intact brain – the resultant channel has different properties. The current mediated by such channels is a transient A-current. Mutations of the human homolog of Kv1.1 – Kcna1 – have been seen at the clinical level, and at least in some cases are associated with an epilepsy phenotype.

Methods

Animals

For studying structural abnormalities and their relationships to genetic epilepsies, we used mice, in which deletion of a known gene is associated with spontaneous seizure development. In particular, both the animals described earlier (p35$^{-/-}$ and Kv1.1$^{-/-}$ mice) exhibit a limbic, TLE-like seizure phenotype, and both show hippocampal neuropathological features (see later). Wildtype animals – of the same genetic background as the mutated mouse – are used for control observations and comparisons.

Seizure Behaviors and Electroencephalographic (EEG) Monitoring

To characterize functional abnormalities (e.g., changes in seizure susceptibility) related to structural abnormalities and/or gene loss, animals are tested for seizure threshold using the volatile convulsant flurothyl to provoke seizure activity. Latencies to onset of myoclonic jerks and to generalized tonic-clonic seizures are recorded and used as a measure of seizure susceptibility. Video/EEG monitoring is used to record simultaneously both clinical behaviors and underlying electrical phenomena (i.e., electroencephalographic (EEG) activity). Animals are implanted with surface and depth (hippocampal) electrodes, and EEG recordings are made at different times of development to obtain data regarding interictal and ictal episodes in freely-moving animals.

Histological and Immunocytochemical (ICC) Methods

Anatomical features of hippocampus – from normal (age-matched) brains (controls), brains from $^{-/-}$ animals exhibiting little seizure activity, and brains from $^{-/-}$ epileptic mice – are described using various histological procedures for visualization of neurons and glial cells. Quantitative assessments of neuronal cell loss, reactive astrogliosis, and neuronal degeneration of neuronal cell bodies and synaptic terminals are based on Nissl staining, GFAP immunocytochemistry, and silver degeneration procedures. Immunocytochemical techniques are used to determine and compare normal and pathological distribution patterns of chemically-different neuron populations (e.g., interneurons). Reorganization of axonal connections (i.e., mossy fiber sprouting) is visualized using Timm staining. Specific protein localization (e.g., for Kv channel subunits) is determined via immunocytochemical methods, using subunit-specific antibodies.

Electron Microscopy and EM-ICC

Electron microscopic techniques – combined with ICC – are employed to provide ultrastructural details of immunocytochemical localization of Kv channel subunits (e.g., Kv1.1 and Kv1.4 subunits) to various neuronal compartments, cell types, and/or axonal projections, as well as to detect potential compensatory changes in expression levels of other subunits in knock-out mice. Electron microscopy provides a strong anatomical assessment of synaptic connectivity, and a basis for understanding changes in synaptic connectivity (i.e., synaptic reorganization) associated with dysplasia and/or seizure activity. Three-dimensional reconstruction of single cells

(e.g., labeled with an electron-dense marker) from epileptic animals provides insight into reorganization of axons and dendrites.

Hippocampal Slices – Electrophysiology and Single Cell Labeling

Extra- and intracellular recording has been carried out in hippocampal slices – prepared from epileptic and control animals – to study cellular electrophysiological characteristics of neurons in normal and reorganized neuronal circuits. These functional data, in combination with the anatomical observations, allow us to hypothesize about what structural/functional features give rise to the epileptic abnormality. In single cell studies, electrophysiologically-identified neurons are intracellularly labeled (with biocytin), and processed for light- and electron microscopy to assess structural features (including dendritic and axonal arbors) of cells of known function/dysfunction.

Recent Results

Clinical Features (Behavioral and EEG)

p35$^{-/-}$ mice

Deletion of the *p35* gene causes disruption of cortical migration and hippocampal dysplasia in all $^{-/-}$ animals, as well as spontaneous behavioral and electrographic seizures in a subpopulation of the p35$^{-/-}$ mice. Spontaneous (tonic-clonic) behavioral seizures were observed in 25% of the animals as early as 2 months postnatal. Spontaneous epileptiform activity was electrographically verified in 75% of the animals (ages 3–5 months). Most of the recorded spontaneous seizures were strong, generalized tonic-clonic episodes that appeared to occur simultaneously in cortex and hippocampus. Flurothyl seizure testing revealed a significantly greater seizure susceptibility of p35$^{-/-}$ mice as compared to age-matched controls. Since the characteristic neuropathological feature of these animals is seen soon after birth (see below), the fact that spontaneous seizure activity was not observed until 'adolescence' suggests that the genetically-determined structural abnormality precedes (and is a cause of?) hyperexcitability. That is, the seizure-related activity is not a direct expression of the gene mutation (e.g., as mediated through an abnormal cellular discharge properties), but rather a secondary consequence of the structural/developmental abnormalities.

Kv1.1$^{-/-}$ mice

The Kv1.1$^{-/-}$ mouse exhibits spontaneous seizures early in development – typically starting during the third postnatal week. The onset of seizure activity corresponds to the time period - in wildtype mice - of rapid increase in Kv1.1 protein expression in axons of hippocampal and neocortical neurons. Thus, in the Kv1.1$^{-/-}$ animal, seizures appear to correspond with the absence of normal Kv1.1 expression. Early seizure behavior consists of such features as twitching of vibrissae, forelimb paddling, and periods of immobility; as the animal matures, seizures exhibit a more 'typical' phenotype with forelimb clonus and generalized tonic-clonic motor seizures. Generalized tonic-clonic seizures appear 4–5 weeks postnatal; they start with tonic arching and tail extension, followed by forelimb clonus (or rearing and forelimb clonus), and then generalized synchronous forelimb and hindlimb clonus. These seizures are often followed by a period of post-ictal depression. This seizure sequence has the characteristics of typical limbic seizures as observed in experimental animal models of TLE (e.g., kainate, pilocarpine), suggesting a critical role of hippocampus and related structures. Seizure-associated EEG recording confirms that these behaviorally-obvious seizure behaviors are always accompanied by an ictal EEG pattern. Typical EEG recordings of a generalized tonic-clonic seizure event show characteristic changes over time: there is usually an initial pre-seizure interictal spike-like event, followed by an abrupt voltage depression, then a period during which low-voltage fast activity progresses into high-amplitude spiking. At seizure cessation, there is an abrupt loss of high voltage spiking and EEG flattening, corresponding to post-ictal depression. Interestingly, ictal EEG patterns are sometimes observed in the absence of obvious behavioral changes.

Kv1.1$^{-/-}$ mice, along with Kv1.1$^{+/-}$ animals and wildtype controls (Kv1.1$^{+/+}$), have also been tested for seizure susceptibility (using flurothyl) at different ages. This seizure threshold testing confirmed that immature/juvenile Kv1.1$^{-/-}$ mice (at P15) have significantly shorter latencies to flurothyl-induced seizures as compared to wildtype and Kv1.1 heterozygous littermates. The heterozygous mice have latencies between Kv1.1$^{-/-}$ mice and wildtype animals. Electrophysiological data from Kv1.1$^{-/-}$ hippocampal slices have shown that this tissue is seizure-sensitive, and the major cellular abnormality involves axonal conduction (and perhaps synaptic release).

Neuropathology and Epilepsy

p35$^{-/-}$ mice

The deletion of the *p35* gene results in disrupted cortical development in which the neocortex is inverted, and additional abnormalities are exhibited in the hippocampus, including heterotopic pyramidal neurons in the CA1 and CA3 subfields and dispersion of dentate granule cells into the hilus and molecular layer (**Figure 1(a–c)**). The dispersed dentate granule cells exhibit aberrant dendritic orientation (e.g., misoriented dendrites, basal dendrites) (**Figure 1(e)**). Perhaps most importantly, aberrant granule cell axons – i.e., mossy fibers – 'sprout' into the regions

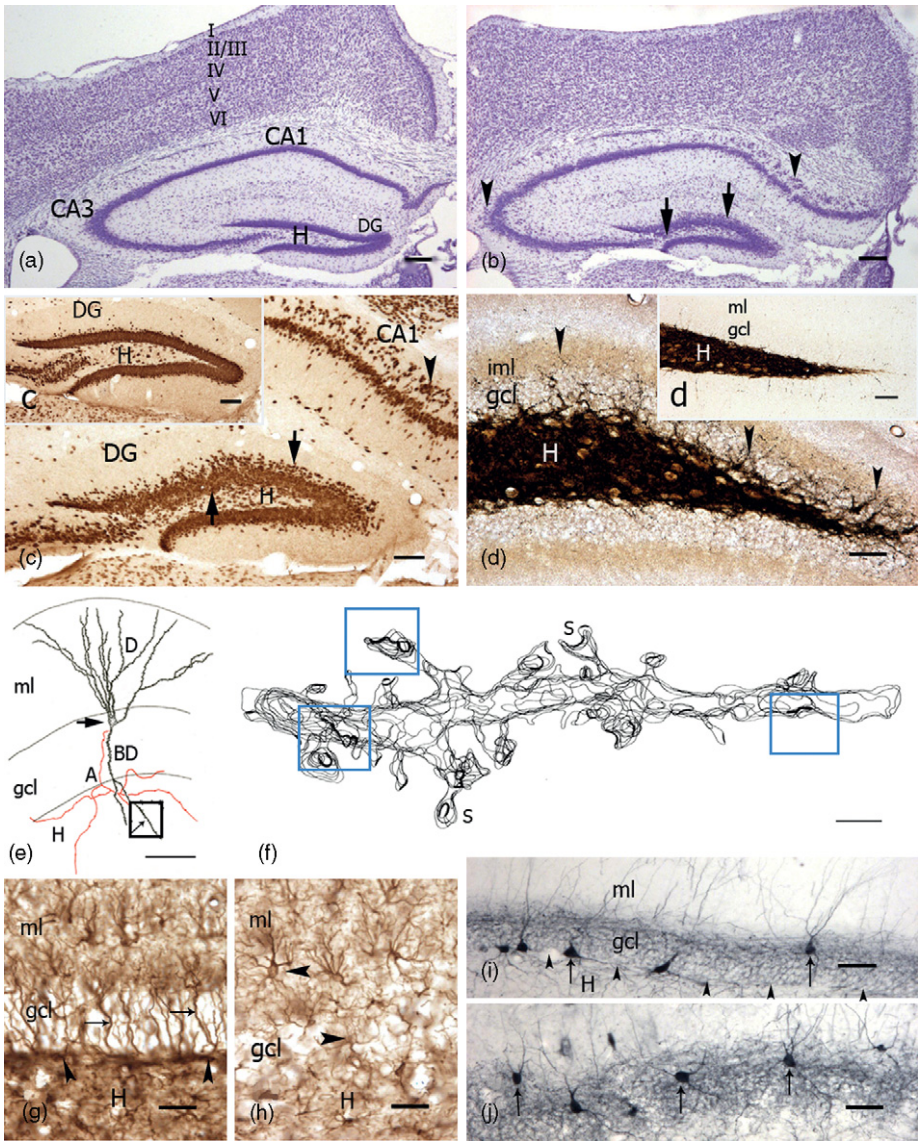


Figure 1 p35 gene deletion is associated with structural abnormalities. (a, b) Transverse sections through neocortex and hippocampus from a p35 wildtype (a) and a p35$^{-/-}$ mouse (b) (cresyl violet). The normal cortical lamination patterns in panel a contrast with the disorganized neocortex and the cell dispersion in hippocampus (b) (arrows show granule cells, arrowheads show pyramidal cells of CA1 and CA3). I–VI – neocortical layers; CA1, CA3 – hippocampal pyramidal cell subfields; DG – dentate gyrus; H – hilus. Scale bars: 200 μm. (c) NeuN-immunocytochemistry in transverse hippocampal sections reveals a normal lamination pattern in the wildtype dentate gyrus (inset c), and aberrant dispersion of CA1 pyramidal cells (arrowhead) and dentate granule cells (arrows) in the p35$^{-/-}$ mouse. Scale bars: 50 μm. (d) Timm histochemistry for zinc in mossy fiber boutons shows the normal staining pattern in the hilus of a wildtype mouse (inset d), and extensive mossy fiber sprouting into the granule cell layer (gcl) and inner molecular layer (iml) (arrowheads) in the p35 knockout mouse. Scale bars: 50 μm. (e) Camera-lucida drawing of a single biocytin-filled granule cell (arrow) from a p35 knockout mouse showing abnormal dendrites (D) emerging from the apical and hilar granule cell pole, and forming basal dendrites (BD). Scale bar: 100 μm. (f) Two-dimensional reconstruction of a representative basal dendritic segment (boxed area in panel e) based on serial section electron micrographs, showing numerous dendritic spines (s) (i.e., postsynaptic synaptic sites); spine synapses with mossy fiber boutons are indicated by blue boxes. Scale bar: 1 μm. (g, h) GFAP-immunostaining of the dentate gyrus from a wildtype mouse (g) presents the typical pattern of radial astroglial cells – located at the granule cell/hilar border (arrowheads) – and their radial fibers (arrows) ascending perpendicularly through the granule cell layer (gcl). In the p35$^{-/-}$ mouse (h), this pattern is absent; astrocytes (arrowheads) and their processes are diffusely distributed. Scale bars: 25 μm. (i, j) Immunocytochemistry for parvalbumin illustrates abnormal migration of interneurons into the dentate of the p35$^{-/-}$ mouse (j) as compared to the wildtype mouse (i). In the normal dentate gyrus (i), parvalbumin-positive neurons (arrows) are mainly located at the granule cell-hilar border (indicated by arrowheads); in the p35$^{-/-}$ dentate gyrus, the borders are disrupted and interneurons (arrows) are diffusely distributed across the layers. Scale bars: 50 μm.

now occupied by aberrant granule cell dendrites (i.e., the molecular layer and the hilus) (**Figure 1(d)**). These abnormal granule cell features in $p35^{-/-}$ mice are also associated with developmental abnormalities of the astroglia. Radial glia are prominent structural features lining the border of the granule cell layer and the hilus, and normally provide guidance for the migrating granule cells. However, in the dentate gyrus of $p35^{-/-}$ animals, these radially-oriented glial cells are absent (**Figure 1(g)** and **1(h)**) (although astrocytes are present and the glial processes run through the granule cell layer in an irregularly oriented pattern).

Studies have suggested that the $p35^{-/-}$ abnormality in neuronal positioning is linked to dysfunction or loss of reelin-containing Cajal Retzius cells, and/or to the absence of radial astroglial cells. Histological investigations of the dentate gyrus of $p35^{-/-}$ mice, using immunocytochemistry and electron microscopic techniques, also show abnormal positioning of dentate interneurons and aberrant projections of their axons (**Figure 1(i)** and **1(j)**).

In $p35^{-/-}$ animals, aberrant mossy fiber collaterals form synaptic contacts with the apical and basal dendrites of misplaced granule cells (**Figure 1(f)**) – both neighbors and, in some cases, their own dendrites (autapses). The morphological results are reflected in the physiological data; hyperexcitability of dentate granule cell responses to stimulation has been demonstrated in vitro. The data are consistent with the hypothesis that formation of recurrent excitatory circuits in the dentate gyrus provides a basis for hippocampal hyperexcitability, and may be primarily responsible for epileptogenesis.

It is significant that this pattern of dysplasia and synaptic reorganization in the dentate gyrus has been found as a consequence of experimentally-induced seizure activity in animal models of TLE, as well as in hippocampi of patients with mesial TLE. It should be emphasized that granule cell dispersion in $p35^{-/-}$ mice is a characteristic aberrant *developmental* feature of the dentate, a result of a migration disorder. Indeed, this granule cell dispersion and related changes in circuitry appear relatively early in postnatal development; by postnatal day 5, granule cell dispersion is evident, as are abnormalities in interneuron structure. That is, while the structural features of the $p35^{-/-}$ mouse resemble some of the pathologies frequently seen in animal models of TLE and epileptic hippocampi from patients with TLE and mesial Ammon's horn sclerosis, these features of $p35^{-/-}$ mice are seen well before seizure onset- and therefore may contribute to (cause) the development of the seizure phenotype.

Mutations in the p35 have not been linked to epilepsy in human. However, there are associations between the Cdk5 signaling pathway (of which p35 is a part) and several genes involved in cortical malformations and epilepsy (e.g., DCX, Lis-1). Further, gene mutations characterized in other mouse models – e.g., the reelin-, Lis-1-, doublecortin mutants – are associated with disruption of normal cortical development. The reelin mutation, in particular, has recently been associated with a rare developmental seizure-related disorder in humans.

Kv1.1 knockout

Kcna1 deletion is associated with characteristic neuropathology and epilepsy. However, in Kv1.1 knock-out mice, the development of these neuropathological alterations is dependent on seizure occurrence and severity. Characteristic neuropathological features, e.g., neuronal cell loss, astrogliosis, and mossy fiber sprouting – normally associated with human MTLE – are consistently seen in animals with documented status epilepticus (**Figure 2**).

These animals exhibit severe neuronal cell loss in pyramidal cell layers of hippocampal CA1 and CA3 subfields, and extensive axonal terminal degeneration across the hippocampal layers (**Figure 2(a–e)**). In the dentate gyrus, cell loss is most apparent in the population of mossy cells and among interneurons. The selective loss of vulnerable neuron populations after prolonged/intense seizures – especially in CA3 and hilus – is the basis for the sprouting hypothesis (**Figure 2(g)** and **2(h)**). Prolonged generalized seizures are always followed by extensive astrogliosis throughout the hippocampus, as well as in other brain regions (e.g., neocortex, thalamus) (**Figure 2(f)**). In contrast to this pattern of neuropathology, $Kv1.1^{-/-}$ mice that experience less severe seizure activity also show limited structural abnormalities (i.e., little or no cell loss, regionally-restricted gliosis, and low levels of mossy fiber sprouting). Thus, even in older $Kv1.1^{-/-}$ mice (>12 months of age), little pathology is evident if the animal has not experienced severe seizure episodes. Interestingly, immature $Kv1.1^{-/-}$ mice show an up-regulation of GFAP expression (in astrocytes) which is relatively restricted to the dentate gyrus and the hippocampal CA3 subfield – precisely those regions of hippocampus in which Kv1.1 is normally expressed at high levels.

Morphological studies on Kv1.1α subunit expression – using ICC and EM techniques – have shown a pattern of specific localization in the mouse brain, not only with respect to brain region, but also with respect to subcellular compartments. Potassium channels containing the Kv1.1α subunit are localized to the juxta-paranodal region of myelinated axons and to fine unmyelinated axons (such as mossy fibers). In normal animals, prominent Kv1.1-immunoreactivity (indicative of Kv1.1α subunit localization) has been found in axons of the major excitatory pathways of the hippocampus (**Figure 2(i)** and **2(j)**). There is little protein expression in somata of hippocampal principal cells; however, somata of subpopulations of hippocampal interneurons stain intensely for Kv1.1α subunit protein. Recent studies on functional consequences of the mouse *Kcna1* deletion have shown that there is

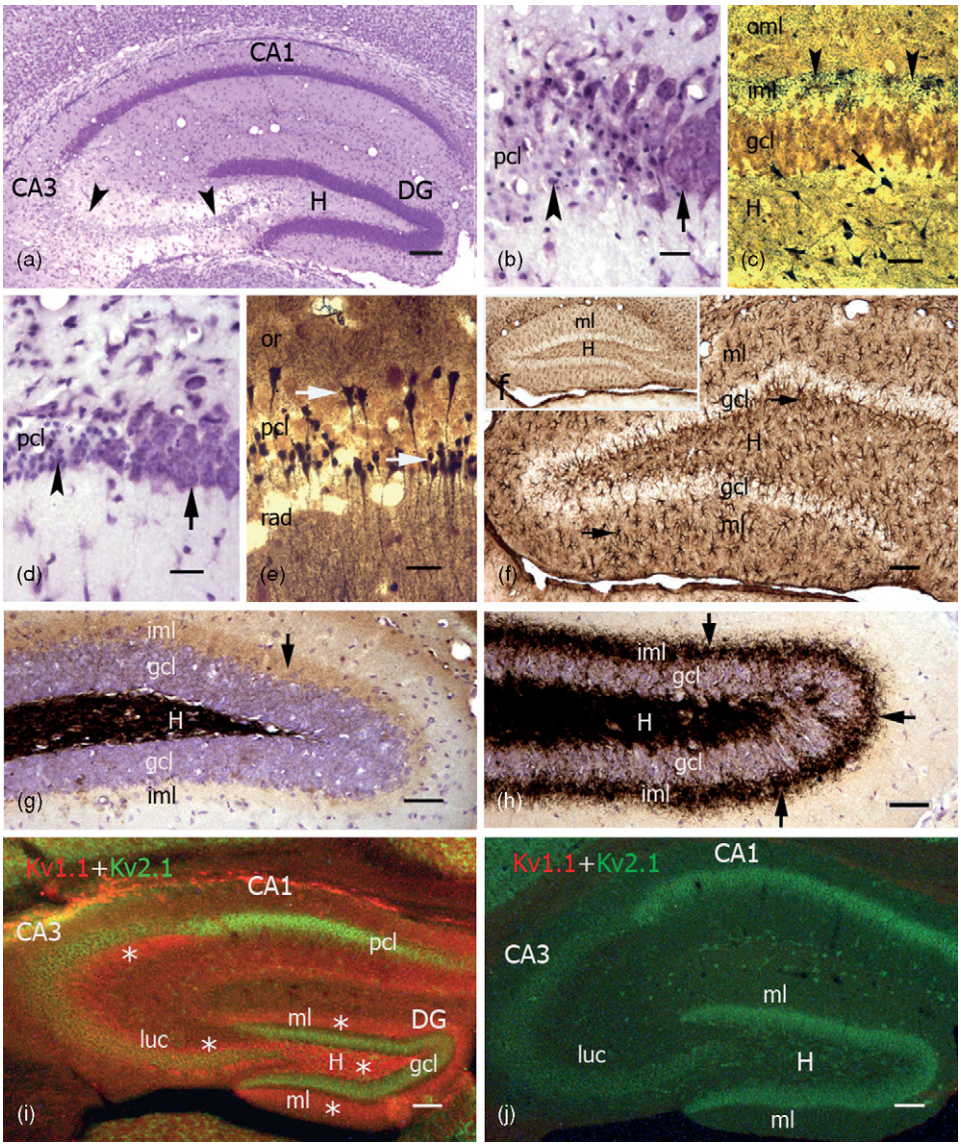


Figure 2 Chronic seizures in the Kv1.1$^{-/-}$ mouse are associated with severe pathology. (a) Transverse section of a Kv1.1$^{-/-}$ mouse shows extensive loss in the CA3 pyramidal cell region (arrowheads) following status epilepticus (cresyl violet). CA1, CA3 – hippocampal subfields; DG – dentate gyrus; H – hilus. Scale bar: 200 μm. (b) Higher magnification of the CA3 pyramidal cell layer (pcl) in A illustrates preserved pyramidal neurons (arrow) and an area with extensive cell damage/loss (arrowhead) (cresyl violet). Scale bar: 25 μm. (c) Higher magnification of the dentate gyrus, showing degenerated neurons (black cells; arrow) within the hilus (H) and terminal degeneration within the inner (iml) and outer (oml) molecular layers (Fink-Heimer silver degeneration staining). Arrowheads in the iml indicate terminal degeneration reflecting damage/death of hilar mossy cells. gcl – granule cell layer. Scale bar: 25 μm. (d) Higher magnification of the CA1 pyramidal cell layer (pcl) showing an area of normal pyramidal neurons (arrow) and an area with damaged (pyknotic) neurons (arrowhead) (cresyl violet). Scale bar: 25 μm. (e) Fink-Heimer staining in the CA1 pyramidal cell layer (pcl), showing degenerated CA1 neurons (black cells; white arrows) and extensive terminal degeneration in strata oriens (or) and radiatum (rad). Scale bar: 25 μm. (f) GFAP immunocytochemistry of the dentate gyrus from a wildtype mouse (inset f) illustrates normal astrocytic cell staining patterns in hilus (H) and molecular layer (ml). In the Kv1.1$^{-/-}$ mouse (f), high levels of GFAP immunoreactivity are present in the hilus (h) and molecular layer (ml), indicating reactive astrogliosis (arrows) following chronic seizures. Scale bar: 100 μm. (g, h) Timm histochemistry for zinc in mossy fiber boutons shows normal staining pattern in the dentate hilus (H) of a wildtype mouse (g) and no/or little staining in the granule cell layer (gcl) and inner molecular layer (iml) (arrow). In the Kv1.1$^{-/-}$ mouse, there is extensive mossy fiber sprouting into the granule cell layer (gcl) and inner molecular layer (iml) (arrows) following chronic seizures and/or status epilepticus. Scale bars: 50 μm. (i, j) Double immunofluorescence of potassium channel subunits (red: Kv1.1; green: Kv2.1) in transverse sections of the hippocampus from wildtype (i) and Kv1.1$^{-/-}$ (j) mice. In the wildtype hippocampus, Kv1.1 is highly expressed (*; red staining) in hilus (H), dentate middle molecular layer (ml) and stratum lucidum (luc), reflecting its localization to entorhinal pathway axons and granule cell axons (i.e., mossy fibers). Kv2.1 immunoreactivity (green) is localized to somatic and proximal dendritic membranes of principal neurons (pcl, gcl) and interneurons. Note the complete absence of Kv1.1 immunofluorescence (red) in the knockout hippocampus in panel j, and preservation of Kv2.1 staining (green) in principal neurons and interneurons. Scale bars: 200 μm.

virtually no compensatory changes in other related subunit (e.g., Kv1.2 and Kv1.4) expression.

Voltage-gated potassium channels are critical regulators of neuronal excitability. In the hippocampus of Kv1.1$^{-/-}$ mice, the A-type potassium current in axons and terminals is lost, leading to broader (more slowly repolarizing) action potentials and the propensity for repetitive discharge and enhanced transmitter release. The resulting hyperexcitability is expressed directly as seizure activity, before any neuropathological features develop. Thus, the Kv1.1$^{-/-}$ mouse reflects a loss of function mutation that results in a TLE-like seizure phenotype, with secondary neuropathological abnormalities. In this case it is the genetic disorder, not structural disorganization, that is the direct cause of the epilepsy.

Future Goals

The animal models of epilepsy discussed here provide investigators with an opportunity to study the structure–function relationships associated with epileptogenesis. In particular, they mimic seizure and structural abnormalities known to be associated with genetic mutations, and provide a context for exploring the causal factors that lead to the epileptic phenotype. The p35$^{-/-}$ and the Kv1.1$^{-/-}$ mice exhibit similar seizure phenotypes, and both animal models are associated with neuropathologies commonly seen in temporal lobe epilepsy – i.e., hippocampal cell loss, granule cell dispersion, mossy fiber reorganization, and gliosis. Current data support the view that the seizure phenotype is primary in the Kv1.1$^{-/-}$ mouse, and the neuropathology develops secondarily to the seizures. In contrast, in the p35$^{-/-}$ mouse, the structural abnormalities appear as the direct result of genetic mutation, and the seizures evolve as a product of reorganization of circuitry due to the aberrant structure. Given this contrast, these models provide an opportunity to investigate a number of interesting and potentially important questions:

1. Although, the current data suggest a temporal lobe (limbic-like) seizure phenotype for both models, the site of origin of seizure generation/initiation must be determined. Neuropathological features are found not only in hippocampus, but also in other brain regions. Further, the genetic mutation itself is common to all cells – and thus could support a 'generalized' seizure type. Thus, these models provide a means of gaining further insight into the presumed 'partial' nature of some idiopathic epilepsies.
2. Gene expression is invariably a developmental process. Absence of a gene normally involved in brain developmental patterning will lead to a developmental abnormality. In p35$^{-/-}$ mice, the early observation of the structural consequences of gene deletion can be examined alongside the maturational expression of the seizure phenotype – thus allowing the investigator to study the relationships between structural alterations and seizures (e.g., which structural changes are most closely linked – causally related to? – seizure activity). More generally, this mouse presents as a model in which to test for functional consequence of early seizures associated with a developmental malformation.
3. Similarly, the Kv1.1$^{-/-}$ mouse shows seizure-dependent pathogenesis, providing a means for exploring the feedback consequences of seizure-associated neuropathology on further seizure propensity and intensity.
4. Recent studies have focused on the interaction of p35 protein with other signaling proteins in normal neuronal migration patterns. The p35$^{-/-}$ mouse offers an opportunity to study such interactions, particularly with reelin and the role of radial glia, in the pathogenesis and developmental basis of seizure disorders such as TLE.
5. A major goal in working with animal models of epilepsy is to elucidate the underlying mechanisms of seizure activity, ranging from the molecular/cellular level to the complex structural organization of neuronal circuits. In the Kv1.1$^{-/-}$ mouse, the precise mechanism by which Kv1.1α subunit deletion gives rise to tonic-clonic seizure activity is not fully understood. Recent studies have yielded insight into the normal structure and function of Kv1.1-containing channels, and the importance of Kv1.1 interactions with related subunits (e.g., Kv1.4) and other gene products (e.g., LGI1). The functional nature of these interacting molecules in the absence of Kv1.1 may provide insight into the molecular basis of the seizure phenotype.
6. Finally, both models provide intriguing windows for exploring the potential for early intervention for preventing epileptogenesis. For example, interference with aberrant circuit organization (i.e., sprouting) in the p35$^{-/-}$ mouse may provide a basis for preventing seizure development. Alternatively, gene replacement therapy can be examined in the Kv1.1$^{-/-}$ mouse as a means of treating epilepsies associated with loss-of-function gene mutations.

See also: **Development/Malformations**: Epilepsy-Associated Reelin Dysfunction Induces Granule Cell Dispersion in the Dentate Gyrus; Focal Malformations of Cortical Development: Pathogenesis and Pathways; **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; **Models**: Model Characterization in Relationship to Human Disorders; **Neuropathology**: Neuropathology of Epilepsy; **Pediatric Epilepsy**: Animal Models of Catastrophic Epilepsies of Childhood; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy.

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TRANSPORTERS

Contents

Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures
GABA Transporters: Regulation of Tonic Inhibition
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Neuronal Cl Transport: Setting the Stage for Successful Inhibition
Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma

Function of Cell-Surface Glutamate Transporters in the Brain: An Important Role for Development and Preventing Seizures

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Introduction

Glutamate is the main excitatory neurotransmitter of the central nervous system. It is synthesized by neurons and stored in synaptic vesicles localized in axonal terminals. An action potential arriving at the terminal triggers a cascade of events leading to the fusion of the vesicle with the membrane and the release of glutamate into the extracellular space, where its concentration increases from few nanomoles to 1 mM. In less than 1 ms, glutamate binds to specific receptors (AMPA, kainate, or NMDA receptors) located on either the postsynaptic or

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Introduction

Glutamate is the main excitatory neurotransmitter of the central nervous system. It is synthesized by neurons and stored in synaptic vesicles localized in axonal terminals. An action potential arriving at the terminal triggers a cascade of events leading to the fusion of the vesicle with the membrane and the release of glutamate into the extracellular space, where its concentration increases from few nanomoles to 1 mM. In less than 1 ms, glutamate binds to specific receptors (AMPA, kainate, or NMDA receptors) located on either the postsynaptic or

presynaptic neuron (and also on astrocytes). These receptors are coupled to ionic channels that are permeable to Na^+ and K^+, and in case of NMDA receptors also to Ca^{2+}. Activation of these glutamate receptors leads to depolarization, and in some case, the genesis of action potentials in a postsynaptic cell. In addition to their action on these ionotropic receptors, glutamate also binds to receptors coupled to G protein (metabotropic receptors). Activation of the latter receptors modulates different ionic channels via second messengers.

Through the activation of these receptors, glutamate plays a major role in many aspects of brain function, including cognition, learning, and memory. During development, glutamate also contributes to cell migration, cell differentiation, and synapse formation and elimination. However, glutamate may have deleterious actions; it is intrinsically involved in many neurodegenerative diseases (e.g., amyotrophic lateral sclerosis, Alzheimers disease) and in epilepsies. The influence of glutamate function in the brain (physiological or pathological) is determined by the spatiotemporal distribution of its concentration in the extracellular space – which will influence the amplitude, the duration, and the number of receptors activated by the transmitter. The intensity of receptor activation, and the area covered by glutamate in the extracellular space, is strongly regulated by the cell surface GluTs. The main action of these proteins is to rapidly uptake and remove glutamate from the extracellular space. GluTs are important elements of regulation of the neuronal network activity, and transporter dysfunction can be the cause of severe neurological disorders. In this article, we examine the main properties of GluTs and discuss the role of these proteins during development, particularly in view of recent findings showing that inhibition of these transporters in the immature brain produces seizures with a 'suppression burst' EEG pattern.

Background

Glutamate Transporter Subtypes

The uptake of glutamate from the extracellular space is performed by cell-surface GluTs that are localized on both neuronal and glial cells. Five subtypes of GluTs have been identified and cloned. They show a high degree of conservation across species. In human brain they are called excitatory amino acids transporters (EAAT1–5). In rodent, the nomenclature is somewhat different: GLAST (glutamate-aspartate transporter) is the human analogue of EAAT1 and is located on astrocytes; GLT1 (glutamate transporter 1) is the human analogue of EAAT2 and also located on astrocytes; EAAC1 (for excitatory amino acid 1) is the analogue of EAAT3 and located on neurons (synaptic terminals, dendritic spines, and somas). These transporters are expressed in the neocortex, the hippocampus, the cerebellum, the retina, and the basal ganglia. EAAT4 and EAAT5 are also located on neurons but specifically expressed on the cerebellar Purkinje cells and in bipolar and photoreceptors cells of retina, respectively.

Functions of GluTs

GluTs play a major role in maintaining glutamate homeostasis in the brain. By transporting glutamate from the extracellular to the intracellular space, they contribute to many functions: (1) maintenance of nM concentration of glutamate in the extracellular space, in order to prevent neurotoxic effect of the transmitter; (2) reduction of glutamate receptors desensitization; (3) synthesis of GABA. With regard to GABA synthesis, GluTs are present on GABAergic terminals, and take up glutamate that is converted into GABA by glutamic acid decarboxylase (GAD); (4) limiting diffusion of glutamate out of active synapses (spill-over) and subsequent activation of glutamate receptors located at extrasynaptic sites and/or at neighbouring synapses. In this way, GluTs help maintain specificity of information transfer from cell to cell; (5) detoxification of NH_4^+/NH_3 in astrocytes, where glutamate is converted into glutamine by glutamine synthetase in the presence of NH_3. This often-overlooked reaction is fundamental to cell health, since hyperammonemia can lead to brain damage and neurological disorders. Glutamate present in the cytosol is also transported into mitochondria by mitochondrial glutamate/H^+ symporters (glutamate carriers GC1 and GC2) or exchange with aspartate (by the aspartate/glutamate carriers, AGC1 and AGC2). AGCs are present only in neurons, while GCs are present in both neurons and astrocytes. In the mitochondria, glutamate is converted by glutamate dehydrogenase into α-ketoglutyrate and NH_3. α-Ketoglutyrate to provide ATP for the cells via the tricarboxylic acid cycle. The glutamate dehydrogenase reaction in the mitochondria is the major source of NH_3, which in astrocytes will be used for the formation of glutamine. Therefore, GluTs function is important for the metabolism of glutamate.

Mechanism of Uptake

The transport of glutamate is an electrogenic process that is accompanied by an increase of positive charges in the cells. Thus, glutamate transport is coupled to the cotransport of three Na^+, one H^+, and counter-transport of one K^+. Na^+ is necessary for the binding of glutamate on the transporter, and K^+ is necessary for transport. In addition, the transporters facilitate anion (Cl^-) flux across the membrane. This flux, which is stimulated by the binding

of glutamate and/or Na^+ to the transporter, follows strictly its own transmembrane electrochemical potential gradient. The amplitude of the chloride conductance varies between different transporters subtypes; the glutamate-elicited current in EAAT5 and EAAT4 is carried largely by chloride ions, in contrast to the small chloride conductance associated with GLAST, GLT1, and EAAC1. Glutamate-induced anion conductance may prevent the depolarizing action of glutamate transport by neutralizing the transported positive charge. It has also recently been shown that the anion current associated with the transport of glutamate by EAAT5 (located at the presynaptic terminal of the rod bipolar cell of the retina) hyperpolarizes the axon and inhibits synaptic transmission between these cells and the AII amacrine cells.

The affinity of glutamate for the transporters is high ($EC_{50} = 4$–$30\,\mu M$) and close to the affinity of glutamate for NMDA receptors or glutamate metabotropic receptors. Moreover, kinetics studies indicate that transporters bind glutamate as rapidly as AMPA receptors (~ 1 ms), allowing a signification pool of transporters to be engaged during glutamatergic synaptic transmission.

In the cortex, 80–90% of the transport is performed by astrocytes, which often ensheath synapses. In contrast to neurons, astrocytes possess many features that are optimized for efficient uptake, including high expression of transporters compared to neurons. In addition astrocytes have a very hyperpolarized resting membrane potential (~ -90 to -100 mV), increasing the gradient concentration of Na^+ and K^+ that drives transport of glutamate into the cells. They have also a low input resistance and do not fire action potentials, thus reducing the likelihood of large depolarizations and allowing the maintenance of the gradient of concentrations of Na^+ and K^+. Moreover, the astrocytic cytosolic concentration of glutamate is low because the transmitter is either converted into glutamine or transported into the mitochondria by symporters. In contrast to astrocytes, neurons can be more easily depolarized because of an higher input resistance (hundreds megaohms), their ability to generate action potentials, and the presence of large numbers of synapses (which give rise to ongoing synaptic events). In addition, the intracellular concentration of glutamate is much higher in glutamatergic neurons than in astrocytes because glutamate is not converted into glutamine or GABA.

Factors Affecting the Function of GluTs

Several factors have been shown to regulate the function of GluTs. This regulation may directly affect the synthesis and expression of the transporters at different levels: DNA transcription, RNA splicing, and turnover and trafficking of transporters from the endoplasmic reticulum to the membrane. The capacity of glutamate uptake may also be indirectly reduced when the gradient of Na^+, K^+, or H^+ is not maintained (e.g., during an hypoxic–ischemic episode when pH and intracellular ATP levels fall), or when glutamate accumulates in the cytosol.

GluTs and Epilepsy

Several studies performed in patients suffering from mesial temporal lobe epilepsy have shown that extracellular glutamate concentration rises at or before seizure onset, especially in the hemisphere where the seizures originate. Similar results have been obtained in chronic seizure animal models (e.g., kindled seizures, genetically epilepsy prone rats). These data have led to the hypothesis that the elevated extracellular concentration of glutamate is related to alterations of GluTs expression and function. Several studies have subsequently been performed in order to test this possibility, both in human temporal lobe epilepsy and animal models. These studies have given rise to some contradictory results. For example, studies of distribution of EAAT1 and EAAT2 in resected human sclerotic hippocampi (compared to non-sclerotic hippocampi) show a reduction of EAAT2, a slight or no reduction of EAAT1, or no change in these transporters. An increase or no change in EAAT3 mRNA (or protein) has also been described in hippocampal surgical specimens from patients with temporal lobe epilepsy. Similarly, there is no clear change in mRNA and protein expression of transporters in animal models of temporal lobe epilepsy (induced by kindling or by neurotoxins such as kainate or pilocarpine). An increase, a decrease, or no change of neuronal (EAAC1) and glial (GLT-1, GLAST) glutamate transporter expression has been reported. These contradictory results can probably be explained by rapid postmortem proteolysis of GluTs. Another approach to this issue, based on the deletion of the transporter genes (knockout mice) or the inactivation of the proteins (antisense treatments) has also been used in order to establish a link between an alteration of glutamate uptake and the generation of seizures. These experiments have also given rise to conflicting results; seizures were seen in the experimental animal groups in some studies but not in others. For example, the GLT-1 KO mice display spontaneous recurrent seizures and are more susceptible to pentylenetetrazol – sensitivities that have been attributed to an elevation of extracellular glutamate concentration. However, chronic administration of antisense nucleotide of GLT1 or GLAST in adult rats produced elevated extracellular levels of glutamate (associated with excitotoxicity) but no seizures. Similar results were seen with nucleotide antisense for the neuronal EAAC1 transporter; while antisense treatment resulted in epileptic seizures (hypothesized to be a consequence of decrease in GABA synthesis); the EAAC1 KO mice did not develop seizures. The reasons for these discrepancies are unclear.

GluTs Function is Essential for Normal Brain Development

A recent study has provided convincing evidence that glial glutamate transporter function is essential for normal brain development. In that study, deletion of both GLAST and GLT-1 genes in mice reduced cell proliferation and radial neuronal migration (leading to a disturbed laminar organization of the cortex). In addition, neuronal differentiation appears to be strongly affected in these mice. This cortical malformation seen in these animals involved, in part, the activation of both AMPA and NMDA receptors, since the $GLAST^{-/-}/GLT1^{-/-}$ phenotype was partially rescued by injection of ionotropic glutamate receptor antagonists. These results indicate that glutamate may act as a neurotransmitter at a very early stage of development, even before synapse formation; these actions of glutamate must be tightly controlled – by GLAST and GLT1 – for normal brain development. In keeping with this view are the following observations: (1) Immunoreactivity for glutamate in the maturing cortex is found in the neuroepithelium during neurogenesis, and later on is present in the intermediate zone (zone of migration), subplate, the cortical plate, and the marginal zone; (2) Functional glutamate receptors are expressed by neuronal precursor cells and neurons, in several structures, at a very early stage of development; (3) Glutamate is released before synapse formation by growing axons and growth cones; (4) GluTs are functional during the perinatal period and contribute to the clearance of glutamate; and (5) In the absence of efficient transport, glutamate diffuses through the extracellular space and activates distant cells in paracrine manner. All these factors suggested that GluTs play an important role in normal brain development, controlling the maturing cortical network activity when synaptogenesis is just getting started. Recent studies support this hypothesis and show that this control is fundamental, as it prevents the generation of seizures.

Methods

For in vitro experiments, we have used whole cell patch-clamp recordings of pyramidal cells and interneurons, together with field recordings, from neocortical (frontal, temporal, occipital) or hippocampal slices of Wistar rats (from embryonic E20 to postnatal day 7). For in vivo studies, we have employed video/EEG monitoring in freely moving rat pups. For the latter experiments, the animal was placed in a stereotaxic frame, and 1 µl of TBOA (5 mM per litre) or saline solution was injected in the left lateral ventricle (ICV injection) with a needle via a peristaltic pump. Two 1 mm-long tungsten electrodes fixed to a copper plug (100 µm diameter) were stereotaxically implanted at the surface of the somatosensory cortex. A reference electrode was implanted in the cerebellum. Two EEG signals were recorded, representing the potential between the reference and the recording electrodes. Signals were amplified (×1000), filtered at 0.16–97 Hz pass, acquired at 256 Hz using a time constant of 0.3 s and analyzed using Coherence 3NT program (Deltamed, France).

Results

GluTs During Development

Most of the studies concerning the function of GluTs and their role in neurologic disorders have been performed in adult brain. In contrast, there are few data available on their function in the developing brain, even though glutamate is known to play important roles in neuronal development. GluTs are expressed early in development, in fetal stages, in both human and rodent brain, suggesting that they may play important roles for brain maturation. Immunohistochemical and immunoblot analysis of EAAT1–4 performed between the seventeenth and twenty-fourth week of gestation in human brain showed that EAAT2 is the most prominent glutamate transporter subtype, highly expressed in cortex, basal ganglia, hippocampus, cerebellum, and thalamus. EAAT2 is rare in cells in the proliferatives zones, but is expressed in the intermediate zone and in the cortical plate. EAAT1 immunoreactivity is found in cortex, basal ganglia, and in the hippocampus, and is present in the proliferatives zones (ventricular and subventricular zones) on radial glial cells, and in the intermediate zone on the radial glial fibers. EAAT1 is also present on astrocytes localized within the cortical plate. EAAT1 and EAAT2 are found in the CA1 field and dentate gyrus of fetal hippocampus, and EAAT3 has been localized in cortex, basal ganglia, thalamus, hippocampus, and the cerebellum. EAAT3 distributes within the subplate and the cortical plate, on pyramidal cells and on the Cajal–Retzius cells of the marginal zone. EAAT4 is present in the cortex, hippocampus, and cerebellum. In rodent, the developmental expression of these four transporters has been found to be generally similar to the human pattern.

GluTs Control Early Network Activity: Their Inhibition Results in Slow Network Oscillations In Vitro

During the perinatal period, when synaptogenesis is just getting started, the ongoing cortical activity is composed of fast synaptic events mediated mostly by GABA (which is excitatory during this period of life) and – to a lesser extent – by glutamate. This cortical activity is often composed of recurrent network driven events of few hundred

milliseconds duration, called giant depolarizing potentials (GDPs). This pattern, observed in wide range of developing structures, reflects a high degree of synchrony in immature neurons in spite of the small number of functional synapses, and is thought to participate in activity-dependent growth and synapse formation. GDPs have been extensively studied in the hippocampus, where they appear to be initiated by a glutamatergic drive, and are mediated mostly by GABA receptors (in the neocortex they are mediated by glutamate receptors). This pattern of activity is developmentally regulated and disappears around the second postnatal week in rats and mice.

Two recent studies demonstrate that GluTs exert a powerful control of the activity of the maturing cortical neurons (neocortex and hippocampus). Using, DL-TBOA, a nontransportable inhibitor of neuronal and glial GluTs, investigators showed that a deficit of glutamate uptake rapidly transforms the ongoing activity into recurrent depolarization of several seconds duration associated with bursts of action potentials (Figure 1). This pattern of activity, referred to as slow network oscillations (SNOs), is generated during the first two postnatal weeks of life (but not thereafter), and is observed in both interneurons and pyramidal cells throughout the neocortex and hippocampus. SNOs display similar features in the two structures and are mediated by both NMDA and $GABA_A$ receptors, but require the activation of NMDA receptors for their generation; thus, SNOs are fully blocked by NMDA receptors antagonists. To summarize published and unpublished observations, the generation of SNOs proceeds as follows: (1) SNOs are initiated by the leakage of glutamate from glial cells. This leakage, unmasked when GluTs are not efficient, give rise to a sustained increase of [glutamate] in the extracellular space; (2) This sustained elevation of [glutamate] tonically activates NMDA and metabotropic glutamate receptors and depolarizes some glutamatergic neurons that display intrinsic recurrent bursting activity; (3) These burst discharging neurons periodically release glutamate into the extracellular space; this glutamate spreads out from synaptic clefts, leading to the activation of extrasynaptic NMDA receptors on both interneurons and pyramidal cells. The periodic diffusion of glutamate in the extracellular space thus leads to the coactivation of large population of cells within the cortical network, enabling these cells to generate bursts (of action potentials) that last for several seconds. From these studies, investigators concluded that GDPs and SNOs are initiated by the same glutamatergic drive, but that a deficiency in the transport of glutamate enlarges the range of action of this drive in the network, due to the diffusion of the glutamate released and extrasynaptic activation of NMDA receptors. This 'spill-over' engages large populations of neurons in rhythmic activities, including cells in which GDPs are ordinarily absent.

GluTs Prevent the Generation of Seizures in Rat Pups in vivo. Inhibition of GluTs Leads to a 'Suppression Burst Pattern' in the EEG and Partial Seizures

The consequence of dysfunction of glutamate transport on electrographic patterns has recently been investigated with video-EEG monitoring in freely moving postnatal

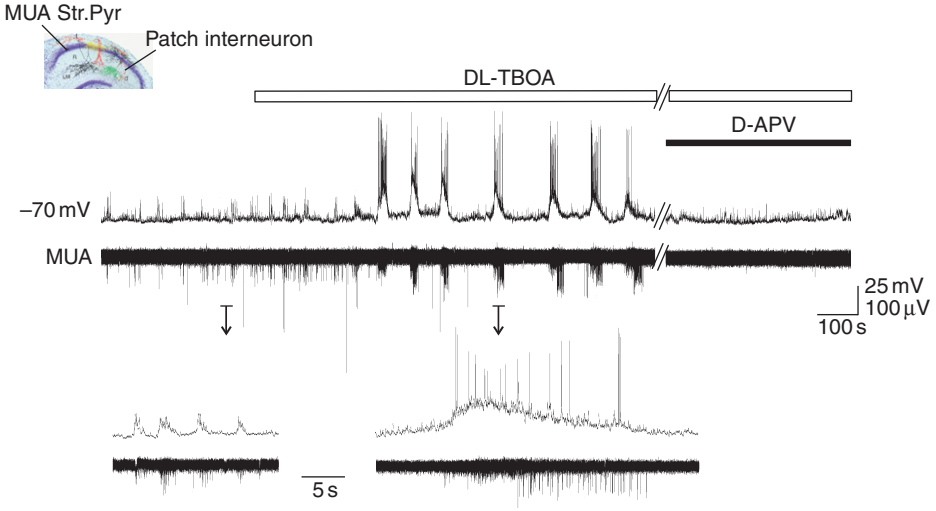


Figure 1 Inhibition of glutamate transporters generates slow network oscillations. Patch-clamp recording of an interneuron from P0 recorded in the stratum radiatum of the hippocampus (upper trace) in current-clamp mode and combined with the extracellular recording of multiple unit activity (MUA) with a pipette located in the stratum pyramidale (Str. Pyr., lower trace). The activity of the CA1 region is composed by GDPs and associated with MUA in the stratum pyramidale (showed at an expanded time scale below the two traces). Bath application of DL-TBOA (100 µM) generates SNOs. This pattern is blocked by D-APV (80 µM), an antagonist of NMDA receptor. The trace is shown 5 min after the application of APV. GDPs recovered 15 min after the washout of the drugs.

day (P)5-P7 rats after intracerebroventricular (ICV) injection of DL-TBOA (a glutamate transporter inhibitor) (see **Figure 2**). In control animals (untreated pups or ICV injection of vehicle), the EEG was composed of bilateral spindle burst (slow delta waves superimposed with faster alpha oscillations). The inhibition of GluTs in rat pups treated with TBOA imposes a singular pattern of activity, composed of recurrent bilateral paroxysmal bursts (a few seconds duration) that alternate with silent periods (also a few seconds in duration). The paroxysmal bursts were sometimes associated with erratic myoclonias. Moreover, partial seizures could also be recorded, as long lasting rhythmic delta–theta oscillatory activities in one hemisphere that were occasionally associated with behavioral manifestation (crawling, tonic posture of one or two limbs, freezing like posture, hypermotor activity). Intraperitoneal injection of NMDA receptor antagonists blocked myoclonias, the 'partial' seizures, and the paroxysmal EEG pattern. These clinical manifestations were not observed after ICV injection of other convulsive agents such as pilocarpine or kainate (which produced a status epilepticus) or after febrile seizures. Therefore, they appear to be related specifically to a deficiency of glutamate transport.

This clinical presentation is observed in some severe forms of early onset epilepsy, such as the early myoclonic encephalopathy with 'suppression burst' pattern. The EEG pattern consists only of complex bursts of spikes and sharp waves, and slow waves lasting 1–5 s, alternating with flat periods of 3–10 s during which there is practically no activity. The paroxysmal bursts can occur either synchronously or independently in the two hemispheres. Partial seizures are also observed in these children. Although the clinical relevance of the study in rat pups to this severe epileptic disorder requires further investigation, the observations do suggest that a deficient transport of glutamate may be a potential cause for early onset epilepsy, particularly those associated with a suppression-burst pattern EEG.

To date the etiology of early epileptic encephalopathy with suppression burst remains elusive. In 50% of patients, cortical malformations are observed with MRI; in others, genetic factors and/or inborn errors of metabolism (leading to a metabolic acidosis, to nonketotic hyperglycinemia, or to a defect of pyridox(am)ine– phosphate–oxydase activity) are thought to play a prominent role. Recently, a mutation in the gene encoding the mitochondrial glutamate/H(+) symporter GC1(*SLC25A22*) has been reported in patients with a suppression burst pattern EEG. The mutation of GC1 has been shown to impair mitochondrial glutamate import, suggesting that a defect of glutamate metabolism may play a major role in this pathology. One might speculate that this GC1 mutation also results in an alteration of glutamate uptake and transport by cell-surface GluTs. Theoretically, a link between the mitochondrial and cell-surface transporters could exist, since cell-surface GluTs are powered by transmembrane gradients of Na^+, K^+, H^+, and substrate, and an abnormal pool of glutamate (that may occur in the GC1 mutated cells) in addition to a rise of intracellular Na^+ concentration may prevent or reduce the uptake of the transmitter from the extracellular to the intracellular space – thus mimicking the action of TBOA.

Conclusions and Future Directions

It is widely accepted that the maturing brain is highly excitable as compared to the adult brain. In human, the

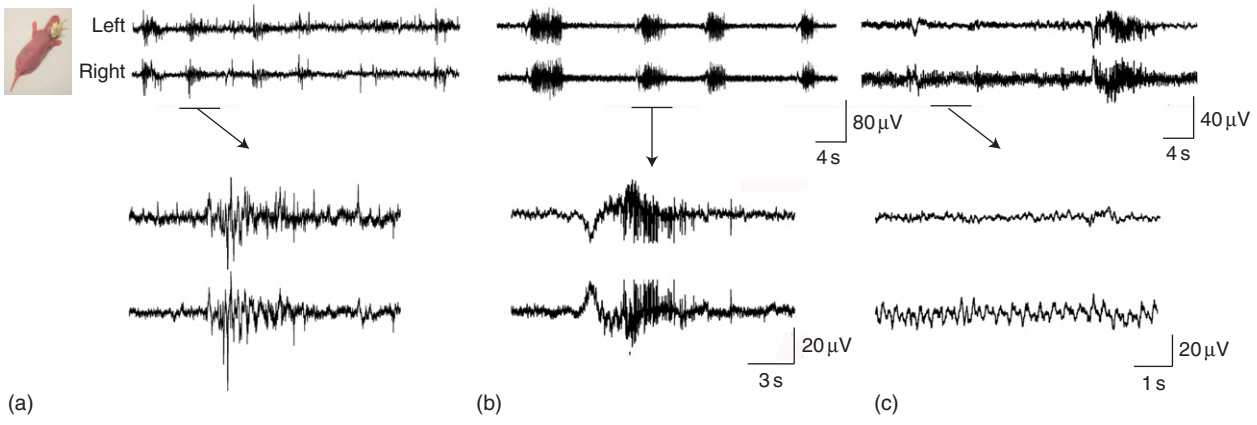


Figure 2 Suppression burst pattern and partial seizures are generated after inhibition of glutamate transporters in rat pups. EEG recording of a freely moving P5 old rat after ICV injection of saline solution (a) or DL-TBOA (b,c). Recording electrodes were placed in the frontal cortex of each hemisphere and the reference electrode was placed in the cerebellum. In the control rat, the EEG is composed of spindle burst (see Khazipov *et al*., 2004). In the TBOA treated rat, the EEG is composed by slow deflections followed by burst of spikes. These paroxysmal bursts occur in recurrent manner and alternate with silent period. (c) Example of partial seizures. Note the presence of slow oscillations only in one hemisphere. This activity was associated with freezing like posture.

incidence of seizures is significantly higher during the first year of life than during adulthood. In rodent, the susceptibility to convulsive agents is higher in the maturing than in adult brain. Studies performed on the brains of developing rodents suggest that several factors may contribute to this preferential hyperexcitability, including excitatory GABA action, high input resistance of cortical cells, and the presence of synchronized network driven events. All these features may be important for the normal development of the brain, but may also favor the generation of seizures. The early expression and function of GluTs are thus fundamental as they appear to tightly control glutamate at these early stages (before synapses are prominent), enabling the correct construction of cortical networks. At later stages of development, when functional synapses are established, these transporters are key to the prevention of paroxysmal activity.

Based on in vitro studies, it has suggested that GluTs reduce the impact of putative glutamatergic 'generators' of rhythmic activity on the network. By preventing or reducing the spread of glutamate released into the extracellular space, GluTs would act to limit the coactivation of neurons and would limit the number of cells that participate in network-driven activity. This control allows the brain to generate a physiological pattern of activity, and prevents specifically, the generation of a suppression burst EEG pattern. This control by GluTs raises the possibility that transporter dysfunction could be a cause of this highly pathological electrical activity in human brain. In this context, it will be important to determine if these transporters are inactivated by direct mutation, or if the metabolic disorders reported in patients suffering from early myoclonic encephalopathy (as a final consequence of their disorder) dysfunction of astrocytes and of GluTs. Finally, our ability to generate in vitro (in cortical slices) recurrent paroxysmal activity that shares similarities with the electrographic pattern in intact brain offers the investigator a rare opportunity to study, in a small circuit, the mechanism leading to the genesis of these recurrent paroxysmal activities. Such studies will lead to a better understanding of the pathophysiological basis of the suppression burst pattern.

Acknowledgements

The authors would like to acknowledge the "Fédération de la Recherche Sur le Cerveau (FRC)" for the financial support.

See also: **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; **Glia/Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters; **Pediatric Epilepsy**: Hippocampal EEG in the Neonatal Rat: Development of Normal and Pathological Activity; **Transporters**: Glutamate Transporters in Epilepsy.

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GABA Transporters: Regulation of Tonic Inhibition

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Introduction

$GABA_A$ receptors are located in the specialized neuronal membrane at synaptic connections, where they respond to GABA released from presynaptic cells (Ca^{2+}-dependent vesicle fusion) by the opening of a Cl^- permeable ion channel. This process underlies fast synaptic, or phasic, inhibition in the brain. $GABA_A$ receptors are also located in the extrasynaptic membrane, where there are no specialized structures for the rapid delivery of GABA to $GABA_A$ receptors, such as exists at synapses. Over the past decade, a great deal has been learned about these extrasynaptic $GABA_A$ receptors. Extrasynaptic $GABA_A$ receptors are molecularly, pharmacologically, and biophysically distinct from those at synapses, and are ideally suited to respond to the low concentrations of ambient [GABA] found in the extracellular space of the brain. The action of ambient [GABA] on these extrasynaptic receptors mediates a tonic conductance, or current, that has been termed 'tonic inhibition.' Tonic inhibition is present in many neurons throughout the brain, including the cerebellum, cortex, and hippocampus.

There are several potential sources of ambient [GABA] responsible for the tonic activation of extrasynaptic receptors, including escape of GABA from synapses after vesicular release, GABA release from nonselective anion channels in glia, and reversal of GABA transporters on neurons and glia. Even when GABA transporters are not working in the reverse direction, theoretical considerations and experimental evidence support an important role for GABA transporters in regulating the ambient [GABA] concentrations that act on extrasynaptic receptors to cause tonic inhibition. This role of GABA transporters is the focus of our discussion.

The expression and function of GABA transporters and the tonic GABA currents they regulate are altered in some epilepsy syndromes and contribute to the actions of many antiepileptic drugs. The continuous inhibitory charge movements and reductions of input resistance produced by tonic inhibition, have significant effects on cellular and network excitability. Indeed, genetic deletion of $GABA_A$ receptor subunits that mediate tonic inhibition in pyramidal neurons of hippocampus results in spontaneous epileptiform discharges. Understanding the physiologic actions of GABA transporters is, therefore, highly relevant to our understanding and treatment of epilepsy and seizures.

Background

GABA Transporter Subtypes

In mammalian brain, there are four cloned isoforms of GABA transporters: GAT-1, GAT-2, GAT-3, and BGT-1 (betaine/GABA transporter). These isoforms have distinguishing pharmacology (see below) and unique cellular localizations. GAT-1 is the primary GABA transporter in the neurons throughout the brain, but is found in glia as well. The expression of GAT-1 is highest in axons and presynaptic terminals. The work of Quick and colleagues has shown that surface expression of GAT-1 can be rapidly regulated by extracellular GABA, and that GAT-1 containing vesicles are mobilized to presynaptic membranes during synaptic events. These findings suggest a dynamic role for GAT-1 in the removal of synaptically released GABA. GAT-3 is primarily expressed in glia. Glial GABA transporters encounter synaptically released GABA when it escapes the synaptic cleft, and contribute to its removal before it diffuses further. GAT-2 expression is most prominent in leptomeningeal cells and ependymal cells, but GAT-2 immunoreactivity has also been identified in the processes of neurons and glia. BGT-1 expression is found in several brain regions, primarily in astrocytes, and in tissues outside the central nervous system including liver and kidney. There exists significant variation in BGT-1 expression patterns across species. We must note that the nomenclature of GABA transporters differs between mice and other species, and this difference can complicate the review of the literature; in mice, GABA transporters are named GAT1-4, and GAT-2 is the same molecule as BGT-1. GAT-2 and GAT-3 in rats and humans are equivalent to GAT-3 and GAT-4 in mice, respectively. In this review we will use the rat (and human) terminology.

Antagonists of GABA transporter function have been indispensable to evaluate the roles of GABA transporters in phasic and tonic inhibition. Commonly used research tools to inhibit GABA transporters include nipecotic acid, SKF-89976a, NO-711, SNAP-5114, and the clinically used anticonvulsant tiagabine. The drugs SKF-89976a, NO-711, and tiagabine are all selective inhibitors of GAT-1. SNAP-5114 inhibits GAT-2 and GAT-3. Nipecotic acid, a structural analog of GABA, blocks the uptake by GAT-1, GAT-2, and GAT-3, but unlike the other agents, it also induces the release of GABA through a process termed 'heteroexchange.' β-alanine is a transportable

substrate for GABA transporters and therefore functions as a competitive antagonist of GAT function. The pharmacology of GABA transporters is discussed more thoroughly elsewhere in this volume.

Biophysical Properties of GABA Transporters and Dynamic Equilibrium

GABA transporters belong to the superfamily of Na^+- and Cl^--dependent co-transporters that harness the potential energy stored in the steep transmembrane sodium gradient (established by the Na^+/K^+-ATPase) to move the substrate from extracellular to intracellular compartments. This secondary active transport process allows a substrate, in this case GABA, to move against its concentration gradient. GABA transporters translocate two Na^+ ions, one Cl^- ion, and one electroneutral, zwitterionic (a zwitterion is a molecule with two or more dissimilarly charged atoms but no net charge) GABA molecule every thermodynamic reaction cycle (see **Figure 1**). Thus, one net charge is transferred to the intracellular compartment during an uptake cycle by the electrogenic GABA transporter. The translocation of GABA, however, may also occur in the reverse direction leading to the release of GABA into the extracellular space. GABA transporters do not function like vacuum cleaners, relentlessly taking up GABA to drive $[GABA]_o$ to zero; instead, they exist in a state of dynamic equilibrium. The direction and magnitude of GABA transport is a direct and predicted consequence of the prevailing thermodynamic conditions,

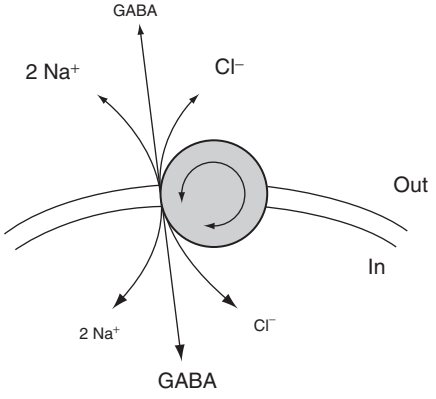


Figure 1 Schematic of GABA transporter. During each transport cycle two sodium ions, one chloride ion, and one GABA molecule undergo coupled translocation. Note that transport can occur in both the forward or reverse direction. The GABA transporters GAT-1, GAT-2, and GAT-3 share this stoichiometry. The betaine/GABA transporter BGT-1 differs in that it translocates three sodium ions per transport cycle. Reproduced from Richerson GB and Wu Y (2003) Dynamic equilibrium of neurotransmitter transporters: Not just for reuptake anymore. *Journal of Neurophysiology* 90: 1363–1374, with permission from American Physiological Society.

determined by membrane potential and the intracellular and extracellular concentrations of Na^+, Cl^-, and GABA. Quantification of the driving force for GABA transport with varying substrate concentrations and membrane potentials is a useful and essential exercise to understand GABA transporter behavior.

The driving force for transmembrane solute movement mediated by transporters and ion channels is a function of the electrochemical potential ($\Delta\mu$) for a given solute, the electrochemical potential being the sum of the electrical potential ($\Delta\psi$) and chemical potential (ΔG). If we were to consider a single molecular or ionic species X, this force is described by the following equation:

$$\Delta\mu_x = \Delta\Psi_x + \Delta G_x = z_x \times F \times E_m + RT \ln \frac{[X]_{in}}{[X]_{out}} \quad [1]$$

where z_x is the valence, F the Faraday's constant, E_m the membrane potential, R the universal gas constant, and T the absolute temperature. When this equation is solved for equilibrium conditions, when $\Delta\mu_x = 0$, it becomes the Nernst equation and describes the equilibrium membrane potential for a given set of intracellular and extracellular solute concentrations. For a GABA transporter such as GAT-1, however, because the translocation of two Na^+ ions, one Cl^- ion, and one GABA molecule are usually linked, the electrochemical potential for this reaction as a whole is described as follows:

$$\Delta\mu_{GAT-1} = 2 \times (\Delta\Psi_{Na} + \Delta G_{Na}) + 1 \times (\Delta\Psi_{Cl} + \Delta G_{Cl}) + 1 \times (\Delta G_{GABA}) \quad [2]$$

Under equilibrium conditions, when $\Delta\mu_{GAT\text{-}1} = 0$ and there is no net force for GABA movement, this equation reduces to:

$$E_m = -\frac{RT}{(2Z_{Na}Z_{Cl})F} \ln \left[\frac{[GABA]_{in}}{[GABA]_{out}} \left(\frac{[Na^+]_{in}}{[Na^+]_{out}} \right)^2 \frac{[Cl^-]_{in}}{[Cl^-]_{out}} \right] \quad [3]$$

This equation defines the reversal potential for GABA transporters, the membrane potential at which GABA movement will change from influx to efflux. The validity of this equation has been experimentally verified. Solving this equation using realistic concentrations of intracellular and extracellular substrate for neurons under normal conditions, we find that the equilibrium potential for GABA transport is −62 mV, close to the resting potential for many neurons (see **Figure 2**). Thus, under control conditions with a membrane potential of −62 mV, there is no net movement of GABA. However, control conditions in neurons are dynamic, and small membrane hyperpolarizations will lead to GABA influx and a subsequent reduction of $[GABA]_o$ towards a new equilibrium value, while small depolarizations produce GABA efflux and a rise in $[GABA]_o$ towards a new equilibrium value.

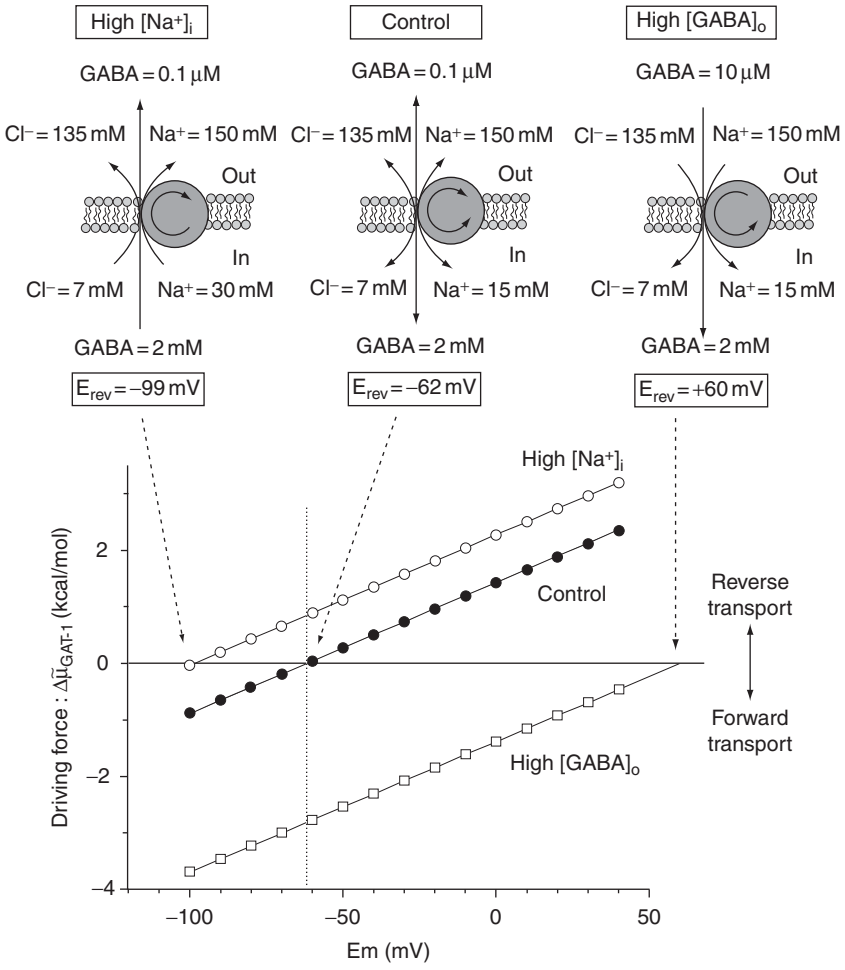


Figure 2 Thermodynamic conditions determined by transmembrane concentration gradients for substrate and membrane potential control the direction and magnitude of GABA transport by GAT-1. The sum of electrochemical potentials for all the transported solutes, μ_{GAT}, represents the driving force for GABA transport. Based on eqn. [2] in the text, this driving force is plotted for three different conditions illustrated at the top of the figure. Under control conditions the calculated reversal potential, the membrane potential at which transport changes direction from influx (forward transport) to efflux (reverse transport), is −62 mV. This is close to the typical resting membrane potential of many neurons indicating that GABA transporter reversal under physiologic conditions will occur in response to small depolarizations. If $[Na^+]_i$ becomes elevated the reversal potential will become more negative and a driving force for reverse transport of GABA will occur at typical resting potentials. In the 'high $[Na^+]_i$' situation this is illustrated as an increase from 15 to 30 mM, in the range of $[Na^+]_i$ changes that occur in hippocampal neurons in response to spontaneous action potentials. Elevations in $[GABA]_o$ will produce positive shifts in the reversal potential and a driving force for forward transport and GABA uptake at normal resting potentials. With $[GABA]_o$ of 10 μM the reversal potential for GABA transport is +60 mV so GAT-1 will always work in the forward direction at membrane potentials that would typically exist in neurons. GABA transporters will dynamically adjust their rate and direction of transport towards new equilibrium values in response to the changes in membrane potential and substrate concentrations accompanying action potentials and synaptic events. Calculations made for 35°C.

Alternatively, increases in intracellular sodium, such as that occurring in neurons during bursts of action potentials or during glutamatergic synaptic transmission, cause the reversal potential for GABA transport to shift towards more negative values, favoring GABA efflux at typical resting membrane potentials. The 'High $[Na^+]_i$' situation illustrated in **Figure 2** shows that an increase in $[Na^+]_i$ from 15 to 30 mM will shift the reversal potential to −99 mV. Changes in the intracellular chloride concentration would have similar, but smaller, effects as those of sodium concentration changes, because only a single Cl^- ion is transported during GABA uptake. In the presence of high $[GABA]_o$, the reversal potential for transport will become more positive – favoring uptake. In the example given in **Figure 2**, $[GABA]_o$ of 10 μM results in a reversal potential of +60 mV. During GABAergic synaptic transmission, when $[GABA]_o$ may transiently reach as high as 1 mM, the reversal potential for GABA transport would be +184 mV. Thus, after synaptic release of GABA there would be a large driving force for reuptake.

These calculations are made for static equilibrium conditions, but the brain does not work as a static system. In reality, GABA transporters of neurons and glia are dynamically adjusting the rate and direction of GABA transport towards new equilibrium values in the face of constant changes in membrane potential and substrate concentrations (resulting from vesicular release of GABA, action potentials, and synaptic potentials). For example, spontaneous action potentials and synaptic transmission can increase intracellular sodium in the soma of hippocampal neurons by as much as 19 mM for durations of several minutes; the rises in intracellular sodium occurring in restricted compartments, such as dendritic spines, may be much larger. This dynamic equilibrium of GABA transporters allows effective uptake of synaptically released GABA to terminate synaptic events and to reclaim GABA. It also permits transporter reversal under physiologic conditions. Biochemical and electrophysiological experiments have demonstrated nonvesicular release of GABA in response to a variety of stimuli (see **Figure 3**). This GABA release is nonvesicular because it can occur after blocking vesicular release of neurotransmitter (e.g., with tetanus toxin or zero Ca^{2+} solution), and is sensitive to GABA transporter inhibitors. GABA transporter reversal is predicted to be augmented under pathologic conditions such as ischemia or seizures, when there is collapse of sodium gradients due to energy failure and widespread depolarization related to high-frequency action potential firing and excessive extracellular potassium accumulation.

Methods

Approaches employed to obtain results on GABA transporters and tonic inhibition described in this review include electrophysiology, biochemical assays, immunohistochemical techniques, as well as the use of transgenic animals.

Electrophysiology

Voltage-clamp experiments to record membrane currents from neurons utilize whole-cell patch-clamp recordings with relatively low resistance microelectrodes (3–5 MΩ) to establish a high-resistance seal (10^9 Ω) between the electrode and plasma membrane. The 'patch' of membrane contained within the electrode tip is then ruptured to allow electrical and physical access to the cell, thus providing control of the composition of both the intracellular and extracellular solutions during the experiment. This methodology has been applied to neurons in tissue culture, neurons in acute brain slices, and cloned $GABA_A$ receptor proteins heterologously expressed in mammalian cell lines. Tonic inhibition is defined as the change in holding current produced by application of $GABA_A$ receptor antagonists (e.g., bicuculline or gabazine). Measurement of tonic inhibition under different conditions allows us to evaluate the contribution of various mechanisms to ambient [GABA] concentrations; these mechanisms (and conditions used to inhibit them) include synaptic release (zero extracellular Ca^{2+}/EGTA) and GABA transport [SKF-86671 (GAT-1) and SNAP-5114 (GAT 2/3)]. Alterations of intracellular concentrations of Na^+ and GABA occur during patch clamp recordings and in response to the influence GABA transporter function; therefore, inclusion of varying concentrations of Na^+ or GABA in pipette solutions, or recording from non-dialyzed cells with perforated-patch clamp methods, is used to overcome this artificial effect of whole-cell recordings on intracellular constituents. GABA transporters are electrogenic, so there are membrane currents associated with their activity that can be detected with voltage-clamp methods.

Biochemical Assays

Measurements of GABA uptake and release may be made with biochemical methods. Exposure of cells to radiolabeled [H^3]-GABA for set periods of time, and then washing cells in ice-cold solution (to remove excess isotope and to attenuate/stop release processes), allows the investigator to quantify GABA uptake by counting emission from the radioisotope. GABA concentrations may also be measured in samples of extracellular fluid with high-performance liquid chromatography. Determination of uptake and GABA concentrations with these methods allows pharmacologic characterization of uptake and release mechanisms. In addition, performing these experiments with zero extracellular calcium, or on cells treated with concanamycin (a H^+-ATPase inhibitor that prevents the pH-dependent packaging of neurotransmitter into synaptic vesicles), provides a means of distinguishing nonvesicular (transporter-mediated) from vesicular release mechanisms.

Results – Regulation of Tonic Inhibition by GABA Transporters

Extrasynaptic $GABA_A$ Receptors and Tonic Inhibition

Spontaneous tonic currents sensitive to $GABA_A$ receptor antagonists exist in cerebellar granule cells (CGCs), dentate gyrus granule cells (DGGCs), and pyramidal cells of hippocampus and cortex. These observations have generated considerable effort from many laboratories to identify and characterize the receptors responsible for this novel form of inhibition. Studies in the cerebellum have

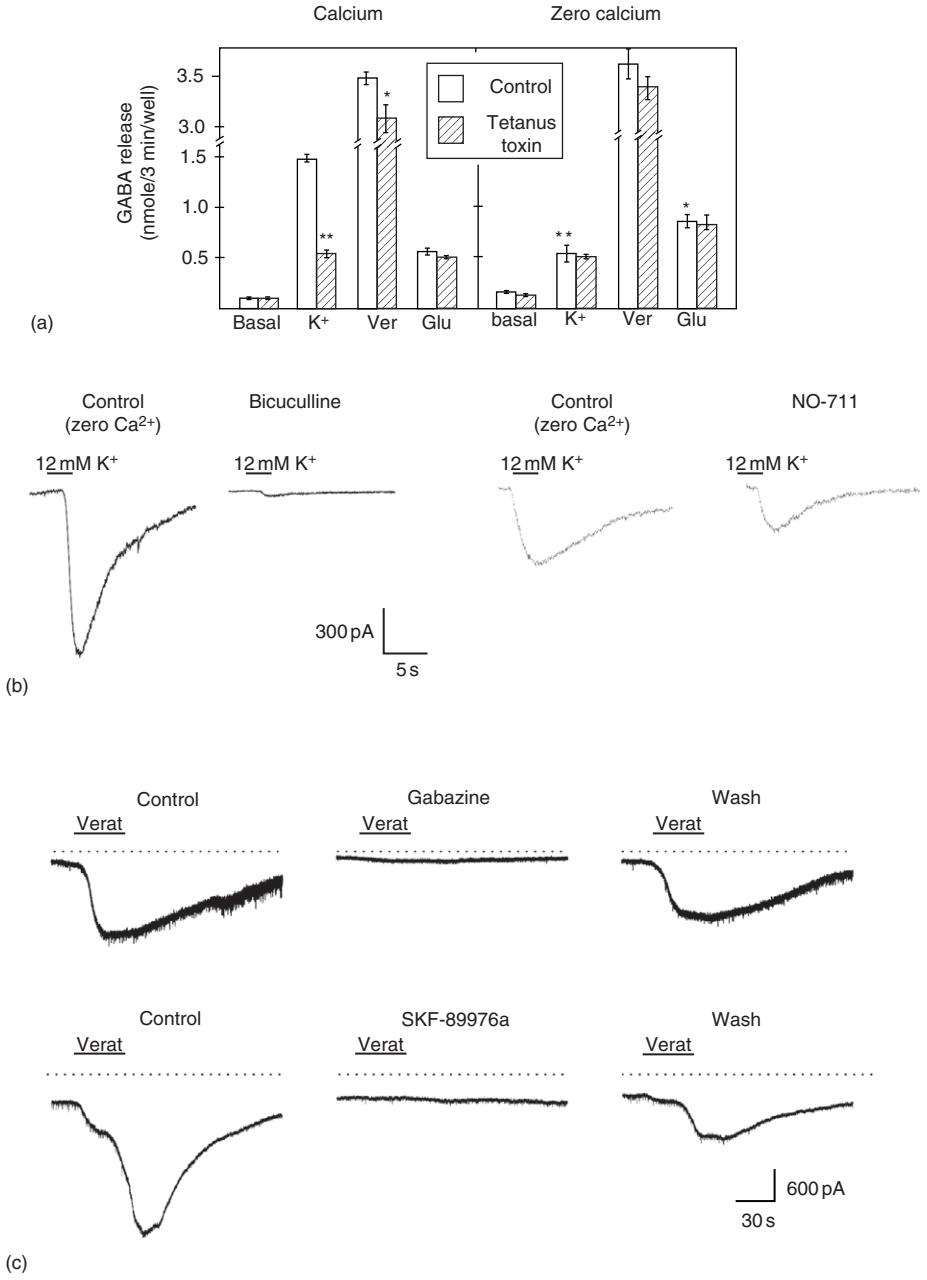


Figure 3 GABA transporter reversal. (a) Biochemical detection of nonvesicular GABA release. With or without extracellular calcium, and/or in the presence of tetanus toxin (maneuvers known to block vesicular release), GABA release from mouse neurons occurred in response to depolarization with 55 mM K^+, the sodium channel opener veratridine (ver), and glutamate. Reproduced from Pin JP and Bockaert J (1989) Two distinct mechanisms, differentially affected by excitatory amino acids, trigger GABA release from fetal mouse striatal neurons in primary culture. *Journal of Neuroscience* 9: 648–656, with permission from Society for Neuroscience. (b) Electrophysiological detection of nonvesicular GABA release in response to depolarization. During patch clamp recordings from hippocampal neurons in the absence of extracellular calcium, application of 12 mM K^+ evoked currents that were completely blocked by bicuculline and partially blocked by the GAT-1 inhibitor NO-711. Transporters other than GAT-1 or other mechanisms may be contributing to the nonvesicular release of GABA seen here in response to elevated K^+. The membrane potential (V_m) was −60 mV and E_{Cl} = 0 mV so $GABA_A$ receptor activation produced inward currents. Reproduced from Gaspary HL, Wang W, and Richerson GB (1998) Carrier-mediated GABA release activates GABA receptors on hippocampal neurons. *Journal of Neurophysiology* 80: 270–281, with permission from American Physiological Society. (c) GABA transporter reversal in response to elevations of intracellular Na^+. Acute application of the sodium channel opener veratridine induced gabazine- and SKF-89976a-sensitive currents in hippocampal neurons. Horizontal bars in panels (b) and (c) indicate period of drug application. These experiments were performed in zero Ca^{2+} solutions. V_m = −60 mV, E_{Cl} = 0 mV. Reproduced from Wu Y, Wang W, and Richerson GB (2006) The transmembrane sodium gradient influences ambient GABA concentration by altering the equilibrium of GABA transporters. *Journal of Neurophysiology* 96: 2425–2436, with permission from American Physiological Society.

been instrumental in advancing our understanding of extrasynaptic receptors that mediate tonic inhibition. Evidence for differential sorting of $GABA_A$ receptor subtypes to synaptic and extrasynaptic membranes came from biophysical studies that identified a uniform single channel conductance of 28 pS for synaptic receptors, in contrast to extrasynaptic receptors which displayed multiple conductance levels. Studies of recombinant $GABA_A$ receptor subunit combinations believed to exist in the cerebellum found specific subunit combinations ideally suited to continuously respond to low levels of ambient [GABA] and mediate tonic inhibition. For example, receptors containing α6 and δ subunits have high affinity for GABA, responding to $[GABA]_o$ as low as 30 nM with EC_{50} values of 0.2–0.4 μM; further, they display little desensitization in the continued presence of agonist. Tonic currents in granule cells and interneurons of the dentate gyrus are also mediated by extrasynaptic receptors containing δ subunits. The δ subunits are of particular interest as these confer unique pharmacology to the receptors mediating tonic currents in some areas of the brain. For example, δ subunits are the target of socially relevant concentrations of alcohol. Low concentrations of ethanol (10–30 mM) potentiate tonic currents in DGGCs and CGCs, and are modulated by neurosteroids. As expected, due to their high affinity for GABA, extrasynaptic receptors and tonic inhibition are blocked by concentrations of the competitive antagonist gabazine that are much higher than needed to completely inhibit phasic inhibition. Additionally, extrasynaptic receptors containing α6 subunits in cerebellum are distinguished by insensitivity to the benzodiazepine diazepam, and to inhibition by furosemide. The main extrasynaptic receptor in CGCs responsible for tonic inhibition is believed to have an α6β3δ subunit composition. Tonic inhibition is present in hippocampal pyramidal cells of areas CA1 and CA3, but is mediated by extrasynaptic receptors with a different subunit combination than that seen in CGCs or DGGCs (not including the δ subunit). The tonic current in these pyramidal cells is insensitive to modulation by low concentrations of alcohol or neurosteroids, and is reduced or absent in mice lacking the α5 subunit.

Tonic inhibition significantly impacts neural processing and behavior. In fact, the total charge transfer due to tonic inhibition is at least threefold larger than that produced by exocytotic release of GABA in response to high frequency stimulation. The firing rate and gain of neurons is modified by tonic inhibition in vitro and in vivo. Modulation of extrasynaptic receptors by neurosteroids has been implicated in catamenial epilepsy and pubertal anxiety. The neurobiological and behavioral effects of socially relevant concentrations of ethanol are due to potentiation of tonic currents mediated by δ subunit-containing extrasynaptic receptors in cerebellum and other areas of the brain. All of these actions of tonic inhibition are ultimately dependent on the ambient [GABA].

Ambient [GABA]

Tonic inhibition is the result of a finite concentration of GABA being present in the extracellular space. Some $GABA_A$ receptors can spontaneously open in the absence of an agonist, but these events occur with a low frequency and are not believed to contribute significantly to tonic inhibition normally recorded in neurons. The primary mechanism of GABA release into the extracellular space is calcium-dependent exocytosis at presynaptic terminals in response to action potentials. The GABA released in this way is meant to rapidly and transiently act on adjacent postsynaptic receptors, causing phasic inhibition. GABA transporters on presynaptic neurons (i.e., GAT-1) and glia (GAT-3) limit the diffusion of GABA away from the synaptic cleft. However, the control of GABA diffusion provided by this system is incomplete, allowing spillover of GABA from synapses where it acts on extrasynaptic receptors to cause tonic inhibition (see the following section). The levels of phasic and tonic inhibition have been experimentally correlated in area CA1 of hippocampus, confirming a contribution of vesicular release of GABA to ambient [GABA] levels. However, tonic inhibition in the cerebellum and hippocampus persists in the presence of TTX to block action potentials and following the blockade of synaptic vesicle release by removal of extracellular calcium, synaptobrevin cleavage with tetanus toxin, or concanamycin inhibition of the vesicular H^+-ATPase that normally establishes the proton gradient responsible for loading neurotransmitter into synaptic vesicles (see Figure 4(a)). In the developing cerebellum, the level of tonic inhibition in young animals is regulated by action potential and calcium-dependent vesicular release, but not in adult animals where nonvesicular release of GABA dictates ambient [GABA]. Nonvesicular release of GABA (and other amino acids) through nonselective anion channels of glia can occur in response to cell swelling or Ca^{2+} waves. Additionally, GABA can be liberated into the extracellular space by reverse operation of GABA transporters of glia and neurons. This latter means of nonvesicular release can occur under physiological conditions, but will be increased during pathologic states such as seizures (when extracellular sodium can decrease by 10–15 mM with concomitant rises in intracellular sodium). The pivotal role of GABA transporters in determining ambient [GABA], and therefore tonic inhibition, is complex and will be discussed in the following section.

GABA Transporters and Tonic Inhibition

In the traditional model of phasic GABAergic inhibition, the time course of an inhibitory postsynaptic potential is determined by the kinetics of $GABA_A$ receptors and removal of GABA from the synaptic cleft. This removal is due to diffusion and uptake by GABA transporters on

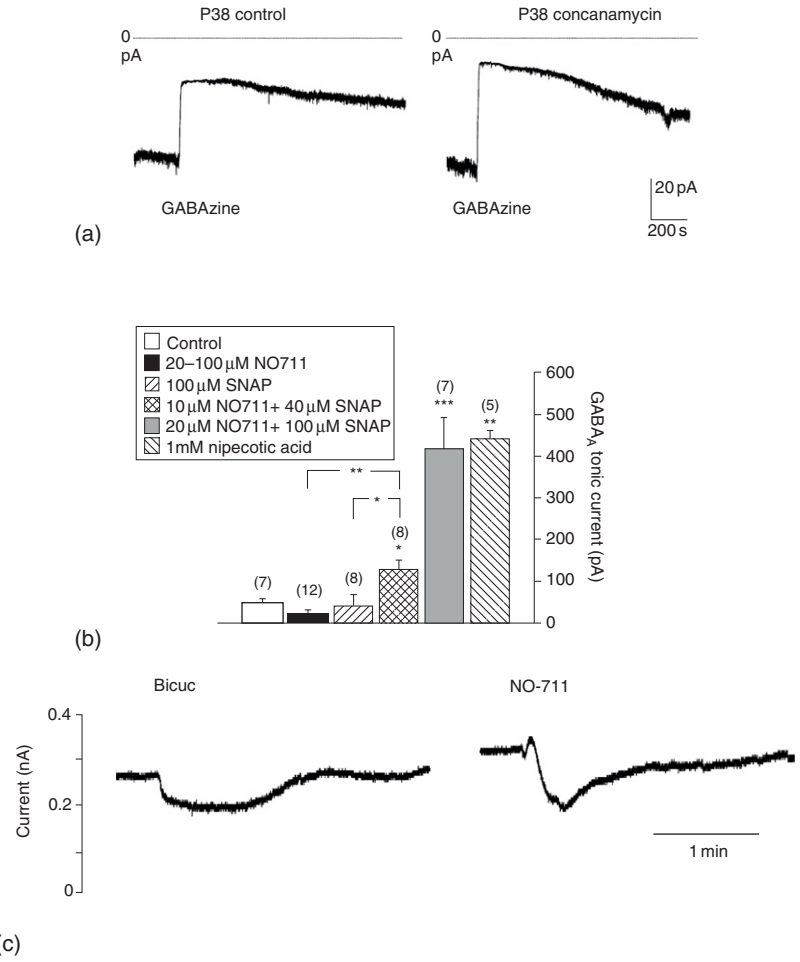


Figure 4 The effects of blocking vesicular GABA release or GABA transporters on tonic inhibition. (a) Patch clamp recordings of cerebellar granule cells from postnatal day 38 rats. Tonic currents recorded at −60 mV in cerebellar granule cells were blocked by gabazine and the amplitude of the gabazine-sensitive tonic GABA current was unaltered following inhibition of vesicular release of neurotransmitter with the vesicular H^+-ATPase antagonist concanamycin. Reproduced from Rossi DJ, Hammann M, and Attwell D (2003) Multiple modes of gabaergic inhibition or rat cerebellar granule cells. *Journal of Physiology* 548: 97–110, with permission from Wiley-Blackwell Publishing. (b) Summary of the effects of GABA transporter antagonism on tonic inhibition in rat neocortex. Tonic currents were not significantly affected by GAT-1 antagonism with NO-711 or GAT-2/3 antagonism with SNAP-5114 in isolation. Inhibition of GAT-1 and GAT-2/3 simultaneously resulted in large increases in tonic current amplitude. Reproduced from Keros S and Hablitz JJ (2005) Subtype-specific GABA transporter antagonists synergistically modulate phasic and tonic GABAA conductances in rat neocortex. *Journal of Neurophysiology* 94: 2073–2085, with permission from American Physiological Society. (c) Gramicidin-perforated patch recording from hippocampal neurons with zero extracellular Ca^{2+}. During these recordings using the perforated-patch configuration, GAT-1 inhibition with NO-711 reduced tonic inhibition, indicating that GABA transporters were working in reverse under these conditions. In these perforated-patch recordings intracellular contents are undisturbed and intracellular Cl^- concentration is low so tonic currents are outward. Reproduced from Wu Y, Wang W, and Richerson GB (2006) The transmembrane sodium gradient influences ambient GABA concentration by altering the equilibrium of GABA transporters. *Journal of Neurophysiology* 96: 2425–2436, with permission from American Physiological Society.

the presynaptic neuron (which must reclaim GABA molecules for repackaging into synaptic vesicles) and perisynaptic membranes (both neurons and glia). This model has fostered the view of GABA transporters as vacuum cleaners that continually operate in the forward direction to bring $[GABA]_o$ close to zero. This model also leads to the conclusion that tonic inhibition is due to GABA molecules that are released at synapses and 'escape' the action of transporters and are thus 'freed' to act on extrasynaptic receptors. The experimental work of many laboratories, demonstrating increased tonic currents after pharmacologic inhibition or genetic deletion of GABA transporters, has strengthened the view that GABA transporters normally serve to limit GABA spillover to extrasynaptic sites and reduce tonic inhibition. The concerted action of presumed neuronal/synaptic GAT-1 and glial/perisynaptic GAT-3 in the control of tonic currents was strikingly demonstrated in neocortex, where inhibition of both types

of transporters was necessary to increase tonic inhibition (see **Figure 4(b)**). However, the persistence of tonic currents in multiple brain regions following blockade of vesicular GABA release compellingly demonstrates that vesicular release of GABA, although an important source of GABA in the extracellular space, is not the sole source of ambient [GABA].

What are the nonvesicular sources of GABA? Astrocytes may release significant quantities of GABA; studies in embryonic hippocampal neurons found that neuronal co-culture with astrocytes, or their treatment with astrocyte-conditioned media, could produce $GABA_A$ receptor currents that were not significantly different from those evoked with 3 μM exogenous GABA. The mechanism for GABA release from glia is not firmly established but it is calcium-independent and may occur through nonselective anion channels, reverse transport, or hemichannels (unpaired gap-junction connexin proteins that may function like an ion channel). GABA release by neuronal transporters is predicted (by eqn. [3]) to occur readily in response to depolarization or intracellular sodium rises that occur under physiologic conditions. Although GABA transporters are often found to limit ambient [GABA] and tonic inhibition, under some conditions – such as during gramicidin-perforated patch recordings, when intracellular contents of cells are undisturbed – blockade of GABA transporters can reduce tonic inhibition (see **Figure 4(c)**). In this situation, reversal of transport contributes to ambient [GABA].

Based on the theoretical considerations discussed above, a more accurate view of the control of ambient [GABA] and tonic inhibition is that GABA transporters set a lower limit for ambient [GABA] governed by the prevailing thermodynamic conditions. Equation [3] can be rearranged to solve for equilibrium values of ambient [GABA], and the solutions to this equation with four different intracellular GABA concentrations is plotted as a function of membrane potential in **Figure 5** (using realistic, experimentally determined values for ion concentrations, as indicated in the figure). These curves demonstrate that the equilibrium value for ambient [GABA], the ambient [GABA] that GABA transporters will dictate even in the absence of vesicular release, is in the range that will activate high-affinity, extrasynaptic $GABA_A$ receptors. Intracellular [GABA] in hippocampal neurons is not known precisely, but values ranging from 1 to 6 mM have been reported for mammalian neurons. If we consider intracellular [GABA] of 2 mM as an average value, ambient [GABA] would range between 0.1 and 0.2 μM for membrane potentials of −60 to −40 mV, respectively. These values are in excellent agreement with results from experiments on tonic inhibition in CGCs, where ambient [GABA] was estimated to range between 90 and 250 nM. Elevation of intracellular GABA, such as occurs following GABA transaminase inhibition with the anticonvulsant vigabatrin, alters the equilibrium of GABA transporters, causing an increase in ambient [GABA] and tonic inhibition.

Summary and Future Directions

Tonic inhibition is a novel form of GABAergic signaling mediated by extrasynaptic $GABA_A$ receptors with distinct

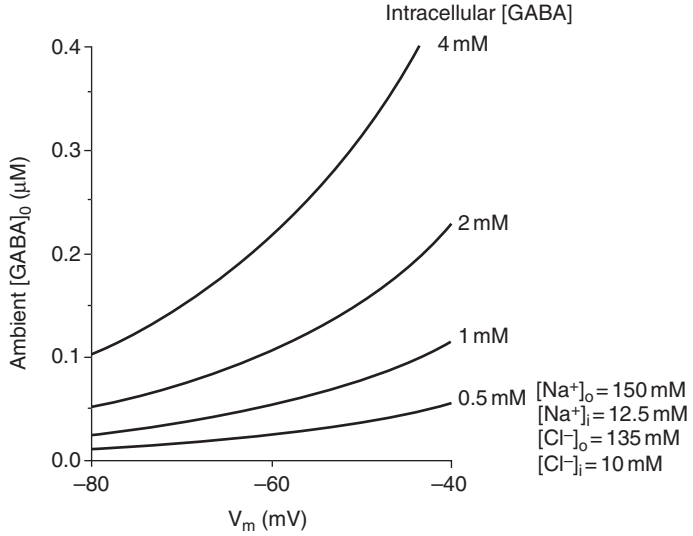


Figure 5 Equilibrium values of ambient [GABA] for GAT-1 as a function of membrane potential. Substrate concentrations are indicated in the figure. Solid lines are drawn for four different values of intracellular [GABA] according to eqn. [3] in text, rearranged for $[GABA]_o$. At −60 mV with $[GABA]_i$ of 2 mM (a low average of published values for $[GABA]_i$ in neurons), GABA transporters at equilibrium would attempt to clamp ambient $[GABA]_o$ at 0.1 μM. This equilibrium value is well within the range needed to tonically activate high-affinity, extrasynaptic $GABA_A$ receptors and consistent with experimental estimates of ambient [GABA].

biophysical and pharmacological properties. Extrasynaptic $GABA_A$ receptors have high affinity and are capable of responding to the low ambient extracellular GABA concentrations in brain extracellular space, on the order of 10^{-7} M. Under physiologic conditions at typical resting membrane potentials, GABA transporters exist near equilibrium and rapidly adjust their rate to take up synaptically released GABA and limit 'spillover' of GABA released at synapses. However, they will also export GABA in response to small depolarizations or changes in ion concentrations. Most importantly, GABA transporter thermodynamics render them incapable of lowering ambient [GABA] below the level needed to activate extrasynaptic receptors and cause tonic inhibition, even in the absence of continued synaptic, vesicular release of transmitter.

As many as 30–40% of patients with epilepsy continue to have seizures despite best medical therapy. The continued expansion of our knowledge of the mechanisms contributing to hyperexcitability – including GABA transporter expression, function, and the regulation of tonic inhibition – has the potential to improve this statistic. GABA transporters remain a promising target in the field of drug discovery for treatment of seizures. Additionally, drugs designed to potentiate tonic inhibition, such as the neurosteroid ganaxolone, have proven effective in several animal models of epilepsy and are currently undergoing phase II trials in humans.

See also: **Antiepileptic Drugs**: GABA Transporters as Targets of Antiepileptic Drugs; **GABA**: Differential Alterations of $GABA_A$ Receptors in Epilepsy; $GABA_A$ Receptor Plasticity in Epileptogenesis; **Hormones and Gender**: Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain; **Transporters**: GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability.

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GABA Transporters: Structure, Oligomerization, Trafficking, and Pharmacology Determine Neuronal Excitability

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Introduction

Physiological Roles of the GABA Transporters

The canonical role of GABA transporters (GATs) is to remove GABA from the synapse following phasic neurotransmitter release, terminating GABAergic neurotransmission. GABAergic transmission is usually inhibitory, because $GABA_A$ and $GABA_B$ receptors activate Cl^- and K^+ channels, respectively (although some young neurons have a high intracellular Cl^- concentration, producing a depolarized Cl^- equilibrium potential and therefore leading to excitatory GABAergic postsynaptic events). Additionally, GATs perpetually maintain the ambient extracellular concentration ($[GABA]_o$) at a nonzero concentration, which dictates the amplitude of tonic GABAergic currents via activation of extrasynaptic GABA receptors. Even after vigorous synaptic activity, $[GABA]_o$ is much less than the intracellular GABA concentration ($[GABA]_i$) in the presynaptic boutons and surrounding glia. To move the neurotransmitter against this concentration gradient, an electrogenic process cotransports GABA with Na^+ and Cl^- down the combined electrochemical gradient of the cotransported ions.

GATs are attractive targets in the treatment of epilepsy, because inhibiting GABA uptake – through either pharmacological or genetic intervention – prolongs neurotransmitter lifetime and increases ambient $[GABA]_o$ to decrease excitability. Importantly, GATs are equally able to transport substrate in either the forward (into the cell) or reverse (out of the cell) direction. Reverse GABA transport is a physiological process that occurs under depolarizing conditions. Consequently, inhibitory interneuron activation can stimulate both quantal GABA release from synaptic vesicles and nonvesicular GABA release via reverse GABA transport. During periods of high-frequency stimulation, synaptic vesicle stores may become depleted but the transporter-mediated GABA release continues. Furthermore, surrounding astroglia can become depolarized and release GABA through reverse transport. Therefore, during a seizure, reverse transport could have an important neuroprotective function.

This article serves as a brief introduction to the several GAT isoforms expressed in the brain, and reviews how their differential tissue distribution, their structure, and their physiological and pharmacological modulation can control neuronal excitability.

Background

Classification and Differential CNS Expression of GABA Transporters

The GATs are part of the neurotransmitter sodium symporter (NSS) transporter family, which includes transporters for the neurotransmitters dopamine, serotonin (5HT), norepinephrine, and glycine. The NSS transporters are also known as the solute carrier 6 (SLC6) family of membrane proteins. Our laboratory helped to clone the first SLC6 transporter, rat GAT-1 (SLC6A1); 21 human SLC6 transporter genes were subsequently identified, including three other GABA transporters: GAT-2 (SLC6A13), GAT-3 (SLC6A11), and the betaine/GABA transporter, BGT-1 (SLC6A12). GAT-2, GAT-3, and BGT-1, respectively, show 51%, 50%, and 48% amino-acid identity with GAT-1, conservation occurring primarily in their predicted transmembrane spanning regions (**Figure 1**). The 'GAT' nomenclature of the GABA transporters is not the same across species (**Table 1**). The SLC6 gene nomenclature offers a standardized taxonomy, but because this volume focuses on human epilepsy, we prefer to use the more descriptive human/rat classification of the transporter proteins.

Differential CNS distribution of GAT isoforms

Expression of GAT-1 and GAT-3 is restricted to the brain, whereas BGT-1 and GAT-2 are found in peripheral tissues and in the CNS. GAT-1 is the predominant GABA transporter in the CNS; it is localized primarily on axons and presynaptic boutons of GABAergic inhibitory neurons but is also expressed in astrocytes.

Our laboratory generated knockin mice expressing GAT-1 transporters fused to green fluorescent protein (GFP). These mice allow direct visualization and quantification of GAT-1 expression in situ, strongly complementing earlier immunohistochemical and radiolabeling studies. The cerebellar pinceaux exhibit impressively high GAT-1 density, containing almost 8 million GAT-1 molecules per pinceau. The Bergman glia in the molecular layer and the axons and boutons of Golgi cells in the glomeruli of the granule-cell layer account for the more diffuse GAT-1–GFP expression elsewhere in the cerebellum. In the hippocampus, GAT-1 is localized to the axons and synaptic boutons of basket and chandelier cells in strata oriens and pyramidale. A bouton in stratum oriens expresses 79% of the GAT-1 volume density observed in a cerebellar

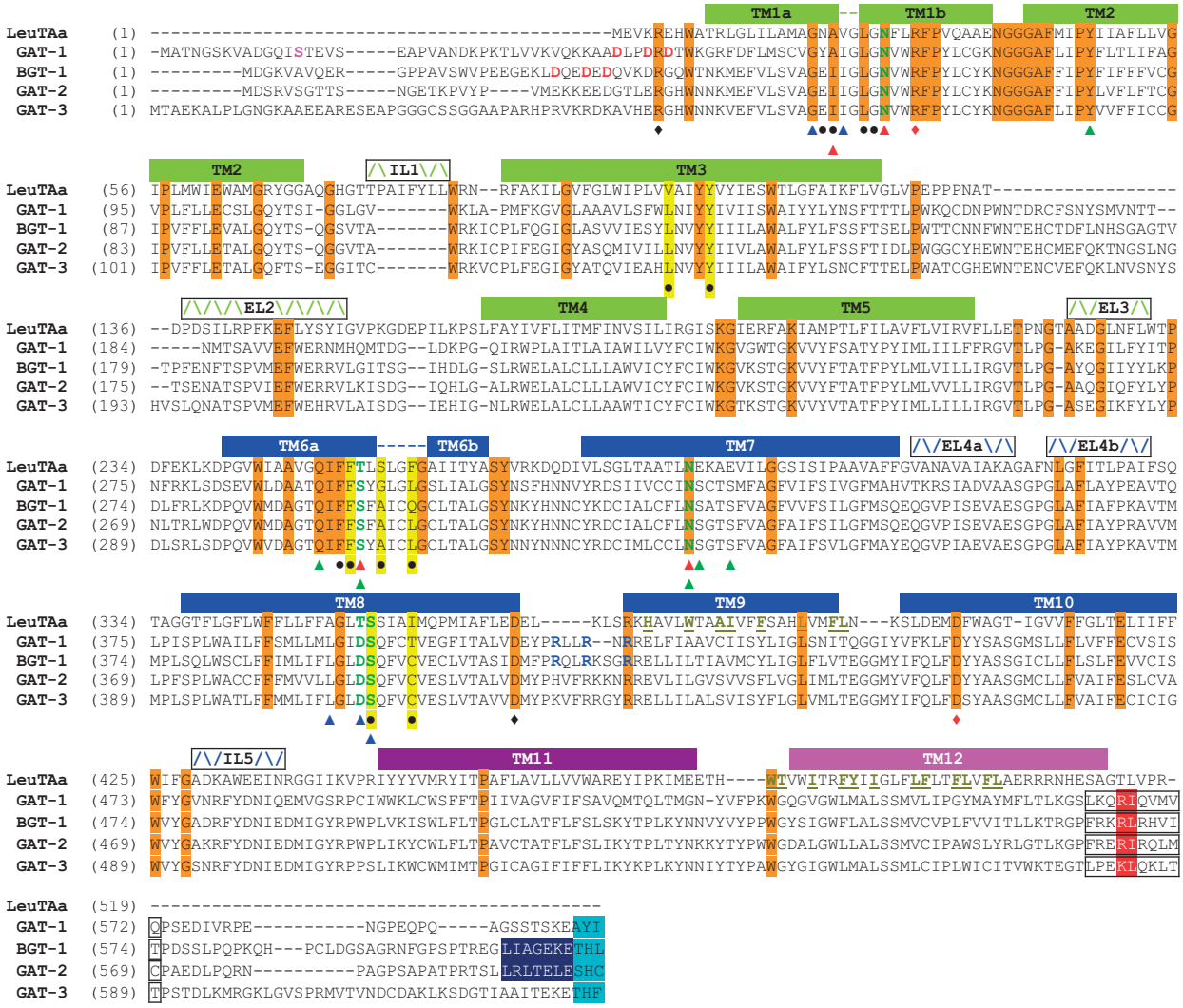


Figure 1 Alignment, secondary structure, and regulatory elements in GABA transporters. A sequence alignment of the four known GABA transporter isoforms with the $LeuT_{Aa}$ bacterial leucine transporter to highlight regions of sequence similarity (presumably leading to structural similarity). Identical residues are highlighted in orange. Transmembrane helices are labeled above the sequences; helices in green (TM 1–5) have pseudo-two-fold symmetry with helices in blue (TM 6–10). TM11 and TM12, which are not part of this symmetry, are colored purple and magenta, respectively. Helical regions in intracellular loops (IL) and extracellular loops (EL) are identified by boxed slashes above the sequences. The red and blue triangles below the sequences highlight residues involved in coordinating ions Na1 and Na2, respectively, and those residues whose side chains interact with the Na^+ ions are shown as green letters. Residues that coordinate the Cl^- ion are marked by green triangles below the alignment. The filled black circles specify the residues involved in binding the leucine substrate in $LeuT_{Aa}$, and residues whose side chains interact with the leucine are shaded yellow. The red and black diamonds indicate the charged pairs at the extracellular and cytoplasmic entrances, respectively. The residues that form the $LeuT_{Aa}$ dimer interface in TM9 and TM12 are shown as underlined brown letters. N-terminal residues involved in syntaxin 1A binding are shown as bold red letters, while IL4 arginines, their interaction partners in the absence of syntaxin 1A, are shown as bold blue letters. The C-terminal sequence implicated in constitutive and PKC-regulated endocytosis is boxed. The R/K-I/L COPII-interacting residues are highlighted in red. Distal C-terminal sequences that mediate somatodendritic (basolateral) sorting are highlighted in dark blue. PDZ-interacting motifs are colored cyan.

pinceau. The relative fluorescence intensity of GAT-1–GFP in the neuropil of hippocampal pyramidal and dentate gyrus cell layers is ~50% of that seen in cerebellar pinceaux but no clear axon or bouton labeling is observed because of high fluorescence in surrounding astrocytes. In human hippocampus, the termini of GAT-1-expressing axon terminals cluster with high density around the dentate

Table 1 Cross-species nomenclature of cloned GABA transporters with official gene symbols

Species	*Nomenclature*			
Human/rat	GAT-1	BGT-1	GAT-2	GAT-3
Mouse	GAT1	GAT2	GAT3	GAT4
Gene name	SLC6A1	SLC6A12	SLC6A13	SLC6A11

gyrus granule-cell somata. GAT-1 expresses at low levels in the cerebellar white matter, striatum and most thalamic nuclei, the latter tissue restricting GAT-1 expression to the astrocytes. Higher expression occurs in the GABAergic thalamic reticular nucleus. In neocortex, GAT-1 immunostaining is associated exclusively with punctate structures resembling axon terminals around the perikarya, and especially in cartridges surrounding the proximal portions of the apical dendrites of pyramidal cells. Cartridges are comprised, at least in part, by the terminals of cortical chandelier and basket cells.

Weak GAT-2 expression is detected in the neurons and astrocytes in cerebral cortex, cerebellum, brainstem, and hippocampus. The greatest GAT-2 expression occurs in the leptomeninges, ependyma, and choroid plexus. Similar to GAT-1 and GAT-3, distal astrocytic processes express GAT-2. However, only GAT-2 is expressed in astrocytic cell bodies and proximal processes. Neuronal GAT-2 expression occurs in axon terminals, mainly in deep layers of rat cerebral cortex, but in areas distant form the synaptic cleft. Weak GAT-2 expression is also detected in the cytoplasm of dendrites from rat cortical neurons. It is unclear if these transporters are ever present at the dendrite surface. Thus, the subcellular distribution of GAT-2 suggests that its primary function is modulating the ambient $[GABA]_o$ rather than terminating GABAergic neurotransmission at the synapse.

The physiological role of GAT-3 is probably similar to GAT-2, as it is exclusively expressed in distal astrocytic processes. GAT-3 is present in the hindbrain, midbrain, olfactory bulb, subfornical organ, hypothalamus, thalamus, and brainstem. Within the basal ganglia, GAT-3 expression is high in globus pallidus, subthalamic nucleus, and substantia nigra but low in the caudate nucleus and putamen. In the neocortex, punctate GAT-3 labeling of distal astrocytic processes forms a continuous sheet around the somata of both pyramidal and nonpyramidal neurons in all layers.

BGT-1 was first identified in kidney, where it accumulates the osmolyte betaine in the hypertonic environment of the renal medulla. It is also expressed in the CNS, although its distribution does not closely correlate with GABAergic pathways despite having a higher substrate affinity for GABA than betaine. Astrocytic cultures from rat brain express BGT-1; monkey BGT-1 expression has been reported in the somata and dendrites of excitatory pyramidal neurons in cerebral cortex and in the CA fields of the hippocampus.

Temporal lobe epilepsy (TLE) has strong ties to GABA transport. Although some GABAergic interneurons are preserved in sclerotic hippocampal tissue from TLE patients, there is a marked reduction in the typical punctate GAT-1 immunoreactivity around the cell bodies of pyramidal cells in the CA regions and granule cells of the dentate gyrus. This pattern reflects reduced GABAergic innervation of these cell types, predisposing them to hyperexcitability. Furthermore, TLE is also characterized by a decrease in glutamate-induced GABA transport reversal. This change is important since seizures are marked by elevated glutamate concentrations, and reduced GAT-1 reversal under this condition would lessen the contribution of reverse GABA transport on seizure inhibition. Regional increases in excitability may be further compounded by general increases in extrasynaptic GAT-3, which could lead to greater GABA removal from the extracellular space, thus reducing GABAergic tone and promoting hyperexcitablity.

Methods

In the Background section, we described research arising from GAT-1–GFP knockin mice that we generated in our laboratory. We have also generated a strain of GAT-1 knockout mice (available from the Mutant Mouse Regional Resource Centers; the knockin mice are generated from this strain by crossing to a strain expressing cre recombinase). More recently we have been studying the oligomerization and trafficking of GAT-1 by generating fusion proteins of the transporter with cyan and yellow fluorescent proteins (CFPs and YFPs, respectively). Besides being fluorescent, these fusions were engineered to be identical to wild-type GAT-1 in all their quantifiable functional properties. Fluorescent GAT-1 fusions were expressed in the neuroblastoma 2a (N2a) cell line, which because of its neuronal origin, possesses much of the necessary cellular machinery to process and traffic GAT-1 correctly. We assessed the lateral mobility and membrane confinement of a fluorescent GAT-1 by fluorescence recovery after photobleaching (FRAP) using an inverted laser scanning confocal microscope. GAT-1 FRAP experiments were performed by photobleaching regions of interest (ROIs) in both the cell footprints and perimeters. We have investigated tethering of GAT-1 to the cytoskeleton by depolymerizing actin with 5 μM of latrunculin B or microtubules with 10 μM of nocodazole and by analysis of the GAT-1 mobile fraction. The mobile fraction was calculated by normalizing the magnitude of fluorescence recovery normalized to the total possible fluorescence intensity.

Förster resonance energy transfer (FRET) was employed to study oligomerization of GAT-1 and also its other intermolecular interactions. If a quantifiable energy transfer was detected between coexpressed proteins tagged with CFP and YFP, it would reflect an intimate intermolecular interaction between the two proteins (<10 nm separation). We employed a spectral laser scanning confocal microscope that has an array of photodetectors that simultaneously yield a full fluorescence emission spectrum at each pixel, allowing the experimenter to deconvolve the individual CFP and YFP spectra without

excessive photobleaching. FRET efficiency was calculated by assessing the magnitude of CFP dequenching during incremental YFP photobleaching.

Recent Results

Structure, Function, and Regulation of GABA Transporters

Insights from recent crystal structures

When GAT-1 was first cloned in 1990, its primary sequence predicted that the transporter possessed 12 transmembrane (TM) spanning segments topologically oriented in the plasma membrane with the carboxy (C−) and amino (N−) termini residing in the cytoplasm. Functional characterization determined that GABA transport was dependent on the cotransport of two Na^+ ions and one Cl^-. In 2005, Gouaux's laboratory reported the crystal structure of a functional leucine transporter, $LeuT_{Aa}$, a homolog of the mammalian NSS transporters (20–25% sequence identity) isolated from the bacteria *Aquifex aeolicus*. The $LeuT_{Aa}$ crystal structure provides a model on which all future structure–function research on NSS transporters can be based. Importantly, features were revealed that could not have been predicted from 15 years of previous experimental data. TMs 1–5 and TMs 6–10 form a structural repeat arranged around a pseudo-two-fold axis in the plane of the membrane (**Figure 2(a)** and **2(b)**). TM1 and TM6 run antiparallel to each other at the core of the protomer, and the center of their helices unwind in the middle of the plasma membrane (**Figure 2(a)**). The exposed main-chain carbonyl oxygen and nitrogen atoms afford hydrogen bonding partners that form most of the contacts with the bound substrate (**Figure 2(c)**). The unwinding of

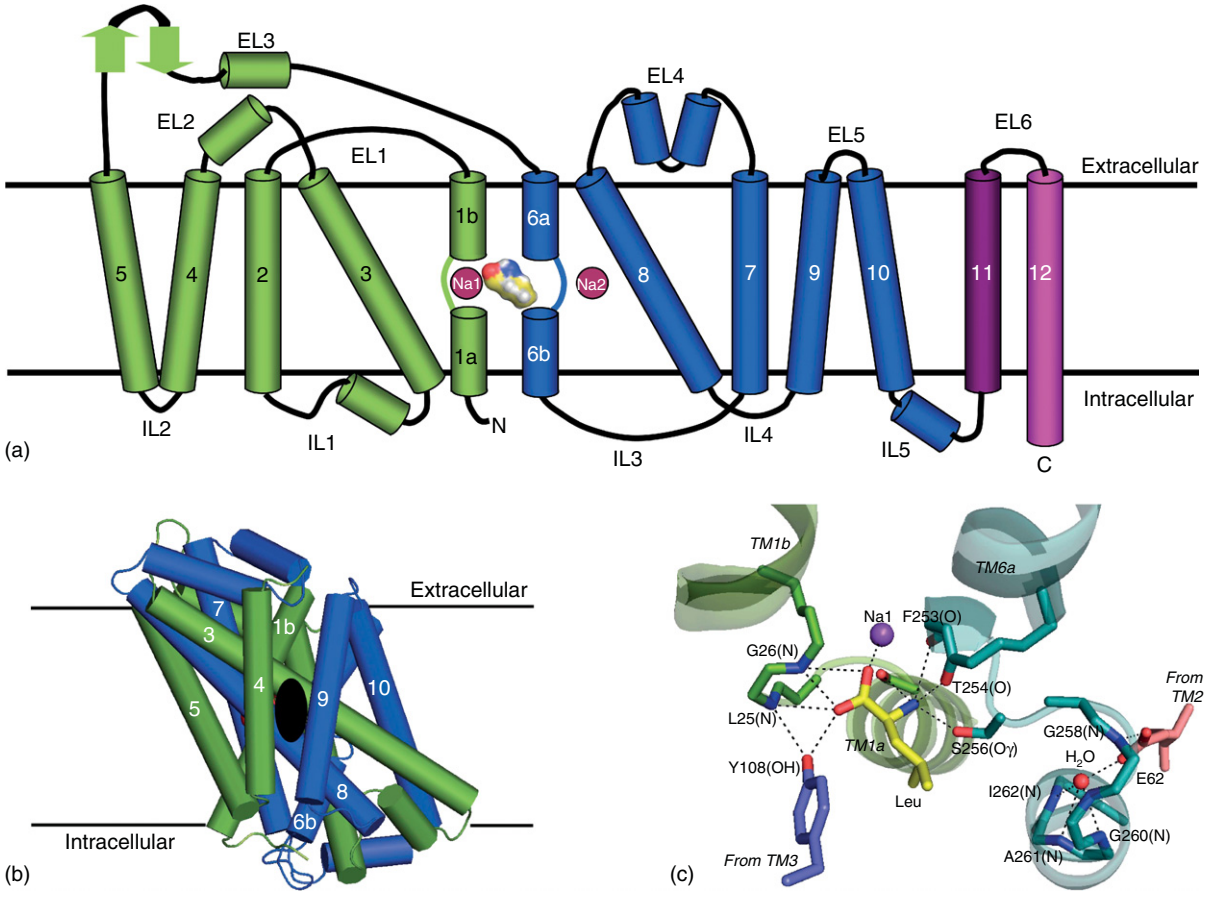


Figure 2 Topology, folded structure, and substrate-binding site in the $LeuT_{Aa}$ bacterial homolog of the mammalian GABA transporters. Panel (a) shows the membrane topology of the SLC6 transporters as exemplified by $LeuT_{Aa}$. Leucine substrate is shown coordinated in the center of the unwound regions of TM1 and TM6; the two Na^+ ions are shown as purple circles. IL, intracellular loop; EL, extracellular loop. Green arrows highlight region of β-sheet structure; the α-helical TM domains are labeled 1–12 and colored according to their pseudo-two-fold symmetrical arrangement in the membrane, which is displayed in panel (b). The black ellipse in panel (b) marks the rotation axis for this symmetry. Panel (c) depicts the hydrogen bonds and ionic interactions that coordinate the substrate (leucine) and the Na^+ ion in the transporter-binding site, which makes direct contact with the leucine. Ion spheres are 25% actual size in order to display all the interactions. Image was generated from the 2A65 PDB $LeuT_{Aa}$ crystal structure using PyMOL v0.99 (DeLano Scientfic, San Francisco, CA).

TM1 and TM6 further stabilizes the substrate. The negative α-helical dipole moments of TM1a and TM6a are placed adjacent to the amino group of the leucine, and the positive moment of TM1b is located next to its carboxy group. The two Na^+ ions cotransported with substrate stabilize the unwound helices at the transporter core for high-affinity substrate binding. Na1 makes direct contact with the carboxyl group of the substrate, (**Figures 2(c) and 3(a)**) which at limiting substrate concentrations could determine the apparent Na^+ affinity of substrate transport. Residues from TM3, TM7, and TM8 form the remainder of the dehydrated substrate/Na^+-binding core. The selectivity for Na^+ as the cotransporting ion arises simply because the pockets are too small to accommodate larger cations such as K^+ (**Figure 3(a) and 3(b)**). $LeuT_{Aa}$ does not require Cl^- cotransport to function, but a Cl^- ion functions as the trigger of the conformational changes that allow substrate to permeate to the cytoplasm in mammalian NSS transporters. The Cl^- is proposed to bind within 5 Å of Na1; the proximity allows the Cl^- to strongly couple with Na1 and stabilize the substrate-binding site. Cl^- is coordinated by the residues that align with GAT-1 Tyr86, Ser295, Asn327, and Ser331 (**Figure 3(c)**). One laboratory also reports the involvement of Gln291. Asn327 is conserved in almost all known NSS members. Ser331 is conserved in all mammalian Cl^--dependent NSSs. Replacing Ser331 or Asn327 (or their aligning residues) in GAT-1, GAT-3, SERT, or DAT (the dopamine transporter), with a carboxylic residue, makes substrate transport independent of Cl^-. Reciprocal mutations in the Cl^--independent $LeuT_{Aa}$ make leucine transport Cl^--dependent. Thus, all NSS transporters require a negative charge (from cotransported Cl^- or an acidic side chain) to be supplied in this pocket during substrate transport.

In the general 'alternating access transport' scheme, a transporter has a cavity that is alternately available for substrate/ion permeation from the extracellular or intracellular side of the membrane; substrate and cotransported ions must all be bound together before translocation across the membrane occurs. Access to the cavity is controlled by intracellular and extracellular 'gates,' which open and close with conformational changes in the transporter. The $LeuT_{Aa}$ structure captures the protein in an intermediate substrate occluded state, in which both these gates are closed. The closed extracellular gate is formed by a water-mediated salt bridge between Arg30 and Asp404, and beneath that, the large hydrophobic side chains on Tyr108 and Phe253. The occluded structure and sulphydryl accessibility studies on mammalian NSS suggest that the extracellular gate opens via movements in TMs 1, 3, 6, and 10 moving Arg30 and Asp404 apart, coupled to a major rearrangement of extracellular loop 4 (ECL4) whose V-shaped tip forms a lid that dips into the extracellular cavity when the gate is closed. The closed cytoplasmic gate is more complicated, comprising 20 Å of packed protein between the substrate and the cytoplasmic face of the protein. Part of this is formed by the backbones of TM1a, TM6b, and TM8 below the substrate-binding site, an Arg5–Asp369 charge pair, an Arg5–Ser267 H-bond, and an Arg5–Tyr268 cation–π interaction at the cytoplasmic face. Trp8 inserts between the backbones of TM1 and TM6b and anchors the N-terminus,

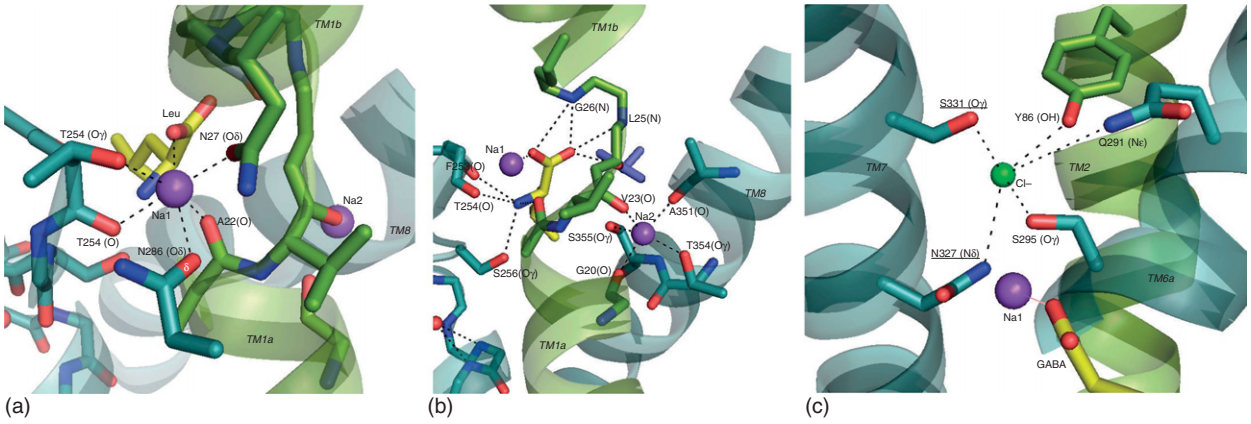


Figure 3 Residues that coordinate the binding of the cotransported Na^+ and Cl^- ions in NSS transporters. (a) Na1 in $LeuT_{Aa}$ coordinated by residues from TM1a, TM1b, TM6a, and TM7 together with the bound leucine are shown. The location of Na2 is shown in the background. (b) TM1a and TM8 residues from $LeuT_{Aa}$ that coordinate Na2 are shown, with the location of Na1 and leucine in the background. (c) Residues from TM2, TM6a, and TM7 that coordinate Cl^- in the GAT-1 transporter. Residue numbering is for GAT-1 in this panel. Underlined residues are almost completely conserved in all NSS transporters. In all panels, ion spheres are 25% actual size in order to display all the interactions. We include the interaction between Gln291 and the Cl^- ion reported by Zomot *et al*., which is not present in the homology models of Forrest *et al*. Panels (a) and (b) were generated using PyMOL v0.99 from the 2A65 PDB $LeuT_{Aa}$ crystal structure. Panel (c) was generated using PyMOL v0.99 from a homology model of GAT-1 based on $LeuT_{Aa}$ kindly provided to the authors for the purpose of this review by Dr. L.R. Forrest and Dr. B. Honig (Howard Hughes Medical Institute, Center for Computational Biology and Bioinformatics, and Department of Biochemistry and Molecular Biophysics, Columbia University, NY).

including Arg5. These residues are conserved throughout the NSS family, and mutation of the aligning amino acids in mammalian NSS transporters impairs transport.

Experiments on SERT have begun to define the pathway through which the substrate diffuses from the transporter core to the cytoplasm. When cysteine residues are substituted at positions that correspond to $LeuT_{Aa}$ residues Gly190, Ile191, Phe194, Ile197, Ala198, and Thr201 in the cytoplasmic half of TM5, the cysteines become extremely reactive to sulphydryl reagents in the presence of 5HT. This indicates that these residues, which are buried in the $LeuT_{Aa}$ occluded structure, become available in a substrate-bound state. They are protected from MTSEA reaction in the presence of cocaine, which traps the SERT in the extracellular-facing conformation.

Trafficking – Modulating transporter number at the plasma membrane

The number of GATs at the plasma membrane shapes neurotransmission and controls excitability. Quantifying the fluorescence intensity in presynaptic boutons and axons in the cerebellum and hippocampus of GAT-1–GFP knockin mice determined that the membrane density of GAT-1 molecules ranges from 800 to 1300 μm^{-2}. Of this value, 61–63% of these molecules are inserted in the plasma membrane, and the remainder are just below the membrane in cytoplasmic vesicles (see later). However, the interplay of constitutive and functional regulatory elements controls the density of transporter at the cell surface at any given point in time.

Constitutive transporter trafficking

Several laboratories, including our own, have measured FRET between transporters fused with CFP and YFP to demonstrate the constitutive oligomerization of transporter protomers. Although single transporter protomers are transport-competent, mutations that prevent oligomerization eliminate FRET, and the fluorescently tagged transporter proteins are retained in intracellular compartments. $LeuT_{Aa}$ crystallized as a dimer, with TM9 and TM12 probably providing the assembly interface. However, this structure likely represents only a low-order oligomerization state for these transporters, as evidence exists for the further oligomerization of transporter dimers. FRET between YFP-tagged Sec24D, a fluorescently tagged component of the COPII vesicle coat, and a CFP-tagged GAT-1 demonstrates that after oligomerization export from the endoplasmic reticulum (ER) occurs via COPII-coated vesicles. Sec24D interacts with a conserved motif (R/L-I/L consensus sequence) in the C-terminal of all NSS transporters. Transporters lacking this proximal C-terminal motif are retained in the ER and exert a dominant negative effect when coexpressed with wild-type protein. Additionally, the ER retention of oligomerization-deficient mutants in which the Sec24D interaction is preserved demonstrates that COPII vesicle-coat interaction is necessary but not sufficient for ER export.

GAT-1 transporters are delivered to the plasma membrane via a population of vesicles, ~50 nm in diameter, which are distinct from the neurotransmitter-containing vesicles. This distinction probably allows regulated transporter expression independent of neurotransmitter release. These vesicles insert their cargo into the plasma membrane under control of the exocyst. The three most C-terminal residues of GAT-1 conform to a consensus sequence for a type II PDZ-interacting motif (**Figure 1**; PDZ, postsynaptic density 95/discs large/zona occludens 1). This sequence mediates transporter interaction with the exocyst by association with PDZ-domain-containing proteins and stabilizes the transporter in this location once inserted. Importantly, NSS transporters are primarily located intracellularly unless they interact with a PDZ-domain-containing protein at their distal C-terminus. For example, a truncated GAT-1 lacking the PDZ-interacting epitope exhibits impaired plasma membrane expression in vitro. Similarly, GAT-1–GFP knockin mice, which express the same amount of total GAT-1 protein as wild-type mice, exhibit a three-fold decrease in cell surface-expressed transporter, because the GFP disrupts the endogenous PDZ-interacting epitope (-AYI). Interestingly, the GAT-1–GFP mice do not phenocopy GAT-1 knockout mice, which have a constant 25–32-Hz tremor in their limbs and tail and display ataxia and anxiety. Thus, there appears to remain a default exocyst-independent pathway through which sufficient GAT-1–GFP can be delivered to the cell surface to maintain wild-type-equivalent GABAergic tone.

Live-cell imaging of GAT-1 molecules reveals that plasma membrane GAT-1 is tethered to the actin cytoskeleton via a multicomponent protein assembly that is mediated by the GAT-1 PDZ-interacting epitope, a PDZ protein, and ezrin. Through FRAP, we recently established that GAT-1 exhibits 30% greater lateral mobility in the plasma membrane when the PDZ-interacting domain is disrupted. Furthermore, FRET determined that the efficiency of the interaction between GAT-1 and ezrin was significantly reduced on depolymerization of the actin cytoskelton. Therefore, interaction with PDZ proteins mediates transporter anchoring in the membrane.

A candidate PDZ-domain-containing scaffold protein that mediates these properties is the membrane-associated guanylate kinase (MAGUK) PALS1 (protein associated with LIN-7, subtype 1). The PALS1–GAT-1 interaction is dependent on the C-terminal-AYI residues, and its coexpression with GAT-1 promotes increased transporter surface expression, presumably by stabilizing the residence of membrane-inserted GAT-1. Neuronal expression of PALS1 and GAT-1 does not perfectly overlap, and it follows that both molecules almost certainly interact with other partners in some cell types and in some

subcellular compartments. The interaction of GAT-1 with PDZ-domain-containing MAGUKs may play a cell-specific and compartment-specific role in regulating transporter availability, location, and stability.

GAT-2, GAT-3, and BGT-1 possess PDZ-type-I-like interacting motifs at their C-termini (Figure 1). GAT-2 and BGT-1 interact with PDZ proteins, including $G_{\alpha i}$-interacting protein COOH terminus (GIPC) and LIN-7, respectively, to stabilize their presence in the plasma membrane. Deletion of these motifs results in redistribution of transporter to intracellular regions but does not perturb the sorting of GAT-2 or BGT-1 to the appropriate cellular compartments. GAT-2 and BGT-1 sort to the basolateral surface when expressed in Madin-Darby canine kidney II (MDCK) cells, a well-established expression system to study polarized sorting of proteins into cellular compartments. The seven amino acids immediately proximal to the PDZ-interacting motifs in GAT-2 and BGT-1 comprise a motif unique to these particular transporters that dictates their delivery to the basolateral surface of MDCK cells (Figure 1). In general, basolateral sorting in epithelial cells corresponds to somatodendric sorting in neurons, while apical sorting, which is observed for GAT-1 and GAT-3, corresponds to axonal (and consequently presynaptic) sorting – although not all proteins fit this paradigm. Truncation of the entire GAT-1 C-terminus does not perturb its apical sorting, but removing the three C-terminal residues from GAT-3 is sufficient to result in mixed apical and basolateral sorting. The THF terminal amino acids of GAT-3 resemble, but do not rigidly conform to, a Type-I PDZ-interacting motif (X-S/T-X-V/L) and reportedly bind the PDZ-domain protein CAP70. For GAT-3 at least, the PDZ interaction may therefore play an important role in both axonal sorting in neurons and stabilizing its expression in the plasma membrane.

Constitutive endocytosis of NSS transporters from the plasma membrane is controlled in large part by the nonpolar residues within the nonclassical endocytic motifs present in their proximal C-termini (consensus sequence L(X)ERLAY(X)IT, Figure 1). Regulated endocytosis and exocytosis occur through pathways distinct from the constitutive processes.

Regulated transporter trafficking

One-third of the total cellular GAT-1 resides in a pool that acutely recycles between the plasma membrane and cytoplasm. In the basal state, one-third of this pool (11% of total GAT-1) resides in the plasma membrane. However, protein kinase and phosphatase activity serves to modulate the transporter cytoplasmic/plasma membrane distribution equilibrium through multiple and distinct pathways. The motif in the proximal C-terminus responsible for constitutive internalization is also implicated in protein kinase C (PKC)-induced transporter endocytosis. PKC-mediated endocytosis is absolutely dependent on the L(X)ERL portion of the L(X)ERLAY(X)IT region, and is located immediately downstream from a conserved putative serine/threonine phosphoacceptor residue. Stimulation of PKC promotes a relative decrease in surface transporter levels, attenuating transporter recycling and redistributing transporter to the endosomal compartments. Stimulating tyrosine kinase activity has the opposite effect. When high neuronal activity elicits large amounts of quantal GABA release, the cell may benefit from redistributing transporters from the intracellular pool to the plasma membrane in a rapid homeostatic response to maintain the local [GABA] close to equilibrium $[GABA]_o$. As substrate permeates the transporter, conformational changes present tyrosines at the cytoplasmic ends of TM2 or TM7 for modification. The phosphorylated side chains signal for reduced transporter internalization, resulting in substrate-induced transporter up-regulation. Analogously, direct inhibition of tyrosine kinases results in rapid attenuation of uptake by GAT-1. NSS transporters therefore exist in mutually exclusive PKC or tyrosine kinase phosphorylated states, and the relative abundance of each of these states dictates the proportion of the recycling transporter pool that is present at the plasma membrane. Nonetheless, direct transporter phosphorylation is often insufficient to drive redistribution. PKC-mediated regulation is still reported even after removal of all consensus phosphorylation sites in the transporter. Protein phosphatases (PPs) also regulate transporter function; in general, PP1 and PP2A inhibition down-regulates transporter activity. Elevated $[Ca^{2+}]_i$ is also reported to decrease GAT-mediated uptake via calmodulin- and calpain-sensitive pathways.

The SNARE complex protein syntaxin 1A is present in the distinct vesicle population that delivers GAT-1 to the cell surface; its coexpression with GAT-1 results in increased transporter numbers on the plasma membrane. A paradoxical feature of this interaction is that syntaxin 1A also serves as negative regulator of the transporter's intrinsic properties. Syntaxin 1A binds to three negatively charged aspartates in the GAT-1 N-terminus (Figure 1), but a cluster of four arginines in GAT-1 IL4 competes with syntaxin 1A at this binding site. Syntaxin 1A interaction with GAT-1 N-terminus releases IL4, which inhibits GABA transport. However, chronic exposure to substrate relieves this inhibition by causing dissociation of syntaxin 1A from the transporter, freeing the N-terminus to sequester IL4. The GAT-1–syntaxin 1A-interaction probably forms only part of a multimeric protein complex that modulates transporter function. PKC-mediated phosphorylation of syntaxin 1A-binding partners (e.g., Munc 18) inhibits their interaction with the SNARE proteins and promotes the syntaxin 1A-mediated effects on GAT-1, while SNAP-25, another SNARE protein, both potentiates syntaxin 1A-mediated inhibition of substrate transport and promotes GAT-1 surface expression. GAT-2 and GAT-3 are not modulated by syntaxin 1A, and FRET indicates

that no direct interaction occurs between syntaxin 1A and these transporters, presumably because their N-termini lack the necessary triplet of aspartate residues present in the N-terminus of GAT-1 and other NSS family members (Figure 1).

Pharmacology – Modulation of the GAT by antiepileptics

GATs are an attractive target in anticonvulsive therapy, because their inhibition leads to activity-dependent prolongation of GABA signals in otherwise hypo-GABAergic synapses. Although many GAT inhibitors possess antiepileptic properties in animal models, tiagabine is the only FDA-approved second-generation antiepileptic drug that selectively inhibits a GABA transporter, GAT-1. Tiagabine-mediated GAT-1 inhibition enhances GABAergic inhibition by prolonging phasic GABA currents. The drug also blocks reverse transport, and so it potentially has proconvulsive properties. Cognitive side-effects of tiagabine are minimal in comparison to first-generation antiepileptics such as valproate, which cause general psychomotor slowing. Nonetheless, the major side-effect of tiagabine is tremor, which can be accompanied by ataxia, dizziness, asthenia, sedation, and nonspecific nervousness. Genetic knockout of GAT-1 produces mice with increased $GABA_A$ receptor-mediated tonic conductance in both cerebellar granule and Purkinje cells, and a slower decay of spontaneous inhibitory postsynaptic currents in cerebellar granule cells. These results point to increased extracellular $[GABA]_o$ due to the lack GAT-1-mediated clearance. Importantly, these mice also partially phenocopy several of the clinical side effects of tiagabine, particularly tremor, indicating the side-effects are inherent to a clinical strategy that specifically targets GAT-1 rather than to nonselective actions on other targets.

Gabapentin and vigabatrin, other antiepileptics in clinical use, do not act directly on GATs, but still influence their function. They indirectly enhance reverse GABA transport during high-frequency stimulation, which could be neuroprotective during seizure.

Although primarily localized on astrocytes and in extrasynaptic regions, BGT-1 is emerging as an attractive antiepileptic target. EF1502 is an equipotent, noncompetitive GAT-1/BGT-1 inhibitor. It has a broad-spectrum anticonvulsant profile in animal models of generalized and partial epilepsy, exhibits a superior tolerability profile in comparison to tiagabine, and displays a synergistic rather than additive anticonvulsant interaction when co-administered with tiagabine. The efficacy of EF1502 is likely explained by its inhibition of extrasynaptic BGT-1, increasing GABA-induced tonic conductance. Similarly, co-administration of NO711 (a selective GAT-1 inhibitor) and SNAP-5114 (a GAT-2/-3 inhibitor) in rat neocortex increased GABAergic tone, but application of either drug alone was insufficient to effect tonic currents. Administration of non-GAT-1 transporter inhibitors alone show enhanced efficacy in the generalized epilepsy models but reduced efficacy on partial seizures when compared with tiagabine. To date there is no known compound that specifically targets BGT-1, GAT-2, or GAT-3.

Future Directions and Challenges

Up to 30% of patients diagnosed with epilepsy exhibit resistance to the available antiepileptic drugs. Among those patients who do respond to treatment with the available medications, seizures are often not completely controlled and many experience one or more undesirable side-effects (e.g., tremor or sedation). The development of new compounds or refinement of existing drugs to create GAT antagonists selective for each isoform remains an attractive option for enhancing GABAergic transmission. Administration of such compounds alone or in combination could form the basis of new treatment paradigms for controlling epilepsy or other CNS GABAergic disorders in a regional or cell type-specific manner. The crystal structure of $LeuT_{Aa}$ has greatly enhanced our understanding of how the NSS transporters' molecular structure relates to their function. The recently reported co-crystal structures of $LeuT_{Aa}$ with tricyclic antidepressant (TCA) drugs now indicates that $LeuT_{Aa}$ may also serve as a pharmacological model for NSS transporters. Both studies determined that TCAs bind in a vestibule in the extracellular face of the transporter, 11 Å 'above' (toward the extracellular face) the substrate and Na^+ ions. Functional studies determined that TCAs can bind $LeuT_{Aa}$ in the absence or presence of substrate and that leucine dissociation was dramatically inhibited by clomipramine. Therefore, it would appear that TCAs noncompetitively inhibit $LeuT_{Aa}$, stabilizing the transporter in a conformation with its extracellular gate closed. However, functional studies in mammalian monoamine transporters suggest that TCAs bind a site deeper in the protomer, inhibiting transport competitively. Despite these functional differences for TCA transporter inhibition between $LeuT_{Aa}$ and the monoamine transporters, the identification of a noncompetitive binding site in the bacterial SLC6 homolog is an exciting development. EF1502, the noncompetitive GAT-1/BGT-1 inhibitor, might bind in the analogous vestibule in GAT-1 or BGT-1. Homology models of GAT-1 or BGT-1 based on the $LeuT_{Aa}$ structure could be used in docking studies of EF1502 and similar compounds to determine whether this is possible. Any significant findings could then be substantiated through functional studies on GAT subtypes carrying pertinent mutations. Tiagabine is a competitive GAT-1-specific inhibitor when in the presence of GABA but is not a substrate. Nipecotic acid, tiagabine's parent compound, is both a potent GAT inhibitor and

substrate. At this time we cannot predict whether tiagabine uses the newly discovered extracellular vestibule when binding GAT-1, but the starting point of any tiagabine docking study is apparent: The substrate-binding site, with the nipecotic-acid moiety of tiagabine coordinated in the place of GABA.

To date, there is no reported evidence of any antiepileptic drug binding $LeuT_{Aa}$. However, if functionally viable, $LeuT_{Aa}$/GAT chimeras could be generated and expressed in *Escherishia coli* for use in the screening of libraries of known antiepileptic drugs or novel compounds for GAT isoform selectivity. In analogous studies to the TCA/$LeuT_{Aa}$-binding investigations, drugs that bind $LeuT_{Aa}$ or the hypothetical $LeuT_{Aa}$/GAT chimeras could be co-crystallized with the bacterially expressed transporters to determine which residues confer drug selectivity between the four GAT isoforms.

Finally, our recent finding of a role for ezrin in the tethering of GAT-1 to the actin cytoskeleton may also imply an important function for this interaction in the pathophysiology of epilepsy. Ezrin is expressed in CNS neurons and is upregulated in the hippocampus of patients with mesial TLE. Likewise, in the lithium-pilocarpine-treated rat model of TLE, ezrin up-regulation occurs within 48 h of seizure induction. Epilepsies associated with malformations of cortical development such as focal cortical dysplasia (FCD_{IIb} and FCD_{IIa}) and gangliogliomas are also marked by neuronal ezrin accumulation. Although the role of ezrin up-regulation in seizure progression has not been formally identified, recent evidence points to the role of ezrin as a downstream target in the phosphoinositide 3-kinase (PI3K) pathway. Further investigation is therefore required to establish if the recently identified interactions between GAT-1, ezrin, and actin are adversely affected by aberrant PI3K signaling in epilepsy-associated malformations.

Acknowledgements

Preparation of this review was supported by a grant from the N.I.H. (DA-09121), an American Heart Association fellowship for F. J. M. and from a Schrödinger fellowship (J2486) from the Austrian Science Fund (FWF) for H. J.

See also: **Transporters**: GABA Transporters: Regulation of Tonic Inhibition; **Glia**/**Astrocytes**: Glial Modulation of Excitability via Glutamate and GABA Transporters.

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Glutamate Transporters in Epilepsy

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Introduction

Much evidence exists to support the idea that epilepsy is mediated by an imbalance of GABA and glutamate in the brain. Both the overstimulation of excitatory synapses by an abundance of extracellular glutamate, and the understimulation of inhibitory synapses by reduction of extracellular GABA, can contribute to the pathological events resulting in seizures and cellular injury. The clearance and metabolism of these two neurotransmitters are intricately coupled, and thus drugs modulating these processes provide potential therapeutic avenues for the treatment of epilepsy.

Synaptic transmission in the brain is generally described in three steps: stimulated release, receptor stimulation, and clearance of the neurotransmitter. This third step is particularly important for shaping neuronal responses. Extracellular concentrations of neurotransmitters are regulated by either enzymes that degrade the transmitter into an inactive form or by transporters that carry the neurotransmitters from the extracellular space into neurons and glia. In the human brain, the extracellular levels of both glutamate and GABA are controlled by neurotransmitter transporters.

Background

Glutamate Transporters

Excitatory amino acid transporters, or EAATs, serve as the major mechanism for limiting the action of the excitatory amino acid glutamate in the central nervous system. In humans there are five different isoforms of EAATs. The expression of each of these transporters is independently regulated and unique to different cell types. EAAT1 (GLAST) is found primarily in astrocytes of the cerebellum, while EAAT2 (GLT-1) is found predominantly in astrocytes throughout the brain, as well as on the presynaptic terminals of glutamatergic neurons. EAAT3 (EAAC1) and EAAT4 are found in the postsynaptic membranes of neurons, while EAAT5 is expressed selectively in photoreceptors and bipolar cells within the retina. Other families of neurotransmitter transporters, such as those for the biogenic amines (dopamine, serotonin and norepinephrine) have single isoforms, which are restricted to neurons that release the cognate neurotransmitters, thus enabling reuptake and repackaging for further cycles of release. In contrast, glutamate transporters are widely distributed, with multiple subtypes expressed both pre and postsynaptically with respect to the glutamatergic neuron, in nonglutamatergic neurons, and on glial cells. In fact, most glutamate is cleared from the extracellular space by the astrocytic carriers.

Many studies have examined the relative impact of each glutamate transporter isoform on glutamate homeostasis, neural function, neurodegeneration, and behavior. Mouse strains with gene deletions in EAATs 1, 2, 3, and 4 have been generated, and while EAAT1, 3, and 4 knockout animals do not exhibit any remarkable neurological developmental profiles, EAAT2 knockout animals exhibit spontaneous and often lethal seizures. One report has suggested that changes in EAAT2 can compensate for reduced glutamate clearance observed when both EAAT1 and EAAT3 genes have been disrupted. EAAT1/EAAT2 double knockout animals are embryonically lethal, underscoring the critical requirement for glial uptake in the maintenance of glutamate homeostasis.

Although the impact of both EAAT1 and EAAT3 on brain physiology was not immediately apparent in knockout animals, more sophisticated analyses have revealed a profound role for these two transporters. As EAAT3 knockout animals age, they begin to exhibit behavioral deficiencies, brain atrophy, decreased glutathione content, and related disability to cope with oxidative injury. The neuronal carrier is an important means of cysteine transport, and reduced levels of this substrate for glutathione synthesis most likely contributes to cell death via oxidative stress.

Recently, it was reported that EAAT1 knockout animals show decreased expression of the GABA transporter, GAT1. A decrease in GABA uptake could increase GABAergic tone and attenuate the epileptic tendency in these animals, as observed with GAT inhibitors in clinical use. This compensatory decrease in GAT1 also could provide an explanation for why EAAT1 knockout mice are not obviously seizure prone, in contrast to EAAT2 knockout animals. The relationship between glutamate and GABA, particularly in the epileptic brain, makes this observation particularly intriguing.

Glutamate Metabolism

Astrocytes recycle glutamate via the glutamate-glutamine cycle (Figure 1). During this process, glial cells take up glutamate, convert it into glutamine using the enzyme,

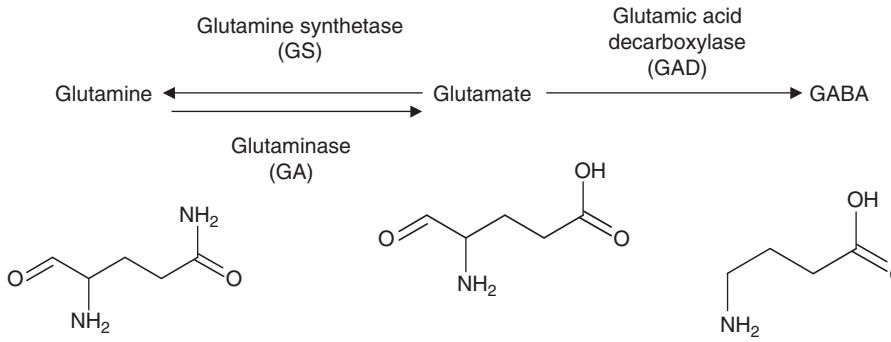


Figure 1 Glutamate and GABA are metabolically coupled. Glutamate can be transported out of the extracellular space into either astrocytes or neurons. In astrocytes, glutamate is converted into glutamine by glutamine synthetase (GS), released into the extracellular space, taken up by neurons and converted back into glutamate by phosphate activated glutaminase (GA). In neurons, glutamate can serve as a source of GABA by conversion via glutamic acid decarboxylase (GAD).

glutamine synthetase (GS), and then release glutamine into the extracellular space through other types of membrane transporters (Figure 2). Neurons take up glutamine and use glutaminase (GA) to resynthesize glutamate and package it into vesicles for synaptic release. A reduction of GS and/or an elevation of GA would lead to an overall increase in extracellular glutamate; indeed, in clinical studies, alterations in GS and GA expression have been linked to several types of epilepsy. However, it remains unclear whether these changes in enzyme levels are the root cause of the pathology or an effect of reduced EAAT activity.

Postsynaptic neurons that express EAAT3 and EAAT4 are often GABAergic. The glutamate that is transported into these cells is converted into GABA by glutamic acid decarboxylase (GAD) and used for inhibitory GABA neurotransmission. Studies inhibiting EAAT activity by a variety of methods have measured a decrease in GABA content and GABAergic activity. This observation reflects the significance of glutamate transport for GABAergic neurotransmission and the content of GABA in the neurotransmitter pool.

Interestingly, GABA is transported from the extracellular space into GABAergic neurons and into astrocytes. Neurons repackage GABA for synaptic release while astrocytes metabolize GABA and effectively remove it from the neurotransmitter pool. Recent investigations of GABA transporter inhibitors as a treatment for epilepsy have shown that inhibition of astrocytic GABA transporters is anti-convulsant, presumably because it preserves GABA in the neurotransmitter pool; however, inhibition of neuronal GABA transporters can be pro-convulsive due to the decrease in inhibitory GABA neurotransmission.

The metabolic pathways for neurotransmitter synthesis and degradation provide a number of potential avenues for therapeutic intervention in the treatment of epilepsy. Several current antiepileptic drugs target GABA transporters, GABA receptors, and glutamate receptors. However, these drugs do not always provide successful treatment, and alternative approaches involving the modulation of glutamate clearance may prove to have utility for limiting the hyperexcitability associated with seizures.

One potential therapeutic strategy directed at glutamate clearance would be to develop drugs that increase the activity of astrocytic and neuronal EAATs, thus reducing the accumulation of extracellular glutamate and limiting its excitatory actions. In addition, increasing the activity of EAATs located on GABAergic neurons could have the beneficial effect of enhancing GABA synthesis and increasing the pool available for release. However, the EAAT isoform, as well as its cellular location and relative abundance, will ultimately determine the therapeutic impact of enhancing EAAT activity.

Another potential means of targeting EAATs for the development of anticonvulsant medications could be through the design of subtype-selective EAAT inhibitors. Although at first this approach might seem counterintuitive, EAAT3 is positioned to regulate the amount of neurotransmitter available to activate metabotropic glutamate receptors (mGluRs). Blockade of EAAT3 would elevate local glutamate concentrations, increase the activation of mGluRs, and trigger mGluR-dependent mechanisms that reduce glutamate release and/or downregulate synaptic glutamate receptors. The neuronal carrier, EAAT3, displays a subcellular distribution that overlaps considerably with mGluRs and thus the selective inhibition of EAAT3 could provide a means of potentiating the homeostatic actions of mGluRs and reducing excitatory tone at nearby synapses.

Currents Associated with EAATs

Glutamate transporters use the sodium gradient to transport glutamate against its concentration gradient – extracellular glutamate concentrations are generally lower than intracellular levels. One cycle of the transporter brings in two or three sodium ions and an hydroxyl ion while glutamate and one potassium ion are expelled as the carrier returns to the outward facing configuration

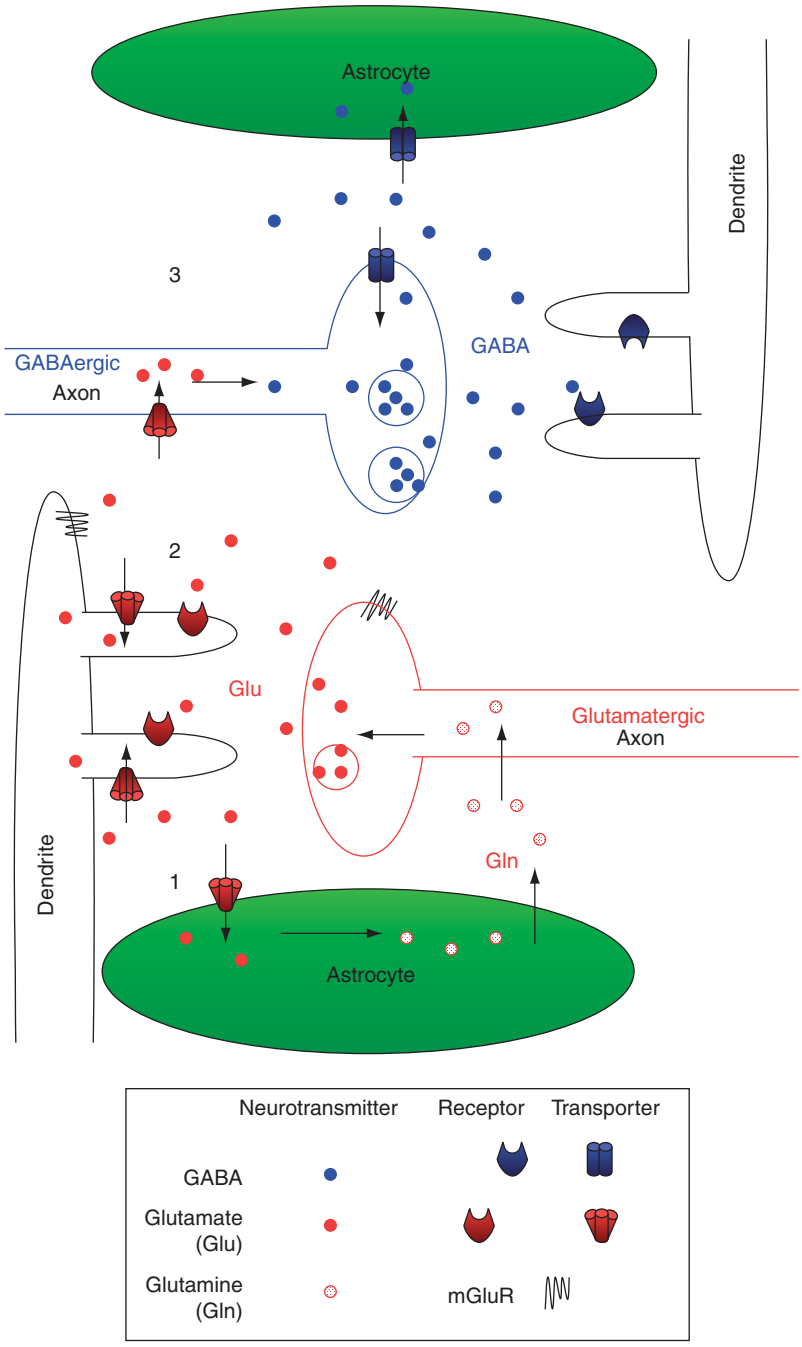


Figure 2 Cellular locations of EAATs and metabolic actions. 1. Glutamate release from the presynaptic terminal activates synaptic glutamate receptors. Synaptically-released glutamate is removed by EAATs located on neurons and astrocytes. Once inside the astrocyte, glutamate is converted into glutamine, which is transported out and taken up by neurons and converted back into glutamate to re-enter the cycle of neurotransmission. 2. Glutamate also diffuses away from sites of release to activate mGluRs. Postsynaptic EAATs can regulate the amount of glutamate available to activate mGluRs. Some of the extracellular glutamate is taken up by neighboring GABAergic neurons where GAD converts glutamate into GABA and enters the GABAergic neurotransmitter pool. 3. GABA is removed from the neurotransmitter pool by GATs on astrocytes or maintained in the neuronal pool by presynaptic GATs.

(Figure 3). Because the substrates and co-substrate carry a net charge, this exchange is electrogenic and a current is generated across the membrane. In fact, monitoring membrane currents is often used to monitor glutamate transporter activity in recombinant systems.

The dependence of glutamate transporters on ion gradients also suggests increased vulnerability of this system when membranes are pathologically hyperpolarized or depolarized. For example, during anoxia, neurons undergo a prolonged state of depolarization, ('anoxic

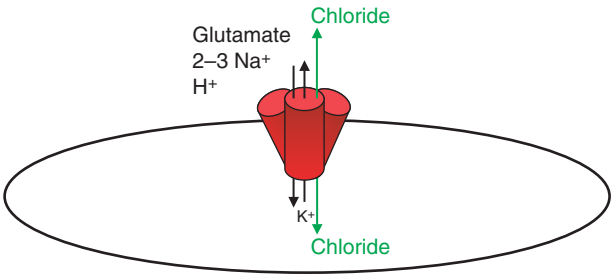


Figure 3 Transport kinetics of EAATs. Under normal, physiological conditions EAATs transport glutamate into cells, against a concentration gradient, by the co-transport of sodium. Hydrogen ions are also brought into the cell and potassium is effluxed. A separate glutamate and sodium gated chloride conductance has also been found, the direction of which is dictated by intracellular and extracellular chloride concentrations.

depolarization'). Depletion of energy stores and the resulting run-down of the sodium pump can lead to a significant reduction in the transmembrane sodium gradient. During this time, glutamate transporters are less functional and may, in fact, transport in reverse. After an ischemic event, extracellular glutamate levels are greatly elevated, in part to EAAT disregulation. The subsequent elevated extracellular glutamate leads to overstimulation of excitatory neurons and glial cells and subsequent cell death. This process is known as excitotoxicity.

In addition to clearing glutamate, some subtypes of glutamate transporter also possess an uncoupled chloride that is activated by substrates and sodium. This secondary role of EAATs as ligand-gated ion channels can complicate the analysis of substrate currents. It has been shown recently for EAAT5 in the retina that these transporter-mediated currents act to hyperpolarize neurons and can directly regulate neurotransmitter release from bipolar cells.

Methods

Initial characterizations of glutamate transport activities in the central nervous system were performed by measuring uptake of radio-labeled substrates in a variety of preparations, including brain slices, synaptosomes (a brain homogenate enriched in pinched off nerve terminals) and primary neuronal and/or glial cultures. To gain a clearer understanding of the energetics and ion requirements of transport, studies were also undertaken in more simplified preparations such as proteoliposomes, in which partially-purified transporter proteins are reconstituted into lipid membrane vesicles. Following the molecular cloning of the various members of the EAAT family, studies of EAATs were extended to include additional molecular and cell-based assays, in which cloned transporter isoforms could be expressed, mutated, and studied in different cellular environments. Two approaches-the measurement of radio-labeled substrate flux and electrophysiological analyses of transport currents-have been used as primary assays of transporter function, regulation, pharmacology, and structure.

The kinetics of uptake can be determined for members of the EAAT family using radio-labeled amino acids. In these assays, a transporter substrate, most commonly tritiated glutamate, is applied to the extracellular side of the cell (or membrane vesicle), and transport is allowed to proceed. Next, the radio-label is washed off, and the incorporated radioactivity is determined. Transport by the EAATs can be described by standard enzyme kinetic equations (Michaelis-Menten), and thus these assays can be used to establish the time-, concentration- and ion-dependence of the process. In addition, the effects of drugs and inhibitors on transport activity can be examined using this approach. In recombinant expression systems, point mutations and other modifications in transporter structure can be made in order to determine the contribution of the primary amino acid sequence to function.

Uptake kinetics can change in two major parameters – apparent affinity or maximum velocity. Changes in apparent affinity can reflect an alteration in the binding sites for substrates or ions within the transporter, whereas changes in maximum velocity usually reflect either a change in the catalytic rate of the carrier (turnover number) or a change in expression on the plasma membrane. Changes in carrier abundance at the cell surface can be assessed with an assay in which surface carriers are covalently modified with a biotin-containing reagent that is added to the external medium. Briefly, a biotin molecule conjugated to a reactive chemical moiety (succinimide) is applied to the outside of the cells where it reacts with exposed lysines. The cells are then harvested and the proteins are isolated by affinity chromatography on avidin-conjugated agarose beads. Western blot analysis of the proteins eluted from the agarose provides a quantitative profile of the membrane expression of the protein of interest. These techniques have been used to demonstrate both increases and decreases in EAAT membrane expression.

Two-electrode voltage clamp recording of currents in *Xenopus laevis* oocytes has been used to measure currents associated with electrogenic transport by the EAATs. Synthetic RNA transcripts of EAATs are injected into the oocyte cytoplasm where the transporter proteins are synthesized and trafficked to the plasma membrane. Once the EAATs appear on the cell surface, substrate-activated currents can be measured electrophysiologically. This approach has enabled investigators to examine the stoichiometry, external ion requirements, and voltage-dependence of the transport process. In addition, because uptake assays can also be done in single oocytes, our group and others have been able to correlate influx of radio-labeled substrates (such as L-glutamate and D-aspartate)

with inward currents, to assess the net charge flux per molecule of substrate.

These methods have provided valuable insights into EAAT function and regulation. Together with approaches from other fields, such as genetic knockdown (RNAi), targeted gene disruption (knockout mice), protein biochemistry, and structural biology (X-ray crystallography), many aspects of the structure and regulation of glutamate transporter function and structure have recently been clarified.

Recent Results

Exciting new studies of EAATs have revealed several distinct structural and regulatory properties of these proteins. A better understanding of the protein's structure and ligand binding is beginning to emerge; the unique features of the protein may indicate directions for devising targeted pharmacological interventions. Unique targeting and trafficking profiles for EAATs, and the potential to manipulate the membrane expression of these transporters, suggest another approach to increasing or decreasing EAAT activity in the human brain.

Pharmacology and Protein Structure

Since the cloning of the EAATs in the 1990s, a variety of useful pharmacological tools have been developed to aid in the characterization of the structure and function of glutamate transporters. Some of these compounds show selectivity for different transporter subtypes and have been used to help establish which transporters are involved in various clinical models of brain injury and disease. A particularly well-studied compound, dihydrokainate (DHK), is a derivative of kainate and exhibits relative selectivity for EAAT2 (**Figure 4**). TBOA blocks all glutamate transporters with comparable potency, but a recently reported derivative, TFB-TBOA, shows selectivity for EAATs 1 and 2. Another recently described compound, NBI 59159, selectively blocks EAAT3. These tools are useful in characterizing the structures of the various EAATs, in experimentally elucidating the contributions of the different isoforms to glutamate homeostasis in the healthy and the injured brain, and in providing avenues for clinical intervention.

Another pharmacological approach to studying glutamate transporters was recently illustrated by a compound isolated from the venom of a spider (*Parawixia bistriata*) that preys on insects. Because insects use glutamate as the neurotransmitter at their neuromuscular junctions, spider venoms often contain components that target glutamatergic systems. Previous studies of the venom from the Joro spider, *Nephila clavata*, have yielded an important antagonist for the study of glutamate receptors. Recent analyses of fractionated *Parawixia bistriata* venom revealed components that altered the activities of glutamate and GABA transporters. This work showed that a component of *Parawixia bistriata* venom, referred to as Parawixin1, appears to act directly and selectively to stimulate glutamate uptake

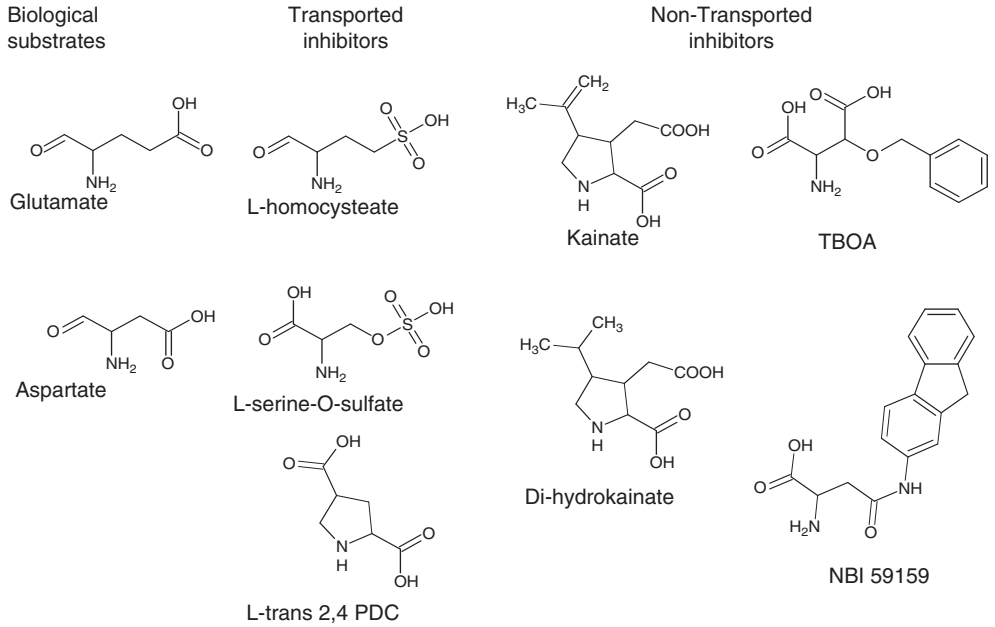


Figure 4 Structure of glutamate transporter inhibitors. Both glutamate and aspartate are substrates for EAATs (left). Transported inhibitors (central column) share some structural similarities. Non-transported inhibitors (right column) are somewhat bulkier and can, as seen with modifications of kainate yielding di-hydrokainate, demonstrate EAAT subtype specificity.

by EAAT2, the major CNS carrier. Studies using EAAT2-enriched proteoliposomes demonstrated that Parawixin1 does not alter the rate of glutamate influx or efflux, but instead facilitates the major rate-limiting step in the transport cycle, the reorientation of the empty carrier to the extracellular surface. An allosteric transport modulator that acts by this mechanism not only provides a research tool for characterization of conformational movements during the reorientation steps in the cycle, but also would have potential utility as a neuroprotective glutamatergic modulatory drug in pathological states characterized by an excess release of glutamate. Consistent with this potential action, Parawixin1 was found to prevent neuronal cell death in a transient ischemia reperfusion model of retinal excitotoxicity. Interestingly, a second component of *Parawixia bistriata* venom, fraction PbTx2, is a GABA transport inhibitor that has an anti-epileptic action.

High throughput screens of chemical libraries have also been a means to identify new modulators of EAAT activity. Recently, a screen of FDA approved reagents found an increase in EAAT2 expression stimulated by beta-lactam antibiotics. Phase 3 clinical trials investigating the potential use of this antibiotic in conditions of long-term glutamate disregulation, such as ALS, are underway.

Understanding the structure of EAATs is another important approach in the development of agonists and antagonists of transport activity. Cloning of the EAATs provided an amino acid sequence that was used to predict membrane topology (**Figure 5**). It appeared that the transporter had 8–12 transmembrane (TM) domains. Topology studies indicate that both the N- and C-termini are on the inside of the cell, and that the transporter is comprised of 8 TM domains with 2 hairpin loops (HP). Functional studies with various mutations have demonstrated that HP1, HP2, TM7 and TM8 are essential for substrate and sodium binding.

Advances in crystallography have yielded important data regarding EAAT structure. A prokaryotic transporter, GLT_{Ph}, has recently been crystallized and illuminates many important new structural features. These studies reveal a trimeric configuration of the transporter, with three subunits associating to form a bowl-like structure. It appears that glutamate can enter into this bowl, essentially moving the substrate half-way across the cell membrane, before any of the transport cycle begins. This crystallized structure also indicates the relative location of the TMs and the HPs. However, data regarding the intracellular N- and C-termini is absent from this model, and other approaches are being used to address their roles in transporter function. These structural features suggest new avenues for agonist and antagonist sites of interaction.

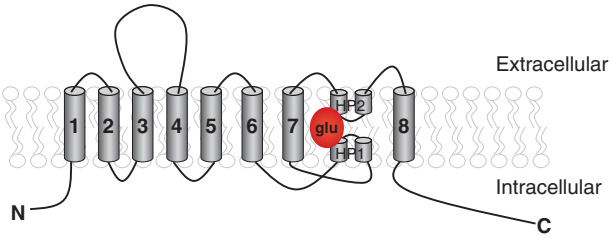


Figure 5 2-dimensional structure of EAATs. EAATs are comprised of 8 transmembrane domains, interspersed with two hairpin loops. Both the N- and C-termini are inside the cell, potentially acting as interactors with other proteins. A large extracellular loop between TMs 3 and 4 appears to by glycosylated, the function of which is unclear.

Cellular Trafficking of Transporters

Membrane trafficking is an important means of regulating transporter function. There are a variety of mechanisms that control the insertion and removal of membrane proteins at the cell surface as a means of increasing or decreasing their activity. For example, an elegant mechanism has been proposed for the regulation of GABA transport in which the accumulation of intracellular GABA through GAT activity triggers a cascade of events that increase GAT surface expression, effectively increasing transporter activity in response to increased synaptic activity.

One robust observation regarding transporter regulation involves stimulation of the protein kinase C (PKC) pathway. EAATs 1 and 2, like the monoamine transporters, are internalized by endocytosis in response to PKC stimulation. However, EAAT3 appears to be regulated differently. Rather than being expressed predominantly at the cell surface, EAAT3 is often found in vesicles just beneath the cell membrane. In response to a PKC stimulus, the transporter is recruited from the intracellular compartment to the cell membrane, increasing glutamate transport activity. The differences in the behavior of the various EAAT isoforms and their cellular locations provide interesting avenues of research.

The cellular mechanisms that govern the insertion and internalization of transporters in neurons and glial cells have not been established. Although many studies support the involvement of kinases and phoshatases in the regulation of transporter trafficking, it remains unclear whether direct phosphorylation of the transporter serves to trigger transporter recruitment or internalization. Several years ago, a number of glutamate transporter associated proteins (GTRAPs) were identified through a yeast-two hybrid screen. GTRAP41 and GTRAP48 interact with, and increase the activity of, EAAT4 through a mechanism involving an increase in the cell surface levels of the transporter. Another of these putative regulatory molecules, GTRAP3-18, interacts with and reduces the activity of EAAT3 by lowering substrate affinity. The LIM-domain containing protein, Ajuba, was identified as an EAAT2 interacting protein; however, as with many of the glutamate transporter-interacting proteins identified

to date, the physiological role of the protein in regulating transporter function remains to be determined.

In addition to having distinct protein binding partners and a variety of mechanisms for regulation, glutamate transporter subtypes display different patterns of subcellular distribution. There is a growing body of knowledge addressing polarized sorting, targeting, clustering and anchoring domains that dictate the localization of carriers to specific regions of the cell. While the EAATs exhibit substantial homology, unique domains in EAAT3 have been shown to be important for its polarized sorting in neurons and kidney epithelial cells, the two major cell types where it is expressed endogenously. For example, in neurons, EAAT3 targets to dendrites and is distributed perisynaptically; in the kidney, EAAT3 is found almost exclusively on the apical membrane of the cell. Using gene constructs to resolve the sequences required for polarized sorting in neurons and kidney epithelial cells, we demonstrated a unique sequence within the C-terminus of EAAT3 that is required for both targeting to apical membranes of epithelial cells and to dendrites in neurons. This sequence was not, however, required for clustering of the carrier in the postsynaptic membrane, suggesting that multiple signals are required to specify targeting and anchoring of the carriers. Targeting to specific subcellular domains can also be facilitated by lipid raft dynamics, short cytoplasmic motifs, glycosyl-phosphatidylinositol (GPI) linkage and/or glycosylation.

Recent studies suggest that EAAT2 localization may be facilitated by targeting to lipid rafts. Lipid rafts are specialized domains of the membrane that are rich in cholesterol and sphingolipids. EAAT2 was shown to be different from other glutamate carriers in its localization within a detergent-resistant lipid microdomain, suggesting that the protein may contain sequences that preferentially target it to raft-like structures. In fact, depletion of cholesterol from primary cortical cultures leads to a decrease in glutamate transport, which is paralleled by a decrease in EAAT2 clustering and membrane expression. It is unclear which domain in the EAAT2 structure dictates lipid raft targeting; however, this distinct targeting profile may explain why EAAT1 and EAAT2 appear in different subcellular compartments in astrocytes and are subject to differential regulation.

Conclusions and Directions for the Future

Epilepsy is characterized by an imbalance of extracellular glutamate and GABA. Neurotransmitter transporters are responsible for maintaining appropriate levels of neurotransmitters in the extracellular space and pose an avenue of potential therapeutic intervention. Inhibition of GABA transport has been used widely in managing epilepsy; however, such treatment does not address all kinds of epilepsies. Modifications of the glutamate system may prove to be another useful avenue of intervention or a complementary treatment to GABA modulation.

Work in our laboratory addresses the structure, function, and trafficking of EAATs. We have been interested in using our current understanding of the transport mechanism and its relationship to EAAT structure to enable the design of agents to modulate glutamate transport in pathological conditions. Increases in extracellular glutamate prior to or at seizure onset have been described, and glutamate has also been implicated in the initiation, spread, and maintenance of seizure activity. Thus, the development and design of compounds that enhance glutamate uptake is an attractive therapeutic strategy, both for epilepsy as well as for neurodegenerative diseases linked to excess glutamate. With this goal in mind, we have investigated the mechanism of action of spider-venom derived compound, Parawixin1, which enhances glutamate clearance by the major glial carrier, EAAT2. This mechanism for enhancing the rate limiting step in the glutamate transport cycle provides 'proof of principle' for the identification of small molecule drugs which increase uptake by similar allosteric mechanisms, and which may prove beneficial in clinical settings.

EAAT activity may also be increased by modifications in membrane expression. Current understanding suggests that changes in the carrier's localization are mediated by phosphatase and kinase activation. These enzymes effect either the transporter directly or a unique binding partner. Identifying and understanding all of the components of the intracellular cascades that contribute to targeting and trafficking pathways will likely lead to other methods for modifying EAAT activity and for developing therapeutic tools to treat epilepsy.

Future challenges include a better understanding of all these features of EAATs, as well as a larger understanding of the overall regulation of glutamate in the human brain. Screens for small molecules may identify compounds that manipulate trafficking, chloride currents, transport efficiency, or even modify GAD or GS. New advances in gene therapy present other tools for examining glutamate homeostasis. Genetic regulation of EAATs is also being explored with investigations of upstream regulators of the transporters.

See also: **Cell Damage/Excitotoxicity**: Excitotoxicity and Neurodegenerative Disease; Molecular Mechanisms of Cell Death After Seizures; **Glia/Astrocytes**: Astrocytic Regulation of Neuronal Excitability; Glial-Mediated Mechanisms of Epileptogenesis in Tuberous Sclerosis; **Neurotransmitter Function**: Consequences of Diminished Function of the Glutamine Cycle on Inhibitory Function in the Epileptic Brain; **Transporters**: Function of Cell-Surface Glutamate Transporters in the Brain: An

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Neuronal Cl Transport: Setting the Stage for Successful Inhibition

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Introduction

The movement of ions through membrane channels is generally thought to be limited by the open probability of the channel through which the ions flow. However, this is not so true for chloride-permeable channels. Although the concentration of chloride in the extracellular space is about 120 mM, the intracellular concentration is only 2–20 mM, depending on the cell type. The rest of the anions inside the cell are negatively charged amino acids such as glutamate and aspartate, either free in the cytoplasm or more commonly as components of proteins. Changing the chloride concentration by just a few millimoles will alter the reversal potential for chloride to be positive or negative to the resting membrane potential of a neuron. In other words, small alterations in chloride concentration will change the direction of chloride flow through channels that open when the cell is at rest. Changing the direction of ion flow changes the effect of channel opening from hyperpolarization of the membrane to depolarization of the membrane.

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The movement of ions through membrane channels is generally thought to be limited by the open probability of the channel through which the ions flow. However, this is not so true for chloride-permeable channels. Although the concentration of chloride in the extracellular space is about 120 mM, the intracellular concentration is only 2–20 mM, depending on the cell type. The rest of the anions inside the cell are negatively charged amino acids such as glutamate and aspartate, either free in the cytoplasm or more commonly as components of proteins. Changing the chloride concentration by just a few millimoles will alter the reversal potential for chloride to be positive or negative to the resting membrane potential of a neuron. In other words, small alterations in chloride concentration will change the direction of chloride flow through channels that open when the cell is at rest. Changing the direction of ion flow changes the effect of channel opening from hyperpolarization of the membrane to depolarization of the membrane.

GABA, the principal inhibitory neurotransmitter in the mature brain, operates an ion channel that is selectively permeable to ions, particularly chloride. Some of

the most effective anticonvulsants such as the benzodiazepines and barbiturates work by increasing the open probability of the $GABA_A$ receptor-operated channel. The efficacy of these anticonvulsants is thus predicated on the gradient for chloride flux. Understanding how this transmembrane chloride gradient is maintained, and the physiological and pathological conditions under which it is reversed, are thus of direct import to epilepsy research.

Background

The neuronal $GABA_A$ receptor is selectively permeable to anions, particularly chloride. The driving force for chloride movement through the $GABA_A$ channel is quite precarious, and can be redirected to drive chloride either into or out of the neuron when GABA binds to the $GABA_A$ receptor. The direction of chloride flow determines whether the opening of $GABA_A$ receptor-operated channels results in the net accumulation or loss of negative charge. When negative charge is accumulated, the net result of $GABA_A$ receptor activation is hyperpolarization of the membrane and inhibition of neuronal firing. When intracellular negative charge is reduced as a consequence of chloride efflux, the net result of $GABA_A$ receptor activation is membrane depolarization, which may excite the neuron by virtue of initiating action potentials or activating voltage-gated calcium channels. Thus, the ion transporters that determine the driving force and direction for GABA-gated chloride flow are important determinants of the function of GABAergic synapses. It follows, then, that chloride transport also determines the effects of anticonvulsants – such as the benzodiazepines and barbiturates – that act by increasing the open probability of GABA-gated chloride channels.

Chloride transporters do not directly utilize ATP to provide the energy to move chloride across its electrochemical gradient. Rather, 'upstream' chloride transport (i.e., movement of chloride against its electrochemical gradient) is energized by coupling the chloride transport to the 'downstream' movement of a cation such as sodium or potassium. KCC2 is a chloride transporter expressed only in neurons that couples the transport of chloride and potassium out of the neuron. Although KCC2 is freely reversible, the transmembrane potassium gradient is maintained by NaK-ATPase such that potassium efflux is strongly favored, so KCC2 is normally a chloride exporter. NKCC1 is a chloride-importing transporter that couples the influx of chloride and potassium to the influx of sodium down its electrochemical gradient. The potassium-chloride and sodium-potassium-chloride transporters are electrically neutral. In other words, an equal number of cations and anions are transported across the membrane in a given direction. Electronuetral transporters are not affected by the membrane potential, nor do they affect the membrane potential or the membrane resistance.

Membrane ion transport must be plastic; that is, it must change to accommodate differing physiological conditions. For example, the cellular response to increases and reductions in intracellular volume is critical to the survival of the cell. NKCC1 is generally activated by decreases in intracellular volume. The electroneutral import of sodium chloride and potassium chloride by this transporter results in osmotically driven increases of intracellular water that act to restore cell volume. KCC2 provides the opposite function, exporting potassium chloride to reduce cell salt in order to drive an osmotic reduction in cell water and volume. These transporters are subject to a variety of regulations, most of which remain poorly understood in neurons. As one example, it is important that both transporters not operate at the same time. Each transporter is capable of moving sufficient ions to alter the intracellular concentration of chloride at a rate of mM/liter/second. If both NKCC1 and KCC2 were active at the same time, they would move similar quantities of sodium and potassium, which would need to be replaced at the same rate by NaK-ATPase. The consequence would be a catastrophic reduction in the ATP concentration over the course of only a few seconds. One mechanism to prevent this scenario is oppositely directed activation sequences. Specifically, NKCC1 is activated by phosphorylation while KCC2 is activated by dephosphorylation.

Immature neurons maintain high intracellular chloride concentrations through the action of NKCC1. In these neurons, GABA is excitatory because opening anion-selective channels results in efflux of negatively charged chloride ions. Perhaps as a consequence, anticonvulsants such as the barbiturates are not very effective treatments of neonatal seizures. NKCC1 is selectively blocked by nanomolar concentrations of the diuretic bumetanide. The block of NKCC1 leaves no active transport in immature neurons, because KCC2 is not expressed in neurons until later in the development. However, even when chloride is passively distributed across the membrane (so that the chloride reversal potential = resting membrane potential), opening chloride channels is inhibitory because of the increase in membrane conductance that effectively shorts out or shunts excitatory currents. Thus, bumetanide has been proposed as an adjunct to the treatment of neonatal seizures with barbiturates or benzodiazepines.

In mature neurons, the intracellular chloride concentration is maintained at low levels by the action of KCC2. Opening chloride-permeable $GABA_A$ receptor-activated channels results in chloride influx and hyperpolarization of the membrane. However, KCC2-mediated chloride transport does not always keep up with GABA-mediated chloride flux. In mature neurons, this 'mismatch' results in depolarizing shifts of the chloride reversal potential during periods of intense activation of $GABA_A$ receptors.

The $GABA_A$ channel is also slightly permeable to bicarbonate, which has a reversal potential near 0 mV at physiological pH. Under some circumstances, bicarbonate efflux through the GABA channel can dominate chloride flux (when KCC2 transport fails to keep up with GABA-gated chloride flux). When this occurs, the net anion flux is outward, resulting in GABA-mediated depolarization of the membrane. Bicarbonate flux can be reduced by inhibiting carbonic anhydrase, the enzyme that catalyzes the hydration of CO_2 to bicarbonate and the dehydration of bicarbonate to CO_2. This action may comprise the anticonvulsant action of the diuretic acetazolamide.

The transport of chloride can change not only physiologically, as occurs during normal development, but also under pathological conditions such as human and experimental epilepsy. The maximal transport rate has been demonstrated to be decreased in two different experimental epilepsy models. This decrease may occur as a consequence of reductions in KCC2 expression due to BDNF activation of TrkB receptors; other potential mechanisms have not been evaluated, such as dephosphorylation-mediated reduction in the activity of KCC2 or an alteration in membrane trafficking. In human temporal lobe removed during surgery for intractable epilepsy, GABA has been demonstrated to be excitatory in some neurons in the subiculum, and corresponding reductions in the expression of KCC2 and increases in expression of NKCC1 have been demonstrated in this brain region. The reduced transport capacity of KCC2 makes it more likely that KCC2 activity can be overwhelmed during periods of intense GABAA receptor activation, while the expression of NKCC1 implies that any level of $GABA_A$ receptor activity may result in membrane depolarization.

One way to sort out the changes in chloride transport occurring during epilepsy is to evaluate the kinetics of chloride transport. The techniques for this approach are described in the following sections.

Methods

In comparison with other cell types, neurons are uniquely equipped for studies of chloride transport because of the $GABA_A$ receptor-operated chloride channel. The $GABA_A$ channel can be activated whenever needed and for as long as is needed by application of ligand. The $GABA_A$ channel can be used to either load the neuron with chloride or deplete the neuron's chloride. Further, the channel can be used to assay the concentration of chloride inside the neuron by testing the reversal potential of the currents flowing through the channel. The ability to alter the concentration of intracellular chloride and assay the changes in chloride concentration provide the tools necessary for experiments in which the neuronal chloride concentration is perturbed and then monitored as the transporters work to drive the chloride concentration back to the steady state. The time course of the chloride concentration changes after the perturbation can be used to derive important kinetic data regarding the active transporter, including the maximum velocity of transport, the affinity of the transporter for chloride, and the driving force for transport.

There are several technical aspects of these kinetic studies that are worth emphasis. The methods used to activate $GABA_A$ receptors can influence the results of the experiment. In brain slice preparations, GABA is subject to rapid uptake, and so does not diffuse far from the point of application. Using agonists that are not subject to reuptake, such as muscimol or isoguvacine, will result in diffusion across the preparation, with the activation of many distant GABA receptors. In kinetic experiments, the volume into which the GABA-gated chloride currents flow is an important variable and using GABA limits the region of activation. If the chloride currents flow into or out of small intracellular volumes, the change in chloride concentration will be proportionately larger than if the currents flow into or out of a larger intracellular volume. Since the change in chloride concentration is what drives the change in transport rate, and is what is monitored by the change in GABA reversal potential, regions with many GABA receptors per unit volume (such as the dendrites) are more easily studied than areas with fewer GABA receptors per unit volume (such as large somata) with these techniques.

When GABA receptors are activated over limited regions of the neuron, the diffusion of chloride to or from other regions of the neuron will act in concert with the active chloride transport to restore the chloride concentration to equilibrium. One way to address the impact of diffusion is to estimate the geometry of the activated region (based on DIC microscopy) and then estimate the rate of diffusion. The diffusion coefficient for intracellular chloride has been published, so these calculations are quite feasible. A more accurate means of separating the effects of diffusion from active transport is to repeat the experiment with active transport blocked. This protocol leaves only diffusion to restore the chloride concentration. The rate of restoration of chloride concentration after transport is blocked can be subtracted from the rate before the block of transport to derive the rate of transport. This approach is particularly useful for NKCC1, which can be selectively blocked by bumetanide. KCC2 and KCC3 cannot be selectively blocked, although in normal adult neurons there is a minimal NKCC1 expression, so a nonselective transport inhibitor such as furosemide can be used.

Gramicidin-perforated patch electrodes that are selectively permeable to cations are commonly assumed to be the best means to study chloride transport. Neurons

expressing NKCC1 are very sensitive to the intracellular sodium concentration, as this concentration determines the free energy available for the import of potassium and chloride against their concentration gradients. Gramicidin electrodes alter the intracellular sodium by virtue of diffusion of sodium into or out of the electrode solution, and because many physiologists leave sodium out of the electrode solution, the resulting intracellular sodium concentration may be artificially low. This situation would result in an excessively large driving force for NKCC1 and a correspondingly high intracellular chloride concentration. Neurons that only express KCC2 are less vulnerable to this effect, because the intracellular potassium concentration is already high in these neurons, and small errors due to diffusion of electrode solution via gramicidin ionophores will not significantly change the driving force for potassium efflux.

Results

Several labs have examined the relaxation of intracellular chloride concentrations after loading or depletion. There is general agreement that this relaxation is a first-order process. Characterizing the relaxation by a rate constant provides some insight into the process, but does not separate the various aspects of transport that contribute to the rate. These contributions include the transporter's affinity for chloride, maximum transport rate, and the driving force for forward or reverse transport. We found that transport by NKCC1 was as reversible as transport by KCC2, suggesting that both transporters are likely to be limited by the driving force or free energy available at the steady-state intracellular chloride concentration. Although Michaelis–Menten kinetics are frequently used to describe membrane ion transport, in the case of neuronal chloride the Michaelis–Menten equation fails to capture the effects of the amount of free energy available to drive transport. This point is important because the action of the transporter changes the chloride concentration and thus the free energy available for transport – while the Michaelis–Menten equation assumes that the available free energy is constant.

We measured the time course of NKCC1 transport after correcting for diffusion by subtracting the rate of chloride relaxation in the presence of bumetanide from the relaxation rate in the absence of chloride. In analogy to Ohm's law, the time course of chloride relaxation was described by an equation containing a conductance term (the transport availability described by the Michaelis–Menten equation) and a term describing the driving force in terms of the total free energy for the transported species, sodium, potassium and chloride. This equation fits the data well despite the fact that the only free variables were the maximum velocity of transport and the steady-state chloride concentration. The affinity of the transporter for chloride was not a useful parameter because the extracellular chloride concentration was high and constant.

These experiments demonstrated that in immature/developing hippocampal neurons, NKCC1-mediated inward chloride transport is limited primarily by the driving force available, so that the resting chloride concentration defines the point at which the free energy for both forward and backward transport is zero. NKCC1-mediated transport was limited by the maximum velocity of transport only at very high rates of GABA-gated chloride flux. The determination of resting chloride concentration by the free energy available for transport has an important implication: changing the concentrations of potassium or sodium, the cations that are cotransported with chloride will change the free energy and thus the steady-state chloride concentration. This relationship led to a surprising finding: changes in NaK-ATPase activity, induced by a series of action potentials, led to changes in the steady state concentration of chloride, presumably by altering the intracellular sodium concentration and thus the free energy available for chloride import. Sodium diffusion into a low-sodium electrode solution in gramicidin-perforated patch recordings will induce similar increases in intracellular chloride, as previously discussed.

Experiments that explore changes in chloride transport in epilepsy are more difficult because it is not easy to compare results across animals and conditions. For example, suppose the density of $GABA_A$ receptors is changed in epileptic animals so that there are fewer $GABA_A$ receptors per unit of membrane area. Under these conditions, a reasonable experimental control – such as ascertaining that the GABA currents used to load neurons with chloride are the same size in control and epileptic animals – may actually distort the results. That is, to elicit the same current, a larger area of membrane will need to be exposed to GABA in the animals with lower densities of GABA receptors; this 'compensation' will lead to smaller changes in chloride, which may be interpreted as better maximum chloride transport, as well as a more significant diffusion component to the relaxation back to steady state. To offset this problem, the volume into which the evoked chloride currents flow can be estimated in each experiment by dividing the total loading chloride flux by the change in neuronal chloride concentration.

The effects of action potential trains on chloride transport, as noted earlier, would certainly suggest that seizure activity may lead to increases in inwardly directed neuronal chloride transport and corresponding increases in neuronal chloride concentration. Increases in neuronal NKCC1, in epileptic tissue, have been demonstrated at both RNA and protein levels, and increases in neuronal chloride have also been reported. It is interesting that this

increase is not at all uniform across neurons in a region. The chloride reversal potential is quite heterogeneous, suggesting that each neuron's transport may be uniquely impacted by the processes that lead to epilepsy.

Future Directions

A great deal of progress has been made in the last decade regarding the physiology of neuronal chloride transport. Characterization of the kinetics of chloride transport has been performed in normal animals, and similarly detailed studies are beginning to be undertaken in epileptic animals. The heterogeneity of chloride transport in epileptic animals will be a challenge, because of the increased number of neurons that will need to be evaluated. Chloride transport studies require 30 min or so of very stable recordings; perforated patch recordings are frequently required. These conditions make electrophysiological analyses of chloride transport particularly labor-intensive, and thus it will be difficult to study the requisite number of neurons in epileptic animals. The development of genetic dual-wavelength chloride-sensitive dyes, such as Clomeleon, may make these studies much easier to undertake. Voltage clamp recordings have the advantage of manipulation of the membrane potential, which greatly facilitates chloride loading and depletion. However, as long as chloride is not passively distributed, it is possible to alter the neuronal chloride concentration by the activation of GABA receptors. The advantages of chloride dyes will rest on the increased number of neurons that can be studied, the ease with which chloride changes can be recorded, and the possibility of assessing anatomical distribution of the chloride changes.

Clinically, pharmacological manipulation of chloride transport is about to undergo clinical trial, as bumetanide is tested as an adjunct to phenobarbital for neonatal seizures. Bumetanide is very lipid soluble, but data regarding its permeability across the blood–brain barrier is limited. Pilot trials of diuretic therapy for intractable temporal lobe epilepsy have been reported in abstract form, with some excellent successes mixed with predictable side effects related to diuresis. Development of strategies to limit the block of NKCC1 to the brain may therefore be particularly useful.

See also: **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; Pathophysiology of Status Epilepticus; **Metabolic Changes**: Role of Metabolic Substrates and Acid-Base Balance in Seizures; **Non-Synaptic Mechanisms**: Changes in Extracellular Space as a Modulator of Excitability and Epileptogenicity; Non-Synaptic Mechanisms in Seizures: The Role of Ion Fluxes and Intrinsic Membrane Properties; **Transporters**: Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma.

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Rapid Degeneration of the Neuronal K-Cl Cotransporter, KCC2, after Trauma

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Introduction – Intracellular [Cl$^-$] Homeostasis

Chloride is one of the most abundant free anions in animal cells, and it clearly has important roles in cell physiology. Since $[Cl^-]_i$ is an important determinant of the Cl^- electrochemical gradient across the plasma membrane, it helps dictate the electrical consequences of anion channel function, and thereby plays a significant role in various cell functions, including cell volume regulation, transepithelial salt transport, and synaptic signaling (depolarizing and hyperpolarizing responses). In addition, $[Cl^-]_i$ is a powerful allosteric regulator of many different proteins, including regulatory proteins and transport proteins. These facts belie the outdated view that $[Cl^-]_i$ is not actively regulated and is distributed at equilibrium across the plasma membrane. It is now well established that Cl^- is actively maintained away from its equilibrium distribution, and is subject to tight regulatory control in virtually all animal cells. Two notable exceptions are skeletal muscle cells and vertebrate red blood cells, both of which have remarkably high Cl^- permeability owing to the constitutive activity of either a high conductance Cl^- channel or a robust anion exchanger.

Like other intracellular ions, steady-state $[Cl^-]_i$ is determined by the relative contributions of active 'pumps' and passive 'leaks.' Most animal cells maintain steady-state $[Cl^-]_i$ above electrochemical equilibrium ($[Cl^-]_i > 30$ mM), implying the presence of a dominant transport pathway that actively *accumulates* Cl^- inside cells. A key exception to this generality is the mature neuron that exhibits hyperpolarizing synaptic inhibition mediated by ligand-gated anion channels. In these neurons, $[Cl^-]_i$ must be below electrochemical equilibrium (i.e., $[Cl^-]_I \sim 5–7$ mM) in order for Cl^- influx to carry hyperpolarizing current; these cells therefore require the presence of a dominant transport pathway that actively *extrudes* Cl^- from the neuron. Thus, depending on the cell type, $[Cl^-]_i$ can be maintained either above or below electrochemical equilibrium by dominant active Cl^- accumulation or extrusion mechanisms, respectively. To date, the most important Cl^- 'pumps' identified are the secondary active cation chloride cotransporters (CCCs), including Na-K-Cl cotransport (NKCC), Na-Cl cotransport (NCC), and K-Cl cotransport (KCC). Through the operation of the CCCs, energy stored in the Cl^- electrochemical gradient can be used by dissipative 'leak' pathways to perform useful electrical and chemical work, such as Cl^- secretion in epithelial cells by cystic fibrosis transmembrane regulator (CFTR) and Cl^--dependent neuronal signaling by $GABA_A$ and glycine receptors ($GARA_AR$ and GlyR).

Background – Developmental and Trauma-Induced Changes in Neuronal [Cl$^-$] Homeostasis

In mature central neurons, GABA and glycine are inhibitory neurotransmitters that mediate fast hyperpolarizing inhibition through activation of anion-permeant $GABA_A$ and glycine receptor/channel complexes, respectively. In immature neurons, however, $GABA_A$/glycine receptor-mediated responses are typically depolarizing, leading to transient increases in intracellular $[Ca^{2+}]$ and often excitation. Such depolarizing $GABA_A$/glycine receptor-mediated responses in immature neurons may provide a critical trophic signal during neuronal development, possibly controlling neuronal proliferation, migration, and targeting. The depolarizing $GABA_A$/glycine receptor-mediated responses are typically replaced by hyperpolarizing responses as neurons mature. The timing of this polarity switch depends on the degree of neuronal maturation, and hence will vary between brain regions and between animal models (e.g., precocious vs. altritial young).

The commonly observed developmental polarity shift (depolarizing to hyperpolarizing) in $GABA_A$/glycine receptor-mediated responses is due to an alteration in neuronal $[Cl^-]$ resulting from a differential expression/activity of CCCs, specifically the NKCC1 and the neuronal K-Cl cotransporter, KCC2. NKCC1 is present in immature neurons where it mediates robust neuronal Cl^- accumulation, leading to elevated $[Cl^-]_i$ and depolarizing $GABA_A$/glycine receptor-mediated responses. In contrast, as most neurons mature, they begin to express KCC2 and reduce NKCC1 expression. Under normal physiological conditions, KCC2 is a Cl^- extrusion pathway, and it plays a critical role in maintenance of low $[Cl^-]$ and hence hyperpolarizing $GABA_A$/glycine receptor-mediated responses of mature neurons.

Remarkably, depolarizing $GABA_A$/glycine receptor-mediated responses are observed not only in immature

neurons, but also in mature neurons shortly after a traumatic insult (e.g., concussion, epileptic seizures, hypoxia/ischemia). Such trauma-induced depolarizing $GABA_A$/glycine receptor-mediated responses in mature neurons are due to a positive shift in Cl^- reversal potential, indicating that there is an alteration in Cl^- transport capacity and accumulation of neuronal $[Cl^-]$. A number of recent studies have examined the expression of the CCCs following various types of trauma, including seizures. Each of these studies observed a marked reduction in expression of KCC2 protein following trauma that was invariably coupled with the appearance of depolarizing and often excitatory $GABA_A$R-mediated responses. Trauma-induced loss of KCC2 protein expression in mature neurons involves both cessation of KCC2 gene expression and degradation of KCC2 protein. A BDNF/TrkB receptor-mediated signaling mechanism has been directly linked to the downregulation of hippocampal KCC2 mRNA and protein after sustained interictal-like activity. In addition to the downregulation of KCC2 gene expression, we have begun to examine the cellular mechanisms responsible for the degradation of KCC2 protein following trauma.

Methods

In our studies, we have made use of two different trauma models, an in vivo whole animal model (i.e., focal cerebral ischemia) and an in vitro cultured model (i.e., glutamate exposure of cultured cerebellar granule cells). Focal ischemia was induced in rats using the middle cerebral artery occlusion (MCAO) technique. Male Sprague-Dawley rats (250–300 g) were anesthetized with ketamine (20 mg per 100 g intraperitoneally) and xylazine (0.1 mg per 100 g intraperitoneally). The left common carotid artery was dissected out. The superior thyroid artery and the occipetal artery were coagulated. The internal carotid artery was dissected out carefully from the vagus nerve, and the pterygopaletine artery was ligated. After ligating the external carotid artery and clamping the common and internal carotid artery, an incision was made in the external carotid artery to insert a suture (3.0 Dermalon-20 mm) with a blunt end. The suture was advanced until the blunt end stopped, which indicated the starting point of the middle cerebral artery (MCA). The clamps were removed and MCA remained occluded for 1 h. The suture was withdrawn from the MCA, and the brain was immediately perfused with 4% paraformaldehyde (pH 7.4) in PBS via the asceding aorta. The brain was dissected, post-fixed for 2 h, and coronal sections (100 μm) were cut for immunohistochemistry.

Cerebellar granule cells (CGC) were obtained in primary culture from 7-day-old rats (Sprague-Dawley) and plated at a density of $\sim 5 \times 10^6$ cells cm^{-2} on dishes coated with poly-D-lysine. CGCs were grown in modified Eagles' minimum essential medium containing 10% fetal bovine serum, 25 mM KCl, 30 mM glucose, 7 μM para-aminobenzoic acid, and 100 mU l^{-1} insulin. Glial cell proliferation was inhibited by the addition of 20 μM cytosine arabinofuranoside 24 h after plating. For in vitro trauma experiments, conditioned growth media was removed and saved, and the CGCs were washed twice in Mg^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM HEPES). CGCs were then brought up in Mg^{2+}-free Locke's solution containing glutamate at 30 μM or 100 μM. After 30 min incubation in a humidified incubator (5% CO_2; 37°C), glutamate-containing solution was removed, and CGCs were washed three times in Locke's solution. After this initial washing, CGCs were returned to their conditioned media in a humidified incubator (5%CO_2; 37 °C). CGCs were sampled at various time points following glutamate exposure (1–24 h) by lysing cells in sample buffer.

We used standard immunological techniques to detect changes in NKCC1 and KCC2 protein expression at specific times points following MCAO or glutamate exposure. Western blotting and immunohistochemical protocols have been previously published from our laboratory. For Western blots, whole cell lysates were separated on 7.5% SDS-PAGE tricine gels, electroblotted to PVDF membranes, and NKCC1 (~150-kDa) and KCC2 (~140-kDa) proteins were quantified using chemiluminescence and phosphorimaging. The antibodies we employed included rabbit anti-KCC2 antibodies and a mouse monoclonal anti-NKCC antibody.

Results

Focal ischemia in rat brains was induced by a one hour occlusion of the middle cerebral artery (MCAO). In this preparation, only those arteries supplying blood flow to the right hemisphere were occluded. The left hemisphere remained unaffected, thus providing an internal control for the focal ischemic insult. To determine those brain regions impacted by the focal ischemic insult, brain sections (2 mm thick) were stained with 2% 2,3,5,-triphenyltetrazolium chloride monohydrate (TTC). Those areas exhibiting injury following one hour MCAO remained unstained by TTC and included the right frontal, parietal, piriform, and perirhinal cortices and striatum (**Figure 1(a)**). Significantly, KCC2 immunoreactivity was markedly reduced in those areas where infarction occurred (**Figure 1(b)**). In contrast, we observed little change in NKCC1 immunoreactivity in control and ischemic regions (data not shown). In sham-operated animals, no significant change was observed for KCC2 immunoreactivity (**Figure 1(c)**). These data indicated that there is a selective and rapid reduction in KCC2 protein following in vivo ischemic injury.

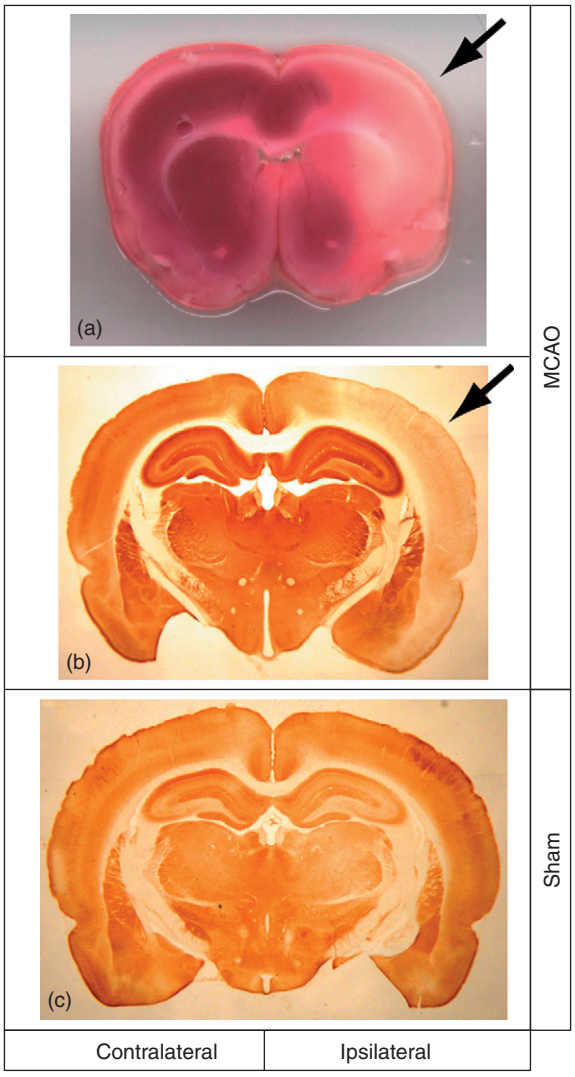


Figure 1 Infarct volume (a) after 1 h MCAO and KCC2 immunohistochemical staining after 1 h MCAO (b) or sham operation (c). Arrows in a and b point to ipsilateral side (right hemisphere) of cerebral cortex where there was significant cerebral injury (a) and loss of KCC2 immunoreactivity (b) after 1 h MCAO. Infarct volume was identified with 2,3,5-triphehyltetrazolium chloride (TTC) on 2 mm thick coronal sections and KCC2 immunoreactivity was visualized with diaminobenzedine on coronal sections (100 μm). Data are representative of 4 (MCAO), 3 (sham), or 2 (TTC) experiments.

To investigate the cellular mechanisms responsible for this rapid reduction in KCC2 protein expression following injury, we established an in vitro trauma model, using cultured cerebellar granule cells (CGCs) exposed to glutamate. We have previously shown that these neurons robustly express KCC2 in the adult rat brain. Furthermore, GCGs are easily obtained in large numbers at high purity (>90%) in primary culture. GCGs express increasing levels of both NKCC1 and KCC2 protein when grown in primary culture. NKCC1 immunoreactivity reached 70% of its maximum by the end of the first week in culture, i.e., 7 days in vitro (DIV). In contrast, KCC2 immunoreactivity appeared much more slowly in culture – not starting until the end of the first week of culturing and reaching a peak at 12 DIV. In order to ensure maximum expression of the cation chloride cotransporters, we used GCGs between 14 and 18 DIV in subsequent glutamate exposure experiments. Cultured GCGs were exposed to glutamate (30 μM or 100 μM) for 30 min and then subsequently washed free of glutamate and incubated in glutamate-free media. As shown in Figure 2, GCGs exhibited a rapid reduction in KCC2 protein expression when exposed to glutamate. Thirty minutes after exposure to each concentration of glutamate, there was a significant loss of KCC2 immunoreactivity; only ~50% of the control KCC2 protein level remained after 100 μM glutamate exposure. We interpret this loss of KCC2 immunoreactivity to be the result of protein degradation. Three hours after 100 μM glutamate exposure, KCC2 protein expression reached 40% of control levels, and this level was maintained for up to 24 h. As with the results for focal ischemic injury, the loss of KCC2 protein following in vitro trauma was selective; we never observed a significant change in NKCC1 protein levels in GCGs at any of the time points (up to 24 h) following cell exposure to 100 μM glutamate. Furthermore, cell viability experiments (trypan blue exclusion) demonstrated that greater than 80% of cells survived 30min exposure to 100 μM glutamate (data not shown). These data indicated that glutamate exposure of cultured CGCs represents a useful in vitro trauma model in which to study the cellular mechanisms responsible for trauma-induced KCC2 protein degradation.

Previous studies have shown that increased levels of intracellular [Ca^{2+}] play an important role in glutamate excitotoxicity. To assess the potential role of extracellular Ca^{2+} influx through *N*-methyl-D-aspartate receptors, we monitored KCC2 levels 3 h after 100 μM glutamate exposure in the presence of either the NMDA receptor antagonist, MK-801 or the Ca^{2+} chelator, EGTA. As shown in Figure 3(a), KCC2 degradation was completely abolished by these two agents, indicating that Ca^{2+} influx via NMDA receptors is likely involved in trauma-induced KCC2 degradation. Further support for the role of extracellular Ca^{2+} influx in trauma-induced KCC2 degradation was obtained from experiments using A23187, a divalent cationophore. Application of this cationophore was able to mimic the glutamate-induced degradation of KCC2 protein (Figure 3(a)). Removal of extracellular Ca^{2+} with EGTA as well as incubation in Ca^{2+} free medium completely prevented the degradation of KCC2 induced by A23187 treatment (Figure 3(a)), confirming that elevated intracellular [Ca^{2+}] is likely a key component in triggering a series of events leading to the loss of KCC2 protein in CGCs exposed to glutamate.

To examine in more detail the mechanisms responsible for trauma-induced KCC2 protein loss, we used inhibitors

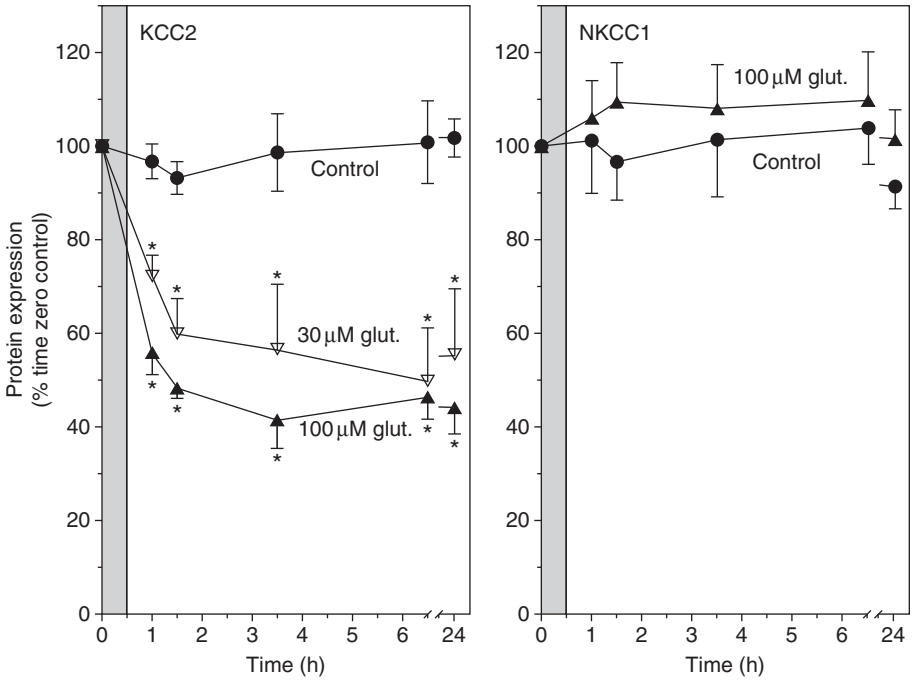


Figure 2 Protein expression of KCC2 and NKCC1 in CGCs after glutamate exposure. GCGs were exposed to glutamate (30 μM and 100 μM) for 30 min after which glutamate was removed and CGCs sampled at various time points (30 min–24 h after glutamate exposure). KCC2 and NKCC1 protein expression in whole cell lysates were quantified by Western blotting and phosphorimaging. Data are mean ± SEM of 4 experiments. *Significance from control value ($p < 0.05$; *t*-test).

of the various cellular pathways of protein degradation. These degradation pathways include lysosomes and proteosomes as well as cytosolic proteases. Neither methylamine nor NH_4Cl, agents, which increase both cytosolic and lysosomal pH, were effective in preventing trauma-induced KCC2 degradation (**Figure 3(b)**), indicating that the cellular mechanism did not likely involve an acidic hydrolase. In contrast, leupeptin prevented the degradation of KCC2 after glutamate exposure (**Figure 3(b)**), suggesting that a cysteine protease(s) was involved. The serine protease inhibitors, PMSF and aprotinin, were unable to rescue KCC2 from trauma-induced degradation. Furthermore, lactacystin was ineffective at preventing KCC2 degradation after glutamate exposure, excluding the possible involvement of the proteosome in the degradation process (**Figure 3(b)**).

Based on the above data, we reasoned that the protease(s) responsible for KCC2 degradation following glutamate exposure was likely a calcium-dependent cysteine protease with optimal activity at neutral pH. One potential candidate that fits these characteristics is the protease, calpain. Significantly, both calpain inhibitor I and calpeptin, which are relatively specific inhibitors of calpain, were able to prevent KCC2 degradation caused by both glutamate exposure (**Figure 3(b)**) and A23187 treatment (data not shown). These results strongly support the involvement of calpain in the process of KCC2 degradation in these conditions.

We examined the possibility of calpain activation in CGCs following 100 μM glutamate exposure by monitoring α-spectrin breakdown products. Non-erythroid α-spectrin (280-kD; α-fodrin) is a well established cellular substrate of caplain. Calpain cleaves two sites of α-spectrin in a sequential manner. The first site is cleaved rapidly once calpain is activated, producing a 150-kD breakdown product, whereas prolonged activation of calpain cleaves the 150-kD fragment to a 145-kD fragment. The appearance of a 150- and 145-kDa doublet of α-spectrin is an excellent indicator of calpain activation. Indeed, we observed a significant increase in the levels of the 150- and 145-kDa doublet of α-spectrin in CGCs exposed to 100 μM glutamate (data not shown). These data confirmed that calpain is activated in our in vitro trauma model and provide further support for the hypothesis that calpain was responsible for KCC2 degradation following glutamate exposure of CGCs. In order to test directly the ability of calpain to proteolyze KCC2, we performed an in vitro cell-free experiment where purified μ-calpain and m-calpain were added to rat cerebellar membranes in the presence of calcium. These two calpain isoforms are classified by their different activation profiles, with μ-calpain being activated in vitro at μM concentrations and m-calpain by mM concentrations. As shown in **Figure 4**, both μ-calpain and m-calpain degraded KCC2 with appropriate activation profiles, demonstrating that KCC2 is indeed an in vitro substrate for calpain.

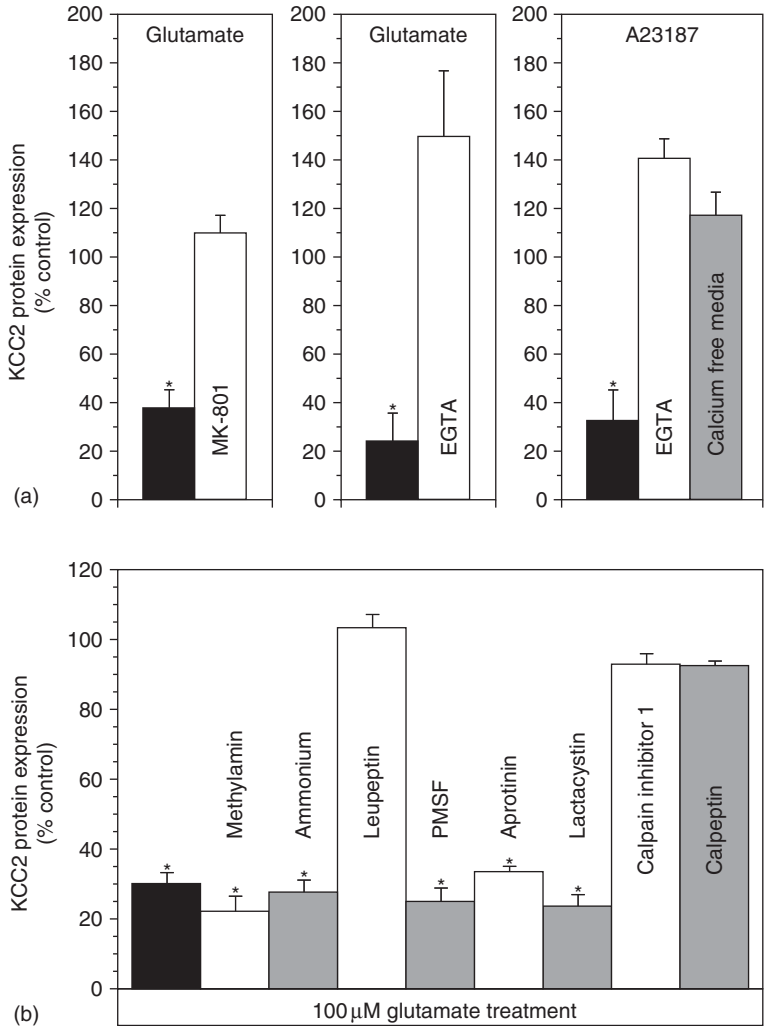


Figure 3 Calcium (a) and protease (b) dependence of KCC2 degradation in CGCs. (a) KCC2 protein expression in whole cell lysates was quantified from Western blots following 2h treatment with 10 μM A23187 or 2–3 h following 30 min exposure to100 μM glutamate. (b) KCC2 protein expression in whole cell lysates was quantified from Western blots 2–3 h after 100 μM glutamate exposure with or without protease inhibitor treatment. Reagents in b were preincubated 30 min prior to glutamate exposure and present in medium throughout incubation time. The concentrations of reagents used: 1 mM methylamine, 10 mM NH_4Cl, 100 μg per ml leupeptin, 1 mM PMSF, 1 μg perμl aprotinin, 10 μM lactacystin, 75 μM calpain inhibitor 1, and 40 μM calpeptin. Data are mean ± SEM of 3–4 experiments. *Significance from time zero control value ($p < 0.05$; *t*-test).

Future Goals

KCC2 clearly plays an essential role in mature neurons through its operation as a major regulator of intracellular Cl^-. Under normal physiological conditions, KCC2 uses the favorable K^+ chemical gradient to extrude Cl^- from the neuron, and thereby helps maintain low intracellular $[Cl^-]$ required for fast hyperpolarizing inhibition mediated by $GABA_A$/glycine receptors. It is important to recognize certain characteristics of this unique transporter, including its robust constitutive activity, its reversible transport capability, and the cell volume changes that will accompany its net ion movement. These are important considerations in epilepsy research, since high neuronal activity as occurs with seizures can result in elevated extracellular $[K^+]$, leading to KCC2-mediated net Cl^- influx (due to reversal of the thermodynamic driving force for KCC2) along with osmotically obliged water (i.e., cell swelling) – both of which have the potential to aggravate seizures.

An important challenge for future research is to understand the factors regulating KCC2 activity. It is now apparent that the operation of KCC2 is controlled through complex regulatory pathways at the level of both the KCC2 protein (i.e., post-translational) and gene (i.e., transcriptional). Recent work indicates that KCC2 activity is controlled through changes in phosphorylation state of the KCC2 protein. Such changes in KCC2 phosphorylation have the potential to modulate the unitary translocation rate of KCC2 and/or the surface KCC2 protein expression (i.e., membrane trafficking). With regards to modulation of gene expression, brain-derived neurotrophic factor (BDNF) appears to differentially

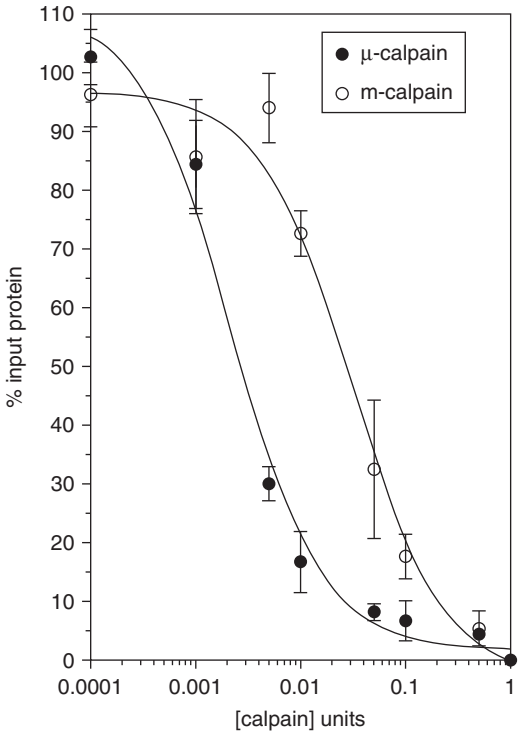


Figure 4 In vitro μ- and m-calpain proteolysis of KCC2 in crude membranes prepared from rat cerebellum. Immunoreactivity of 140-kDa KCC2 was quantified from Western blots and phosphorimaging. Data are mean ± SEM from three experiments.

regulate KCC2 gene transcription, depending upon whether it is acting on developing neurons or mature neurons subjected to traumatic insults. In developing neurons, BDNF acts a trophic factor, leading to upregulation of KCC2 gene expression. In contrast, in mature neurons, BDNF controls the trauma-induced downregulation of KCC2 gene expression. How BDNF can provide such diverse signals for KCC2 gene expression appears to lie in the multiple downstream signaling cascades controlled by the BDNF/TrkB receptor. An essential part in the down-regulation of KCC2 activity following trauma in mature neurons is protein degradation. We have shown that KCC2 is rapidly degraded after trauma via the Ca^{2+}-depended neutral protease, calpain. Thus, once KCC2 gene expression ceases after trauma, KCC2 protein loss can be rapid.

In additon to determining the cellular mechanisms responsible for reduced KCC2 expression/activity in injured neurons, it is equally important to understand the reasons for such a dramatic alteration in neuronal Cl^- homeostasis. The appearance of depolarizing and often excitatory $GABA_A$-receptor mediated responses in mature neurons (after trauma) might at first appear to be detrimental and contribute to the general pathology of the traumatic event. However, one must consider the strong possibility this change is an adaptive response that provides neurons greater flexibility in recovering from the insult by providing a necessary signal for neuronal sprouting and retargeting. After all, depolarizing $GABA_A$-receptor-mediated responses are present and necessary in immature neurons for proper growth and synapse formation during development. Furthermore, the re-emergence of molecular and cellular developmental events following trauma are well documented.

See also: **Cell Damage/Excitotoxicity**: Basic Mechanisms of Neuronal Injury from Seizures and Status Epilepticus; **GABA**: GABA Excites Immature Neurons: Implications for the Epilepsies; **Post-Traumatic Epilepsy**: Posttraumatic Models; **Transporters**: Neuronal Cl Transport: Setting the Stage for Successful Inhibition.

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TREATMENTS (NON-ANTIEPILEPTIC DRUGS)

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Control of Seizure Activity with Electrical Stimulation

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Introduction

Epilepsy is a disorder of the nervous system that has severe consequences for patients and generates a significant social burden. About 3 million people have been diagnosed with epilepsy in the United States and the cost to the society is about $12.5 billion per year. More must be done to enhance the therapeutic treatment of these patients. Although antiepileptic drugs are effective at controlling seizures in most patients, a significant portion of the clinical population is not adequately managed. In these patients, neuroablative surgery can treat epilepsies refractory to conventional pharmacotherapy. However, epileptic foci resection does not guarantee seizure reduction and is not appropriate for generalized epilepsies. Therefore, novel therapeutic approaches, such as electrical stimulation, have been developed for the treatment of epilepsy.

Electrical stimulation has traditionally been used in the nervous system to excite axons and neurons for the purpose of studying basic mechanisms or to replace a function lost following injury. Electrical fields and currents applied to the nervous system generally cause excitation and generate synchronized activity. A more difficult problem is to generate inhibition and/or desynchronization using electrical techniques. Over the past two decades, researchers and clinicians have developed novel methods to treat epilepsy using electrical fields. Electrical stimulation protocols have been designed to directly suppress neuronal firing or to interfere with the synchronization of a population of neurons. Such techniques are extremely valuable in patients suffering from conditions where the neurons and axons fire abnormally, such as in epilepsy and spasticity.

Although several studies have shown that electrical stimulation of the brain can reduce clinical symptoms in patients with epilepsy, the mechanisms of action are still unknown. Despite the lack of clearly identified mechanisms, clinical implementation of electrical stimulation therapies is possible. Recent technological development has led to the formation of several new companies that are investigating the possibility of open-loop and/or closed-loop feedback to decrease the seizure frequency in patients. Electrodes are placed in the epileptic focus of the patient, and stimulation is applied when seizures are detected. Although some reduction of seizure occurrence rate has been achieved with this new technology, therapeutic efficacy of the stimulation paradigm has not yet been established.

The purpose of this article is to review the various methods by which electrical stimulation can be used to suppress abnormal neuronal activity. The focus of the article is on the frequency-dependant suppression of neural activity for seizure control. Some methods use DC subthreshold currents to inhibit action potential initiation, whereas others suppress neural activity with suprathreshold currents at low (1–5 Hz) or high (100–300 Hz) frequencies. These methods may engage different

mechanisms; however, in all cases, the imposed electric fields interact with neural tissue and can modify the excitability of the neural tissue or the synchronized behavior of the network.

Background

Fundamentals of Electrical Stimulation

Electric fields generated by electrodes in volume conductors

Neurons in the CNS are surrounded by an extracellular medium with a relatively low resistivity (80–300 Ω cm). Modeling the current flow around the electrodes is crucial to understanding the effects of electrical fields on excitable tissue. The current density, voltage, and electrical field distributions can be obtained from the quasistatic formulation of the Maxwell equations (since the stimulus frequencies used are usually under 10 kHz). From these equations, it is possible to derive the expression for the voltage generated by several monopolar electrodes in a homogeneous volume conductor with conductivity σ. Assuming n electrodes generating a current I_i located at a distance r_i from the recording point, the voltage is given by

$$\phi = \frac{1}{4\pi\sigma}\sum_{n}\frac{I_i}{r_i} \qquad [1]$$

The voltage difference across a distance Δx generated by large field electrodes producing a uniform field is given by $\Delta V = I \times R \times \Delta x / D$ where I is the current injected, R the resistance between the electrodes, and D is the distance between the electrodes. Voltages generated in nonhomogeneous media can also be calculated using numerical approximations such as boundary element or finite element methods.

Electric fields and excitable tissue

When neurons are placed inside a volume conductor, the current lines generated by external sources will pass through their cell membranes, thereby generating depolarization when current flows outward and hyperpolarization when current flows inward across the membrane. For both uniform and localized fields, positive (anode near soma) stimulation will induce hyperpolarization of the soma. The substhreshold transmembrane voltage generated by the external currents can be calculated using the passive cable equation [2]. (Nonlinear ionic channel conductances can be added in parallel with the membrane resistance and capacitance.) A compartment of length Δx can be modeled at rest by a capacitance C_m in parallel with a series combination of a battery (E_r) representing the resting potential and a resistance R_m simulating the combined resistance at rest of all the membrane channels. Applying Kirchoff's law at each compartment, one obtains the following inhomogeneous cable equation:

$$\lambda^2\frac{\Delta^2 V_m}{\Delta x^2} - \tau\frac{dV_m}{dt} - V_m = -\lambda^2\frac{\Delta^2 Ve}{\Delta x^2} \qquad [2]$$

The space constant of the membrane depends only on the geometric and electric properties of the membrane.

$$\lambda = \frac{1}{2}\sqrt{\frac{R_m^s d}{R_a^s}} \qquad [3]$$

where R_m^s is the specific membrane resistance, R_m^a the axoplasmic specific resistance, and d the diameter of the axon. τ_m is the time constant of the membrane and is given by

$$\tau_m = R_m C_m \qquad [4]$$

The term on the right side of eqn. [2] is called the driving function and is the product of the square of the space constant with the second spatial derivative of the extracellular voltage. Equation [2] predicts that the transmembrane voltage induced in an axon by an external field is proportional to the second-order spatial derivative of the extracellular voltage. Shorter structures such as dendrites are sensitive to the first spatial derivative of the extracellular electric field.

Methods and Recent Results

Direct Current Membrane Polarization

Externally applied currents or fields have been shown to influence excitability in many neuronal systems, such as turtle cerebellum and mammalian Purkinje cells, hippocampal dentate granule cells, and pyramidal and cortical neurons. These studies used uniform fields applied with large field electrodes placed on the surface of the cortex for in vivo experiments, or across the tissue for in vitro studies. These studies have shown that DC electrical fields (with amplitudes similar to those generated endogenously) can produce either excitation or inhibition of neuronal activity, depending on the location, the polarity, and the orientation of the applied field with respect to the dendritic tree. For example, a cathode located near the basal dendrites of hippocampal CA1 neurons produces depolarization of the dendrites and cell body, thereby generating excitation. However, an anode in the same location produces hyperpolarization in that region and can inhibit electrical activity. In addition, the cable equation clearly shows that the transmembrane voltage depends on the extracellular voltages generated along the membrane and is sensitive *only* to longitudinal fields. Therefore, as confirmed by experiments, the orientation of cell bodies with respect to the electric field affects the longitudinal field amplitude, and thus the efficacy of the applied field. Moreover, a cell structure located perpendicular to the induced field lines would not be affected by stimulation.

In vitro studies of direct current effects

The effect of exogenous uniform DC electric fields on epileptiform activity has been tested in several models epilepsy, including the high potassium and low calcium in vitro models. These studies have shown that anodic electric fields (applied with large electrodes) as low as $5\,\text{mV}\,\text{mm}^{-1}$ can completely suppress interictal bursting. Reversal of the field polarity (cathodic stimulation) enhanced the activity as indicated by the number of bursts per minute. The mechanism for activity suppression and enhancement involves membrane polarization, as described earlier, and does not require synaptic transmission. Similar results were obtained in other in vitro models, such as low calcium and picrotoxin (see Figure 1).

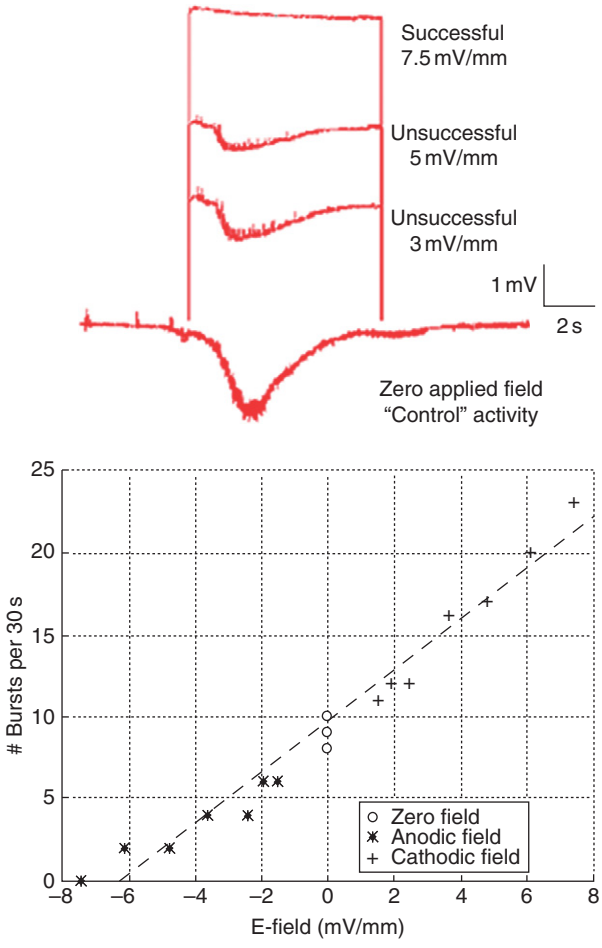


Figure 1 DC current applied to the CA2 region of the hippocampus using large field electrode. The electric is uniform parallel to the dendritic axis of the dendrites. Cathodic current can suppress spontaneous epileptiform activity induced by low-calcium solutions while anodic fields can enhance these events. Modified from Ghai *et al*. (2000).

Effect of low-level DC current in animal models in vivo

Membrane polarization with applied fields at very low frequency and amplitude has been applied in vivo to control seizure activity produced by the tetanus toxin in rats. Small current amplitudes were observed to modulate the seizure activity with an electrode placed centrally in the hippocampus. Low-level DC currents have also been tested in vivo on the kindling model of epilepsy. Currents as low as 5–15 μA applied $15\,\text{min}\,\text{day}^{-1}$ for 14 days to amygdala-kindled animals significantly increased seizure threshold. DC currents are known to generate tissue damage through nonreversible chemical reactions that take place at the electrode surface.

Noninvasive direct current stimulation

Weak direct currents applied noninvasively across the head, using large electrode and employing low voltages, have also been shown to modulate the excitability of cortical neurons in both humans and animals. Currents as low as 100 μA applied for long periods of time (30–60 min) can elevate the threshold of seizures in freely moving rats without any neural damage. Similarly, in patients with epilepsy, 1 mA cathodic current applied for 20 min was shown to decrease seizure frequency. Although the mechanisms for these effects are not known, they are thought to be related to membrane hyperpolarization, as described earlier. Moreover, the target neurons for these effects are unknown. In human patients, the current is applied to the brain through saline-soaked sponges to avoid potential damage caused by DC stimulation. Bipolar stimulation can also avoid the electrochemical damage generated at the interface between the neural tissue and the electrodes, and has been effective to suppress seizures at both low and high frequencies.

Low-Frequency Electrical Stimulation for Seizure Suppression

Effect of LFS on neural excitability in animal models

Low-frequency electrical stimulation has been used successfully to suppress seizure activity in both in vivo and in vitro models of epilepsy. The stimulation is applied using narrow a pulse width and at supra-threshold amplitudes. In vitro studies in the hippocampus have shown that stimulation of the Schaffer collaterals and mossy fibers at frequencies around 1.0 Hz suppress seizure activity and after-discharge duration in rats. Although there are many studies that demonstrate the ability of LFS to suppress seizure activity in various animal models, the mechanisms are still unknown. Effects of stimulation such as increased inhibition and long-term synaptic depression (LTD) have been proposed to explain the decrease excitability.

Effect of LFS on neural excitability human patients

In patients with epilepsy, low-frequency electrical stimulation (at 0.5 Hz, biphasic square waves, 2–4 mA) delivered to different ictal onset zones each day in 30 min intervals, was able to reduce seizure frequency. In addition, stimulation of the caudate nucleus at 4–8 Hz, in 57 patients suffering from intractable epilepsy, also reduced the seizure frequency. The mechanisms for the effects of the caudate stimulation were attributed to the possible activation of inhibitory responses in the network. Although, activation of the ictal zones in patients with intractable epilepsy can decrease seizure frequency, one of the possible side effects of the stimulation is induction of seizures even at these low frequencies.

Noninvasive LFS with transcranial stimulation

LFS can be applied noninvasively through the skull using transcranial magnetic stimulation. Magnetic coils placed on the head of patients are energized with large-amplitude but brief current pulses. The current pulses induce brief electric fields in the neural tissue according to Faraday's law of induction. When applied at frequencies (0.5–5 Hz) similar to those known to generate long-term depression of synaptic transmission (LTD), stimulation can lower the excitability of the cortex. Trains of LFS are applied to coils placed over the vertex for several consecutives days. However, when applied to patients with intractable epilepsy, the results and efficacy of this method to suppress seizures remains controversial. Some studies reported a significant decrease in the frequency of seizures, and but others reported only mild and nonlasting effects. Induction of seizure activity is evident at frequencies greater that 10 Hz. However, at even higher stimulus frequencies, suppression of activity is again observed.

HFS for Seizure Suppression

HFS of the CNS

Deep brain stimulation (DBS) uses HFS applied at frequencies between 100 and 300 Hz, and is an effective treatment for movement disorders such as Parkinson's disease. HFS produces the same effect on clinical symptoms as resection of neural tissue. Therefore, one possible explanation is that electrical stimulation produces a depolarization blockade of neurons within the target neural structures. Yet, recordings obtained downstream from the stimulation electrodes show excitation at or near the stimulus frequency. Therefore, another potential mechanism is a disruption of the neural firing pattern responsible for the disorder's symptoms.

Current DBS paradigms have not been consistently effective for relieving symptoms in patients with epilepsy. Pilot studies show that HFS provides limited therapeutic control of intractable epileptic seizures. Yet, it remains unclear how HFS affects seizure propagation within hippocampal and limbic neural networks. The way in which HFS affects cell bodies, afferent and efferent axons, and axons of passage is likely to be different from mechanisms engaged by LFS; elucidation of those mechanisms remains an important goal. Current research indicates that the nucleus stimulated, its location within the brain, and its neuronal composition are important factors in determining these effects. Our own studies using an in vitro hippocampal preparation show that both local and global application of HFS suppresses cellular synaptic and nonsynaptic epileptiform activities. Although computer simulation studies and in vivo studies confirm that HFS induces suppression of cell bodies, they provide contradictory data on whether HFS excites or inhibits the axons of projection neurons and afferent inputs. Recent experiments have shown that HFS suppresses not only cellular activity but also axonal conduction in vitro. The subthalamic nucleus (STN), basal ganglia, and hippocampus all have important axonal projections vital to the maintenance of seizure (and tremor) loops within limbic and cognitive circuitry. Therefore, HFS modulation of axonal activity would significantly impact the behavior of downstream nuclei and seizure propagation.

The mechanisms of HFS are also unknown. Some evidence shows that voltage-gated ion channels in neurons are impaired by HFS. A more recent report suggests that HFS applied to the subcortical white matter generates a depression of excitatory synaptic currents, while HFS applied in the neocortex produced both synaptic depression and reduced neuronal excitability. The literature also indicates that HFS enhances glutamate release, but does not adversely affect electrogenic pumps, so that glutamate and potassium clearance are preserved during HFS. Our own results show that extracellular potassium concentration is clearly increased during HFS. Measurements showed a correlation between extracellular potassium ($[K+]_o$) accumulation and suppression of synaptic and nonsynaptic epileptiform activity, resulting in depolarization blockade. Moreover, the suppression effects of the HFS can be generated in the presence of synaptic blockers using either sinusoidal or square pulse stimulus waveform trains. These results suggest that the mechanisms involve nonsynaptic phenomena such as depolarization block (see **Figure 2**) or desynchronization. This block may be limited to some localized areas since excitation of other nuclei around the electrodes has been observed and could contribute to the therapeutic effects by disrupting the abnormal neural activity.

DBS electrodes for the control of epilepsy have been located in structures similar to those used for Parkinson's disease. The STN was first used as a target for stimulation, and the results of these initial studies showed that the frequency of seizures was significantly decreased. In the thalamus, stimulation of the centromedian nucleus has



Figure 2 Transmembrane recording obtained by subtracting the extracellular voltage from the intracellular voltage from a CA1 neurons during sinusoidal stimulation at 50 Hz. The signal has been filtered with a 10 Hz low pass filter to eliminate the stimulation artifact. Note that cell fires at the onset of the stimulation and remains depolarized during the stimulus without firing. Note also that the filter significantly reduced the amplitude of the actions potentials normally around 80 mV. Modified from Bikson *et al*. (2001).

been shown to be effective in tonic–clonic seizures but not complex–partial epilepsy. More recently, other structures such as the anterior thalamus, substantial nigra pars reticulata, hippocampus, and cortex have also been targeted. Although DBS for epilepsy appears to be safe, and can decrease the number of seizures in patients with intractable epilepsy, the optimum location and stimulation parameters for such stimulation still need to be determined.

HFS of the peripheral nervous system (vagus nerve stimulation)

HFS of the peripheral nervous system can also affect seizure frequency. In particular, high-frequency vagal nerve stimulation (VNS) can inhibit seizures in canines and interfere with CNS excitability. Subsequent studies showed this paradigm to be effective in other animals, including rats and monkeys. Animal studies have shown that while high-frequency (>70 Hz) VNS can generate EEG desynchronization, low-frequency (20–50 Hz) stimulation induced EEG synchronization.

There are now close to 10 000 patients per year implanted with high-frequency VNS systems for the treatment of epilepsy. The VN stimulator received FDA approval in July 1997. Because of the pattern of vagus nerve innervation of the heart, the vagus nerve can only be stimulated unilaterally (i.e., the left side only). Typically, the treatment routine consists of adjusting the current amplitude to tolerance (0.25–4 mA in 0.25 mA steps), using a 30 Hz frequency, 0.5 ms pulse width, for 30 s ON time and 5 min OFF time. The stimulation can be manually triggered by placing an external magnet over the patient's chest. In multicenter, randomized, double-blind studies, patients receiving high-intensity VNS (30 Hz, 0.25–4 mA, 0.5 ms pulse width, 30 s ON time and 5 min OFF time) experienced a ~30% reduction in seizure frequency. In contrast, patients receiving low-intensity stimulation (1 Hz, 0.13 ms pulse width, 30 s ON time and 180 min OFF time) experienced a ~10% reduction. Stimulation of the trigeminal nerve can also produce significant reductions in the number of seizures when the frequency of stimulation is greater than 50 Hz and with current amplitudes between 3 and 11 mA.

Although VNS is currently the only commercially available electrical stimulation modality for seizure control in patients with epilepsy, the mechanisms underlying the VNS effect have not been elucidated. However, VNS is known to alter the vagal afferent activity and the neuronal firing patterns in the reticular activation and noradrenergic systems, leading to an increase in the seizure threshold of cortical tissue. The effectiveness of this method of stimulation is limited.

Future Work

Several electrical stimulation protocols can either suppress or interfere with abnormal neuronal activity. This suppression has been demonstrated in several animal models of epilepsy and in humans. Unlike surgical resection, the effect of electrical stimulation is generally reversible. Unlike drugs, electrical stimulation can be applied to specific regions of the brain and the 'dosage' of stimulation can be varied easily and instantaneously. By simply changing stimulation parameters, clinicians could potentially screen different protocols. However, there are serious problems to be overcome before these techniques can be implemented routinely. The mechanisms involved in all these stimulation methods are unknown, and unanticipated side effects could potentially induce, rather than suppress, seizures. However, electrical stimulation is potentially a powerful tool to control seizures. Novel approaches in neural imaging, modeling, and electrode design should allow researchers to unravel unknown mechanisms and design more effective stimulation methods to control abnormal activity in the brain.

Acknowledgements

Alicia Jensen and David Tang for reading the manuscript, and the Whitaker Foundation, The National Institutes of Health and the National Science Foundation for providing financial assistance for some of the work mentioned in this review.

See also: **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures; **Pyramidal Cells**: Intrinsic Plasticity of Hippocampal CA1 Pyramidal Cells and its Relevance to Epileptic Discharge and Epileptogenesis; **Treatments (non-Antiepileptic Drugs)**: Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy; Electrical Stimulation.

Further Reading

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Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy

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Introduction

It has been estimated that seizures are uncontrolled in as many as 30% of the patients with epilepsy. Clearly, there is a profound need for new therapies that control seizures but are also safe. Deep brain electrical stimulation (DBS) has the potential to be an effective new therapy for epilepsy. Early clinical reports suggested the efficacy of DBS, but these studies were poorly controlled or limited to individual case reports where the results were mixed and difficult to interpret. As a consequence, research in this area remained dormant for a number of years. However, the successful use of DBS for the treatment of movement disorders, and the application of vagus nerve stimulation (VNS) for epilepsy have led to a renewed research effort to develop electrical stimulation as a therapy for epilepsy.

Before electrical stimulation can become an accepted treatment for epilepsy, the fundamental questions of where and how to stimulate need to be answered. Two general approaches have been taken: (1) stimulus activation of seizure-gating networks or (2) direct stimulation of an identified seizure focus. Parameters such as stimulus frequency, intensity, duration, and waveform may all be critical as to whether a given stimulus will successfully interfere with seizure activity. While the majority of studies have tested the efficacy of high-frequency stimulation (HFS) (similar to protocols used to treat movement disorders), a number of recent reports – based on in vivo experimental seizure models and in vitro preparations – have determined that low-frequency stimulation (LFS) can also interfere with or prevent seizure activity. Once safe and effective stimulation paradigms are identified, the goal will be to combine that therapy with seizure detection technology, in an implantable device that will be capable of delivering stimulation therapy at the optimal time to prevent or disrupt a seizure. This approach will allow for therapy to be customized to the specific needs of a given patient.

Background

Clinical Studies

With all studies in this area, the results are highly dependent on where and how the stimulation was delivered, as well as on the nature of the patient population included in

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Background

Clinical Studies

With all studies in this area, the results are highly dependent on where and how the stimulation was delivered, as well as on the nature of the patient population included in

the study. Interpretation is also complicated by the fact that most of the studies are not controlled or limited to case reports. As a consequence, the results from clinical studies are mixed and often conflicting. Acute clinical experiments that demonstrated that direct cortical stimulation caused a flattening of the EEG – accompanied by a decrease in spontaneous epileptiform discharges – were encouraging. However, the results from studies that examined cerebellar stimulation were inconsistent. Some early studies reported that cerebellar stimulation had no effect on seizure frequency in patients with intractable epilepsy, while a recent limited trial reported that cerebellar stimulation induced a decrease in the frequency of motor seizures. The first controlled clinical trial, in 1992, found that centromedian thalamic stimulation failed to decrease seizure activity. More recently, investigators reported that brief bursts of direct cortical stimulation were found to terminate afterdischarges induced during the process of cortical mapping. High-frequency stimulation of the anterior thalamus, hippocampus, and motor cortex has also been reported to significantly decrease seizure frequency in a limited number of patients. However, it is difficult to know the true significance of these results since very few double-blinded controlled studies have been performed.

To date most clinical studies have used an 'open-looped' stimulation protocol in which stimulation is delivered on a fixed schedule independent of the patient's physiological state. The efficacy of these stimulation paradigms may improve if delivered using closed-looped stimulation – i.e., stimulation that is delivered in direct response to the detection of abnormal epileptiform activity. Combining the appropriate stimulation therapy with seizure detection technology will allow for this approach.

Animal Studies – Activation of Seizure-Gating Networks

Animals studies have identified several brain areas that, when stimulated, appear to activate seizure-gating networks that raise seizure threshold or interfere with seizure propagation. These structures include the basal ganglia, cerebellum, anterior thalamus, subthalamic nucleus, mamillary bodies, locus coeruleus, substantia nigra, and most recently the hypothalamus. Although the mechanism underlying these effects is unclear, the anticonvulsant effect of stimulation of the striatum, subthalamic nucleus, and substantia nigra appears to be mediated through disinhibition of deep layers of the superior colliculus (SC). Activation of the SC has been shown to suppress seizures induced by electroconvulsive shock and spontaneous absence seizures in the GAERS rat. It has been hypothesized that suppression of seizure-induced hind limb extension is likely mediated through projections from the SC to brainstem, while suppression of cortical seizure activity may be mediated through ascending projections to the parafascicular and centromedian nuclei, which have connections with the cortex.

The anticonvulsant action of another seizure-gating network may depend on the activation of ascending noradrenergic pathways. Direct stimulation of the locus coeruleus has been shown to decrease seizure activity in a number of experimental models, whereas locus coeruleus lesions are proconvulsant, and depletion of norepinephrine can accelerate kindling. While the majority of these studies used high-frequency pulsatile stimulation, similar to the paradigms used successfully in clinical treatments for movement disorders, several recent studies have reported efficacy using low-frequency stimulation delivered to structures distant from the seizure focus.

Animal Studies – Direct Stimulation of the Seizure Focus

The effect of direct stimulation of a seizure focus has been examined primarily in the kindling seizure model. Electrical stimulation delivered directly at the kindling focus has been shown to raise afterdischarge threshold, delay kindling acquisition, decrease afterdischarge (AD) duration, and decrease the incidence of seizures in response to the kindling stimulus. However, the efficacy of different stimulation paradigms appears to be dependent on whether the therapeutic stimulation is delivered before, during, or after the seizure.

In Vitro Studies

Several laboratories have demonstrated that field stimulation and direct stimulation can decrease the incidence of epileptiform activity in several different slice preparations. Low- and high-frequency pulsatile stimulation have been shown to decrease epileptiform burst activity induced by 4-AP, bicuculline, ferric chloride, and low magnesium in neocortical, hippocampal, amygdala-perirhinal, and hippocampal-entorhinal slices. Low-frequency stimulation has also been shown to prevent the transition from interictal to ictal activity in several of these preparations.

Vagus and Trigeminal Nerve Stimulation

Currently, vagus nerve stimulation (VNS) is the only approved therapy for the treatment of epilepsy that uses electrical stimulation. Continuous stimulation of the left vagus nerve provides effective seizure control in a small population of patients. The efficacy of VNS has been estimated to be equivalent to that of a new antiepileptic drug, but not as effective as surgical resection. The mechanism by which this therapy works is unknown. It has been hypothesized that VNS eventually activates the locus

coeruleus, which has widely distributed noradrenergic projections that could raise seizure threshold and decrease the likelihood of a seizure. There are some drawbacks to this therapy. It is impossible to predict which patients will benefit from this therapy before surgery and stimulation effects are directly assessed. In addition, besides the potential complications of surgery, patients have complained of hoarseness, voice changes, and dyspnea. A second surgery may be required to remove the device if it proves to be ineffective. Given the relatively small population of patients who benefit from this therapy, it does not obviate the need for new therapies.

The success obtained with VNS has led to an examination of other peripheral nerves as targets for stimulation. The trigeminal nerve has received attention because the trigeminal nucleus receives projections from the locus coeruleus (and, as indicated earlier, it has been postulated that the anticonvulsant effect of VNS is mediated through activation of the locus coeruleus). Unlike VNS, the trigeminal nerve can be stimulated through cutaneous electrodes and is therefore a noninvasive procedure. In one experiment, trigeminal nerve stimulation decreased PTZ-induced seizure activity in the rat. In a preliminary clinical trial, two patients reported a decrease in the number of complex partial seizures after trigeminal nerve stimulation.

Selection of Stimulation Parameters – HFS versus LFS

Stimulation frequency appears to be a key parameter in the activation of seizure-gating networks. Many of the studies that report a decrease in seizure activity after stimulation of the basal ganglia and thalamic nuclei used HFS. HFS (100 Hz) of the anterior thalamus of the rat raised the threshold for PTZ-induced clonic seizures, but 8-Hz stimulation was proconvulsant. HFS of the solitary nucleus, a site distant from the kindling focus, interfered with kindling acquisition. These results suggest that HFS may be effective at activating seizure-gating networks. It has been suggested that HFS induces a functional lesion at the stimulation site due to depolarization block or preferential activation of inhibitory neurons. Inhibition of the stimulated structure leads to activation of other structures within the seizure-gating network through disinhibition. Activation of these normally inhibited structures leads to a net increase in the inhibitory output from the network. It is interesting that direct stimulation of the locus coeruleus at frequencies of 20–50 Hz effectively suppressed penicillin-induced epileptiform activity. In this case, the nucleus was likely activated and not suppressed by the stimulation.

LFS using pulsatile or sinusoidal waveforms may be more effective when delivered directly at the seizure focus. LFS is effective when delivered at the kindling focus and in in vitro slice preparations. It has been suggested that LFS may have a greater potential clinically since the effects of the stimulation last well beyond the duration of the stimulation. This hypothesis is supported by the observations that LFS induced an increase in kindled afterdischarge threshold that lasted for days, and that LFS induced a decrease in bicuculline-induced epileptiform discharges in the hippocampal slice that continued after stimulation was discontinued. LFS mimics the frequency of interictal spikes. It has been hypothesized that the interictal spike may be inhibitory, and that an increase in interictal activity may decrease the likelihood of an ictal event. LFS delivered to CA3 of the hippocampal–entorhinal slice decreased the number of ictal events induced by 4AP and zero magnesium. Recently, LFS has been reported to decrease interictal and ictal events in patients with epilepsy. The mechanism underlying the anticonvulsant effect of LFS is unknown. The term 'quenching' has been used to describe the decrease in kindled seizures after LFS – an effect that later proved to be due to long-term exposure to direct current. Low-frequency pulsatile stimulation is similar to stimulations used to induce long-term depression (LTD) or depotentiation, and investigators have speculated that an LTD-like mechanism underlies its efficacy.

Methods

Our laboratory has focused on identifying stimulation paradigms that either alter epileptogenesis or are anticonvulsant. To address these possibilities, we tested the effect of low-frequency sine wave stimulation (LFSWS) on kindling acquisition and in fully kindled animals. The kindling seizure model was selected for these experiments because it offered several advantages over other models. Briefly, kindling is characterized by the repeated spaced presentation of an initially subconvulsive stimulus that, over time, causes a permanent change in brain function that eventually results in the same initially subconvulsive stimulus consistently inducing a limbic seizure. The severity of kindled seizures can be quantified both behaviorally and electrographically. Changes in afterdischarge (AD) threshold, AD duration, behavioral seizure score, and the number of stimulations required for the animal to become fully kindled can be used to assess changes in the epileptogenic process. Once an animal has exhibited three consecutive stage 5 (Racine scale) seizures, it is considered fully kindled. Changes in seizure incidence, behavioral seizure score, seizure threshold, and AD duration can then be used to measure antiepileptic efficacy in fully kindled animals. In addition, knowing when the seizure will occur (it is a triggered event) affords the investigator a great deal of control when testing a potential therapy.

Effect of Preemptive LFSWS on Kindling Acquisition

Initial experiments examined the effect of preemptive LFSWS (1 Hz) on kindling acquisition in the amygdala of the rat. Earlier studies that examined the effect of LFSWS in kindling reported positive effects, but were difficult to interpret and therefore largely ignored. To carefully characterize the effect of LFSWS, experimental Sprague-Dawley rats received LFSWS (1 Hz, 50 μA, 30 s) immediately before each kindling stimulus (60 Hz, 1-ms pulse duration, 400 μA for 1 s). Control animals received only the kindling stimulus. A total of 20 stimulations were delivered to each group.

Effect of Preemptive LFSWS in Fully Kindled Animals

In these experiments, a LFSWS stimulus (1 Hz, 50 μA, 30 s) was delivered immediately before the kindling stimulus (60 Hz, 1-ms pulse duration, 400 μA for 1 s) in rats that had been fully kindled in the amygdala or the hippocampus. The effect of the stimulation on behavioral seizure score, seizure incidence, and AD duration was measured for each animal.

The effect of preemptive low-frequency pulsatile stimulation (biphasic square wave) was also tested in animals fully kindled in the hippocampus. A LTD-like stimulus (1 Hz, 1-ms pulse duration, 50 μA) was delivered for 15 min before the kindling stimulus.

At the conclusion of each experiment, the brains from each animal were processed for histological assessment of electrode location and damage at the stimulation site. Brains were sectioned on a vibratome and the sections Nissl-stained.

Recent Results

Effect of Preemptive LFSWS on Kindling Acquisition

Basolateral amygdala kindling usually requires 9–12 stimulations for the rat to become fully kindled. The acquisition phase is characterized by an increase in AD duration and electrographic complexity accompanied by an increase in behavioral seizure score. During kindling acquisition in control animals, the kindling stimulus rarely fails to elicit an AD. The presentation of preemptive LFSWS immediately before the kindling stimulus significantly decreased AD duration and behavior seizure score during the kindling process (Figure 1). More significantly, preemptive LFSWS profoundly decreased the incidence of AD by 30% in response to the kindling stimulus (Figure 2, $p < 0.0001$, Fisher exact test).

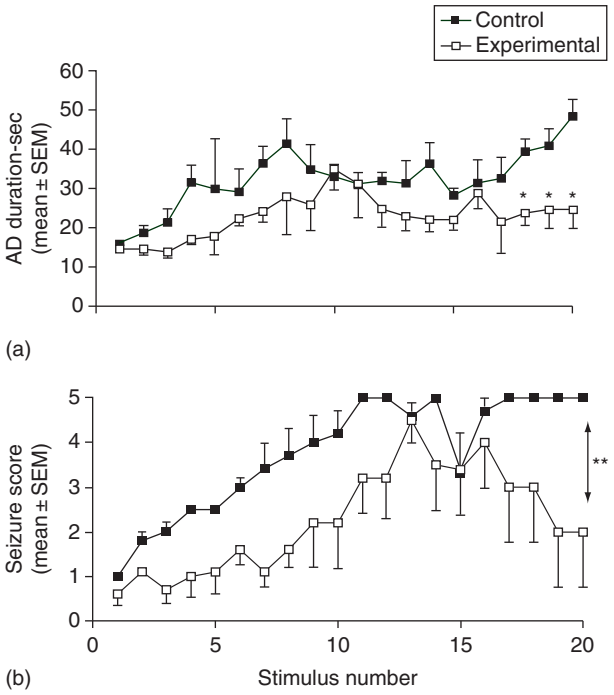


Figure 1 Effect of preemptive LFSWS on kindling acquisition. (a) LFSWS significantly decreased AD duration ($p < 0.01$) and (b) behavioral seizure score ($p < 0.001$). Reproduced from Goodman *et al.* (2005) Preemptive low-frequency stimulation decreases the incidence of amygdala-kindled seizures. *Epilepsia* 46: 1–7, with permission from Epilepsia.

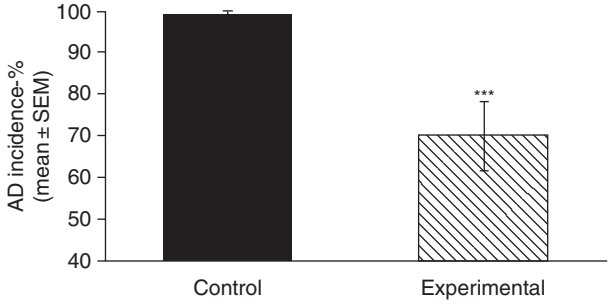


Figure 2 Preemptive LFSWS significantly decreased the incidence of AD by 30% during kindling acquisition ($p < 0.0001$). Reproduced from Goodman *et al.* (2005) Preemptive low-frequency stimulation decreases the incidence of amygdala-kindled seizures. *Epilepsia* 46: 1–7, with permission from Epilepsia.

Effect of Preemptive LFSWS and in Fully Kindled Animals

The effect of preemptive LFSWS was examined in animals fully kindled in the amygdala or hippocampus. Baseline seizure frequency was determined for both groups of animals. In the absence of LFSWS, the incidence of stage 5 seizures was 98–100% in response to the

kindling stimulus. When LFSWS was delivered immediately before the kindling stimulus, the incidence of stage 5 kindled seizures was significantly decreased by 58% in the amygdala (Figure 3, $p < 0.0001$) and by 30% in the hippocampus ($p < 0.0001$, data not shown). Interestingly, when these animals failed to have a stage 5 seizure, there was a complete failure to exhibit an AD or behavioral seizures with lower scores. No change was observed in the incidence of stage 5 seizures in rats fully kindled in the hippocampus when the same experiment was performed using preemptive low-frequency pulsatile stimulation (1 Hz, 1-ms pulse duration, 50 μA, for 15 min). A histological assessment of the stimulation site revealed no evidence of damage that could explain the decrease in seizure incidence in animals exposed to LFSWS.

It is unclear why in this study LFSWS decreased the incidence of kindled seizures while low-frequency pulsatile stimulation had no effect. Other studies have reported positive effects after low-frequency pulsatile stimulation in kindling and in slice preparations. The ineffectiveness of low-frequency pulsatile stimulation in this study may be due to the specific stimulation parameters used. It has recently been reported that LFSWS induced a significant increase in AD threshold that lasted for several days. An elevation in seizure threshold would explain the decrease in seizure incidence observed after LFSWS.

The majority of clinical and basic studies that have assessed the efficacy of electrical stimulation as a therapy for epilepsy have used pulsatile stimulation. Short duration biphasic pulses are charge balanced and less likely to cause damage. But several studies in the 1980s reported decreases in kindling with LFSWS. LFSWS is continuous, with no interpulse interval, so that current is delivered over the entire second of stimulation. The schematic in Figure 4 illustrates that the relative amount of total current per second delivered with LFSWS stimulation is significantly greater than would be delivered with low-frequency 1-ms pulsatile stimulation. This difference raises the possibility that LFSWS may be effective in patients who do not respond to pulsatile stimulation.

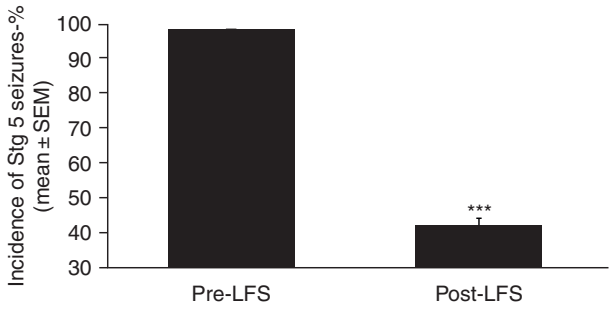


Figure 3 Preemptive LFSWS significantly decreased the incidence of stage 5 kindled seizures from 98% to 42% in rats fully kindled in the amygdala ($p < 0.0001$). Reproduced from Goodman *et al.* (2005) Preemptive low-frequency stimulation decreases the incidence of amygdala-kindled seizures. *Epilepsia* 46: 1–7, with permission from Epilepsia.

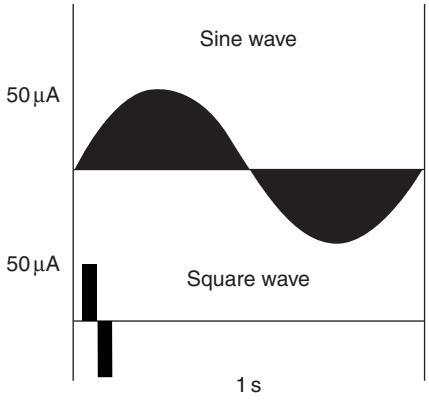


Figure 4 Schematic comparing the relative amounts of current delivered with 1 s of sine wave stimulation versus 1 s of pulsatile stimulation (biphasic square wave, 1-ms pulse duration). Not drawn to scale.

Future Goals

Experimentally, it will be important to determine if stimulation paradigms that successfully decrease kindled seizure activity are also effective in models that produce spontaneous focal seizures. The tetanus toxin model appears to be well suited for this assessment. Tetanus toxin injected into the motor cortex or hippocampus induces spontaneous random seizure activity (similar to that observed in human partial epilepsy) with no evidence of damage. It will also be important to further characterize brain regions that, when stimulated, can activate seizure-gating networks.

The ultimate goal of this research is to combine safe and effective stimulation paradigms with early seizure detection in an implantable stimulator, so that it is possible to intervene and prevent seizures from occurring – or limit their duration and severity. An intelligent implantable stimulator can be programmed to detect abnormal epileptiform activity specific to the individual patient, and then to deliver the appropriate therapeutic stimulus.

See also: **Models**: Characteristics and Mechanisms of Kindling; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Treatments (non-Antiepileptic Drugs)**: Control of Seizure Activity with Electrical Stimulation; Electrical Stimulation; Focal Cooling as a Potential Treatment for Neocortical Epilepsies.

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Electrical Stimulation

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Introduction

Laboratory data and clinical trials have shown that electrical brain stimulation applied to either the central or peripheral nervous system (CNS or PNS) has the potential to reduce seizure frequency in patients with epilepsy. Current standard options for reducing seizure activity include pharmacological intervention, vagal nerve stimulation (VNS), and/or surgical resection. These treatments all have potential drawbacks – e.g., unwanted side effects, risk of infection, potential cognitive impairments, suboptimal efficacy, or drug resistance. In fact, antiepileptic drugs (AEDs) fail to achieve adequate seizure reduction in about 30% of epilepsy patients. For a variety of reasons, not all patients with drug-resistant epilepsy can be considered as candidates for resective surgery. Therefore, the development of novel electrical brain stimulation protocols for suppressing seizure frequency has important ramifications for improving treatment management in specific patients suffering from seizure disorders.

To this end, electrical stimulation of CNS brain structures – either directly involved in epileptic activity (e.g., hippocampus, neocortex), connected via white-matter tracts (e.g., corpus callosum) to epileptogenic regions, or deep-brain structures (e.g., subthalamic nucleus (STN), caudate nucleus) that modulate the activity of cortical structures – has been attempted with some success. Electrical stimulation may, in many cases, have several benefits, including 'reversibility.' In addition, electrical stimulation typically involves no brain tissue removal or destruction. The electrical parameters of the stimulator may be adjusted so as to achieve the best clinical outcome for seizure suppression, and the parameters of stimulation can be individualized for each patient's specific condition.

However, many important questions about electrical stimulation approaches for seizure control still exist. For example, what are the most effective target sites or the optimal electrical parameters? It is also discomforting – but intriguing from a scientific view – that we do not adequately understand the mechanisms responsible for the suppression of epileptic activity achieved by electrical stimulation. Several theories about the mechanisms of action have been put forward, particularly for VNS and deep-brain stimulation (DBS), but none have been thoroughly tested or substantiated. Indeed, that some electrical stimulation protocols have anticonvulsant action on nervous system function is somewhat paradoxical, since

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Electrical Stimulation

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Introduction

Laboratory data and clinical trials have shown that electrical brain stimulation applied to either the central or peripheral nervous system (CNS or PNS) has the potential to reduce seizure frequency in patients with epilepsy. Current standard options for reducing seizure activity include pharmacological intervention, vagal nerve stimulation (VNS), and/or surgical resection. These treatments all have potential drawbacks – e.g., unwanted side effects, risk of infection, potential cognitive impairments, suboptimal efficacy, or drug resistance. In fact, antiepileptic drugs (AEDs) fail to achieve adequate seizure reduction in about 30% of epilepsy patients. For a variety of reasons, not all patients with drug-resistant epilepsy can be considered as candidates for resective surgery. Therefore, the development of novel electrical brain stimulation protocols for suppressing seizure frequency has important ramifications for improving treatment management in specific patients suffering from seizure disorders.

To this end, electrical stimulation of CNS brain structures – either directly involved in epileptic activity (e.g., hippocampus, neocortex), connected via white-matter tracts (e.g., corpus callosum) to epileptogenic regions, or deep-brain structures (e.g., subthalamic nucleus (STN), caudate nucleus) that modulate the activity of cortical structures – has been attempted with some success. Electrical stimulation may, in many cases, have several benefits, including 'reversibility.' In addition, electrical stimulation typically involves no brain tissue removal or destruction. The electrical parameters of the stimulator may be adjusted so as to achieve the best clinical outcome for seizure suppression, and the parameters of stimulation can be individualized for each patient's specific condition.

However, many important questions about electrical stimulation approaches for seizure control still exist. For example, what are the most effective target sites or the optimal electrical parameters? It is also discomforting – but intriguing from a scientific view – that we do not adequately understand the mechanisms responsible for the suppression of epileptic activity achieved by electrical stimulation. Several theories about the mechanisms of action have been put forward, particularly for VNS and deep-brain stimulation (DBS), but none have been thoroughly tested or substantiated. Indeed, that some electrical stimulation protocols have anticonvulsant action on nervous system function is somewhat paradoxical, since

electrical stimulation of nervous tissue has historically been used to excite neurons. Thus, this phenomenon of seizure control remains to be fully explained.

Background

Targets and Frequencies

Electrical stimulation of the nervous system has a long history. The ancient Greeks used electric rays (suborder Torpedinoidei) for applying controlled electrical shocks to those suffering from medical conditions such as headache. In modern times, DBS in human patients with movement disorders has been markedly successful (typically using stimulation protocols with frequencies near ~130 Hz). These protocols have been effective at suppressing neuronal firing and improving symptoms associated with Parkinson's disease, such as tremor, rigor, and bradykinesia. Targets for such procedures include the thalamus, striatum, and the STN.

The use of electrical stimulation for treating epilepsy has included PNS, and more recently, CNS targets. In the PNS, both vagal nerve and trigeminal nerve stimulation have been assessed. VNS has been used for a number of years for seizure control in epilepsy, but only with mixed success. VNS shows benefit, but is not highly efficacious since it only helps about 40–50% of those patients treated. Using the VNS in humans, Olejniczak found previously that a 30-Hz frequency reduced interictal epileptiform activity. A review of the stimulation frequencies used in VNS shows that those above 50 Hz caused major damage to the nerve, and so the U.S. Federal Drug Administration (FDA) has approved frequencies only between 20 and 30 Hz for clinical use. Trigeminal nerve stimulation has been attempted in the PNS of rats, where a decrease in seizure activity has been reported.

In the CNS, stimulation targets have included the cerebellum, hippocampus, the neocortex, and deep-brain structures such as the caudate nucleus, posterior hypothalamus, thalamic nuclei, and the STN. More recently, the substantia nigra pars reticulata (SNr), a popular target for stimulation in Parkinson's disease, has been postulated to be an important player in the pathophysiology of epilepsy. These targets can be divided into two broad categories based on whether the stimulation is directly applied to the epileptic zone or whether the stimulation activates nerve fibers 'upstream' of the epileptic zone. It is not yet clear which method is optimal for clinical applications.

One approach to this issue has been to stimulate an endogenous network that modulates cortical neuronal excitability. The SNr is part of such an endogenous system, inhibiting epileptic seizures through γ-aminobutyric acid (GABAergic) projections to the superior colliculus. The SNr, via this inhibitory output, maintains a constant inhibition of the dorsal midbrain region in the deep layers of the superior colliculus. In this network, tonic glutamatergic input from the STN maintains SNr firing at a high rate. Reduced output of the SNr GABAergic neurons results in loss of inhibitory control over both convulsive and nonconvulsive generalized epileptic seizures. High-frequency stimulation of the STN or SNr has been found to reduce seizure frequency, suggesting that the SNr may be part of a major desynchronizing system that influences the regulation of seizures.

Direct focal stimulation of the hippocampus in human epileptic patients has also successfully decreased seizure activity in patients with intractable temporal lobe seizures. For example, in a preliminary study by Velasco *et al.*, electrical stimulation (130 Hz) was applied prior to a planned anterior temporal lobectomy. Subacute stimulation (i.e., for 2–3 weeks) abolished clinical seizures and significantly decreased the number of interictal spikes at the focus after 5–6 days of stimulation. Moreover, microscopy of the stimulated tissue revealed no obvious histopathological differences between stimulated vs. non-stimulated tissue. Additionally, chronic stimulation (i.e., for 3–4 months) produced a sustained, long-lasting anti-epileptic effect, with no apparent change on short-term memory.

In vitro animal model studies using hippocampal slice preparations have demonstrated that direct stimulation can disrupt experimentally induced epileptiform activity. These in vitro preparations are advantageous, since they allow an examination of potential mechanisms of action under well-controlled conditions, and are manipulated easily. Using this preparation, we have shown that both high- and low-frequency protocols have suppressive effects on seizure-like activity. However, the pattern of effect is quite different in each case (see later). Additional studies on hippocampal slices conducted independently by Durand, Schiff, and others have shown that the application of electric fields also has a suppressive effect; both brief, low-frequency stimuli and direct current high-frequency stimuli suppressed epileptiform activity in these slice studies.

Experiments have also shown that protocols using 1–5-Hz stimulation, similar to those used for studies of long-term depression (LTD), reduce kindling in rats and cats. In kindling studies of this kind, stimulation frequencies of 40–100 Hz are used to elicit the kindling phenomenon. A stimulation of 60 Hz is commonly applied (for 1–2 s) over a period of days to stimulate structures such as the amygdala, which results in the generation of generalized convulsions. Low-frequency stimuli, applied either before or after kindling has begun (to targets such as the amygdala or hippocampus), attenuate the kindled seizure activity. Low-frequency stimulation (1 Hz) in hippocampal–entorhinal rat slices has also been shown to block ictal activity in a 4AP convulsant model of

epilepsy. Inhibitory effects were induced by 1-Hz stimulation in both the subiculum and the entorhinal cortex. Since stimulation patterns used in these studies for suppressing seizure activity are similar to those used for the induction of LTD, mechanisms responsible for LTD (or 'depotentiation') may play a role in the suppression of seizure-like activity.

Mechanism(s)

In general, electrical stimulation – i.e., applied voltage in a local neural network – artificially depolarizes or hyperpolarizes neurons (i.e., modulates membrane potentials). Electrical stimulation can also initiate action potentials, which may propagate orthodromically or antidromically (or even both ways) along the axon. Depending on the intensity of the applied voltage, electrical stimulation can block the propagation of action potentials, thus providing a potential treatment modality for disorders such as epilepsy. However, it is still not clear how electrical stimulation modulates seizure activity. It is likely that there are several mechanisms at work, including perhaps those specific to target site or frequency of stimulation. At the current time, there are two general hypotheses that have been put forth. The first hypothesis, known as 'stimulation-blockade' or 'depolarization-blockade,' posits that electrical stimulation induces alterations in the activation of currents through voltage-gated channels that are in close proximity to the stimulating electrode. According to this hypothesis, electrical stimulation causes a nonspecific suppression of nerve-cell activity via constant membrane depolarization, which in turn inactivates voltage-gated sodium channels that are responsible for the depolarizing phase of the action potential. The second hypothesis, labeled 'synaptic inhibition,' suggests that stimulation effects on seizure activity are mediated indirectly, regulating neuronal output in cells in close proximity to the stimulating electrode, which in turn inhibit excitability at distant sites. In support of this hypothesis, proponents point out the large numbers of GABAergic terminals in structures such as the thalamus and the basal ganglia – i.e., targets for DBS; activation of these axon terminals by high-frequency protocols may inhibit activity in postsynaptic neurons by the summation of inhibitory postsynaptic potentials (IPSPs). The time course of GABAergic IPSPs match well with the optimal summation that occurs at frequencies where DBS is most effective.

With regard to the PNS, several interesting observations have been made, but the precise mechanisms of action of VNS remain unknown. The antiepileptic effects of VNS were initially linked to noradrenaline increases, especially given that locus coeruleus was found to mediate some of the effects of VNS in attenuating seizures; however, more recent data suggest that this explanation is not entirely satisfactory. VNS also appears to change brain function by direct afferent action. Additionally, VNS has been shown to reduce the firing rate of hippocampal neurons in laboratory animals.

Methodological Considerations and Recent Results

Novel electrical stimulation methods include open loop, semiclosed loop, and closed loop protocols. 'Open loop' stimulation is characterized by stimulation that is independent of brain activity; typically, the protocol has a timed on and a timed off period. 'Semiclosed' and 'closed loop' stimulation incorporate feedback from the nervous system of the patient (or experimental animal). In semiclosed protocols, however, the recorded physiological information is only used to switch stimulation on and off rather than to generate a dynamic-state-related stimulation protocol.

Unfortunately, most electrical stimulation protocols have not been evaluated clinically in controlled double-blind clinical trials. The absence of such trials may make efficacy difficult to evaluate, since in some cases patients can sense when stimulation is being induced.

To test the efficacy of DBS on ictal onset in a kindling model of epilepsy (in rat), Wyckhuys *et al.* evaluated the effect of high-frequency stimulation (130 Hz), using a protocol similar to that used by Velasco *et al.* in epileptic patients. These investigators used the 'alternate day rapid kindling' model of temporal lobe epilepsy (TLE), and placed their stimulation electrode in hippocampus (since this structure is often thought to be a seizure focus in human TLE). Electrical stimulation in the hippocampus of epileptic rats was found to modulate the characteristics of evoked afterdischarges in a way that reflects a reduction in excitability of the target region. The beneficial effects of 130-Hz stimulation on evoked afterdischarges were demonstrated to outlast the stimulation for at least 1 week.

Our laboratory compared both high- and low-frequency electrical stimulation protocols (in the same preparation) to determine which protocol was more effective at reducing epileptiform activity. In these hippocampal slice studies, we used convulsant agents such as bicuculline, or bathing medium deficient in magnesium, to generate seizure-like activity. In these paradigms, the epileptiform activity observed in brain slices is similar to the interictal spike discharge seen in EEG recordings of epilepsy patients.

We found that during high-frequency 100-Hz stimulation (for as little as 10 min), an immediate inhibition of extracellular field potential epileptiform discharges was observed (**Figure 1**). The effect was seen within just a few hundred milliseconds of the stimulation onset. In contrast, 1-Hz stimulation demonstrated only a gradual suppression of the complex epileptiform field potential, requiring

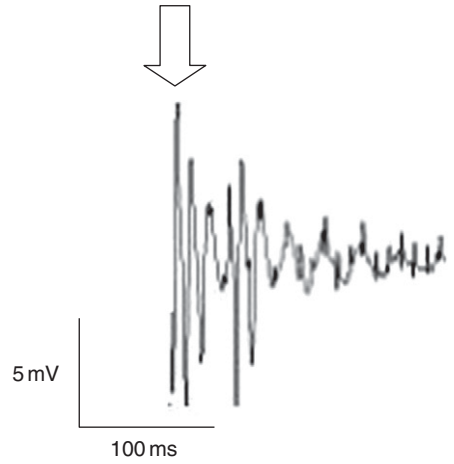


Figure 1 Multiple spiking discharges observed during the application of 100-Hz stimulation in the CA1 rat hippocampal brain slice. The arrow indicates the onset of stimulation. Notice that the extracellular field potential response is greatly reduced in amplitude within just 200–300 ms of the stimulation onset.

30 min for complete suppression. Interestingly, however, once the 1-Hz stimulation was turned off, the beneficial suppression continued for at least 20 min. This longer-term effect was not seen with high-frequency (100 Hz) stimulation, where multiple spiking responses rebounded immediately back to their original amplitudes when the stimulator was turned off. This long-lasting effect of the low-frequency protocol is similar to what has been seen in studies involving LTD.

At the clinical level, Velasco *et al.* recently reported a follow-up study to evaluate the safety and efficacy of electrical stimulation (130 Hz) of the hippocampus, and to assess the impact of stimulation on memory, in patients with refractory mesial TLE. Five of the nine patients had normal MRIs, and stimulation led to seizure reduction of more than 95%. The remaining four patients had hippocampal sclerosis, and stimulation led to seizure reduction of 50–70%. The authors concluded that electrical stimulation provided improved seizure outcome (without memory alteration), particularly in patients with nonlesional TLE. Another clinical study, by Boon *et al.*, prospectively evaluated the effectiveness of long-term DBS in patients with medial TLE. Patients underwent long-term stimulation of medial temporal lobe. After mean follow-up of 31 months, 5 of 10 patients in the DBS group had a seizure-frequency reduction of more than 50%, thus showing efficacy for long-term DBS in medial temporal lobe structures.

Future Goals

Currently, 17 medical centers in the United States are participating in the SANTE (Stimulation of the Anterior Nucleus of the Thalamus for Epilepsy) Trial, using DBS to determine whether bilateral stimulation of the anterior nucleus of the thalamus can effectively and safely reduce seizure frequency in patients with epilepsy. Recent results by Wyckhuys and Velasco (mentioned earlier) and the prospect of new clinical trials using electrical stimulation for seizure control in human studies are encouraging.

Future clinical trials, involving double-blind design, however, will be essential for validating stimulation therapies. The study of electrical stimulation in epileptic patients has unique design problems, particularly the need to make the patient oblivious to differences in the trial (since patients can often detect the stimulation). Other problems include the choice of stimulation parameters and the methods for handling unavoidable variation in seizure rates among patients (i.e., how to evaluate effects when starting from different baselines).

Furthermore, the best targets to stimulate, and the mechanisms underlying stimulation effects, are still unknown. A broad range of novel and existing stimulus protocols still need to be thoroughly tested in potential targets.

Future studies will also likely focus on 'state estimation' of the condition of seizure-prone areas as well as 'state transition.' Progress on prediction and detection systems, which will work hand-in-hand with novel prevention or treatment modalities, will be critical, and the stimulation protocols that could be incorporated into treatments that are based on seizure prediction will be essential for future implementation.

In summary, electrical stimulation is a promising treatment option for a subgroup of patients with intractable epilepsy who are not candidates for surgery. Measures of efficacy, optimal targets, electrical stimulation parameters, and types of devices, as well as patient selection criteria, still need to be characterized.

See also: **Models**: Characteristics and Mechanisms of Kindling; **Networks**: The Role of the Basal Ganglia Circuits in the Control of Epileptic Seizures; **Plasticity**: Synaptic Potentiation and Inhibition after Partial Seizures; **Treatments (non-Antiepileptic Drugs)**: Control of Seizure Activity with Electrical Stimulation; Deep Brain and Peripheral Nerve Stimulation as Potential Therapies for Epilepsy.

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Epilepsy and Gene Therapy: Resculpturing Synaptic Transmission with Neuropeptides

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Introduction

Neuropeptide Y (NPY) is widely expressed in mammalian brain and has been shown to be involved in various functions, such as regulation of blood pressure, circadian rhythms, feeding behavior, anxiety, memory, and cognition. NPY is often colocalized with classical neurotransmitters in neurons, and is predominantly released during high frequency neuronal activity. The effects of NPY are mediated via a number of G-protein-coupled receptor subtypes, including Y1, Y2, Y4, Y5, and Y6 identified so far. The Y1, Y2, and Y5 receptors are expressed throughout the hippocampus, particularly in the mossy fibers and Schaffer collaterals, in the molecular layer of the dentate gyrus, and in granule cells.

Increasing data indicate that NPY might be involved in adaptation mechanisms and/or pathophysiology underlying epileptogenesis. NPY has been shown to exert anticonvulsant action in vivo, when either overexpressed in transgenic rats or injected intraventricularly in different seizure models. In hippocampal slices, NPY has been shown to inhibit epileptiform activity induced by bicuculline, picrotoxin, or zero Mg^{2+} perfusion. In cortical slices, we showed similar inhibitory effects of NPY in the zero Mg^{2+} model. The seizure-suppressant effects of NPY in the hippocampus seem to be mediated mostly via Y2 receptors, but some involvement of Y5 receptors has also been reported. We have shown previously, using NPY receptor Y2/Y5 double KO mice, that activation of both Y2 and Y5 receptors suppress hippocampal seizures in vitro, while activation of Y5 receptors in extrahippocampal regions suppresses generalized seizures in vivo. More recently, hippocampal NPY gene therapy has been shown to effectively suppress seizures in various animal models of epilepsy. To further assess the clinical applicability of such approach, we have investigated how synaptic transmission and plasticity in synapses of the hippocampus is modulated by viral vector-mediated NPY overexpression.

Background

Application of exogenous NPY to cortical and hippocampal slices of the normal brain has been shown to modulate inhibitory and excitatory synaptic transmission, respectively. The most well known action of NPY in the central nervous system is inhibition of excitatory synaptic transmission in the hippocampus. This effect is most likely achieved by decreasing glutamate release via suppressing presynaptic voltage-dependent Ca^{2+}-channels.

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Methods

In our studies, the serotype 1/2 rAAV vector, carrying the NPY gene under the control of the NSE promoter, is injected stereotactically into the hippocampus of rats. Recombinant adeno-associated viral (rAAV)–NPY vector is produced using standard procedures and is obtained via collaboration with Dr. Matthew During from Cornell University, New York, USA. Injection is performed as follows: Animals are anaesthetized and placed in stereotaxic frame, a hole is drilled in the skull according to predetermined coordinates, and glass pipette filled with viral vector solution is inserted into the brain through the hole; injection of 1 µl solution is performed over the course of a few minutes, and the pipette is left in place for another few minutes to prevent backflow of the viral vector into the pipette tract. The vector construct used in our experiments gives a neuron-specific widespread expression of NPY in the hippocampus. Electrophysiological experiments are performed with both field and whole-cell recordings in brain slices prepared three to seven weeks after viral vector delivery. Vibratome-cut slices are maintained in artificial cerebrospinal fluid (aCSF) bubbled with a gas mixture of 95% O_2 and 5% CO_2, and continuously perfused with gassed aCSF in the recording chamber. Field recordings are made with a pipette filled with aCSF, while whole-cell recordings are made with patch-clamp pipettes filled with solutions specifically designed for certain experimental requirements, having pipette tip resistances of 4–6 MΩ. Field or whole-cell potentials or currents are amplified with patch-clamp amplifier (HEKA elektronik, Germany), and the recordings are saved on the computer for later off-line analysis performed with IgorPro and FITMASTER software using appropriate statistical tests. Detailed method description could be found elsewhere (see Further Reading).

Recent Results

Our studies demonstrate that injection of rAAV–NPY vector into the hippocampus of naïve animals leads to high levels of transgene NPY expression, as estimated by increased immunoreactivity in transduced areas of hippocampus. Further, such injections alter excitatory synaptic transmission, predominantly during high frequency neuronal activity (high frequency stimulation – HFS). This effect seems to be due to the release of transgene NPY from neurons, which acts on presynaptic Y2 receptors and suppresses glutamate release. These effects are demonstrated on **Figure 1(a)**, where relative depression of consecutive EPSPs, generated in Schaffer collateral-CA1 cell synapses during the later phase of HFS, is more pronounced in slices from rAAV–empty vector-treated animals

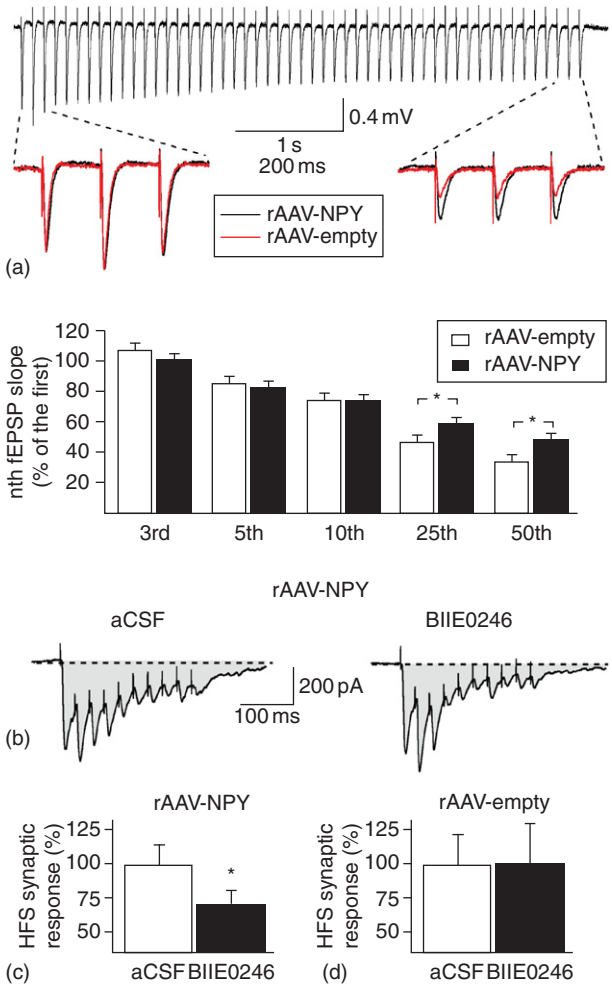


Figure 1 Transgene NPY is released during high frequency stimulation (HFS). (a) Representative trace from a rAAV–NPY treated slices showing a 10 Hz HFS-evoked field EPSPs (fEPSPs) recorded in stratum radiatum of CA1 with AP-5 (NMDA receptor blocker) present in the perfusion medium. Insets: superimposed traces showing more pronounced depression of only last three fEPSPs in rAAV–empty relative to rAAV–NPY treated slices. Average bar plot showing initial slopes of the consecutive fEPSPs (3rd, 5th, 10th, 25th, 50th) expressed as relative percent of the first evoked fEPSP in rAAV–NPY and rAAV–empty treated slices (*$p < 0.05$, *t*-test). (b) Whole-cell patch-clamp recording from a proximal subicular neuron in rAAV–NPY treated slice showing HFS-induced (25 Hz) depression of consecutive EPSCs. Application of Y2 receptor antagonist, BIIE0246, significantly increased EPSC depression in this cell. (c) Average HFS-induced EPSC depression expressed as an integral of the whole response divided by the integral of the first EPSC before (aCSF) and after (BIIE0246) BIIE0246 application. Note the significant decrease of the averaged responses (increased depression) after BIIE0246 application. Such decrease was not observed in rAAV–empty (d) treated slices (*$p < 0.01$, paired *t*-test).

as compared to those treated with rAAV–NPY. In line with this observation, application of the selective Y2 receptor antagonist, BIIE0246, in rAAV–NPY vector treated slices leads to more pronounced depression of consecutive EPSCs induced in subicular neurons by HFS of alveus (**Figure 1(b)**

and 1(c)). BIIE0246 application had no effect on rAAV–empty treated slices (**Figure 1(d)**). These data suggest that transgene NPY is released during HFS, which decreases glutamate release. This initial decrease in glutamate release, in turn, leads to slower depletion of the releasable vesicular pool of glutamate, reflected as less pronounced depression of synaptic responses during HFS.

The fact that transgene NPY is released during HFS is further confirmed by our experiments using two independent pathway stimuli (**Figure 2(a)**). The test pulse-induced EPSC was decreased when HFS stimulation was delivered to another afferent pathway of rAAV–NPY treated animals (**Figure 2(b)** and **2(d)**). This effect was not seen after BIIE0246 application (**Figure 2(c)** and **2(d)**), or in rAAV–empty vector-treated animals (**Figure 2(e)**). Taken together, these data strongly suggest that transgene NPY is indeed released by HFS, and inhibits glutamate release even in neighboring, nonactivated excitatory synapses. Such 'volume transmission' generated by transgene NPY could be a mechanism for reducing/precluding recruitment of nonactivated synapses (and eventually neurons) during high frequency activity. Such an effect would suppress spread of seizures in the hippocampus.

Worryingly, certain types of neuronal high frequency activity in the hippocampus occur not only during seizures, but also during induction of long-term potentiation (LTP), a process related to learning and memory. In rAAV–NPY vector treated animals, we found that LTP in both Schaffer collateral-CA1 (**Figures 3(a)** and **3(b)**) and CA1-subicular cell synapses (**Figure 3(c)**) seems to be attenuated. These findings could raise some concerns about possible interference by transgene NPY in learning and memory process if used in patients with epilepsy. However, behavioral studies indicate that such deficits in learning and memory induced by rAAV–NPY treatment could be transient, and that the animals still learn hippocampal-related tasks – although with some delay. Moreover, our preliminary data suggest that in animals that have been kindled, LTP in Schaffer collateral-CA1 cell synapses is slightly attenuated. The LTP in these 'epileptic' animals does not seem to worsen by injections of rAAV–NPY vector into the hippocampus.

We are currently investigating whether and how NPY affects inhibitory circuits of the hippocampus, and study the possible contribution of NPY-induced alterations of the inhibitory circuitry in the seizure-suppressant action

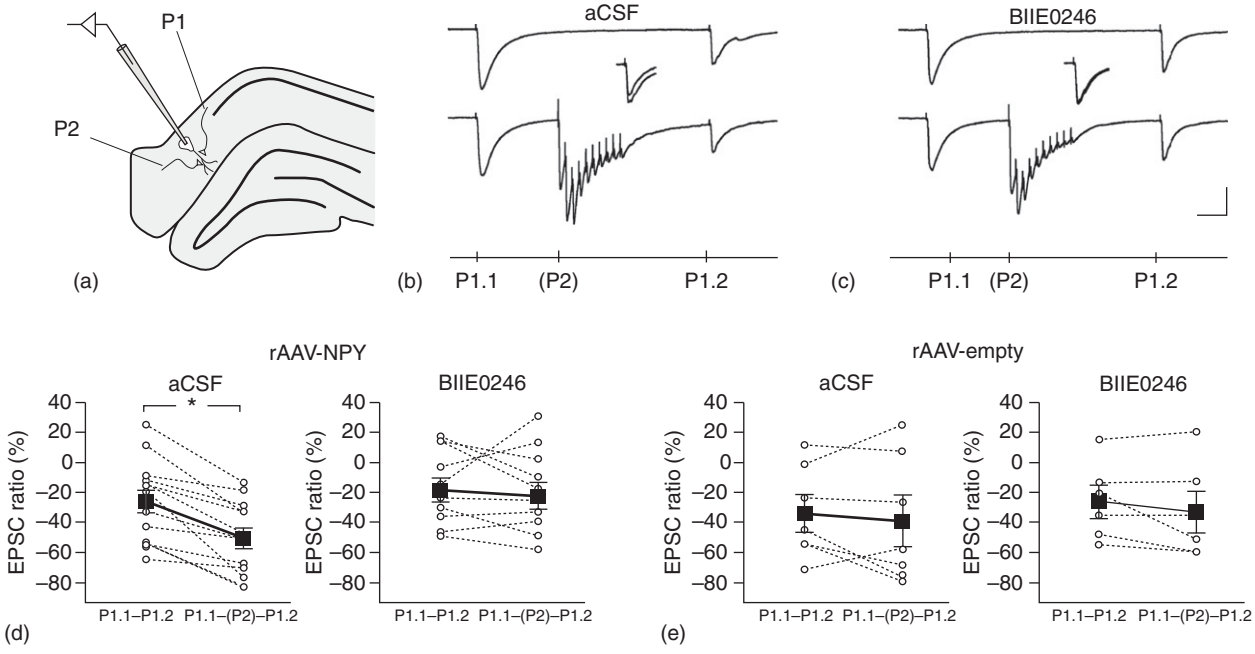


Figure 2 HFS-induced release of transgene NPY suppresses glutamate release in neighboring synapses as a volume transmitter. (a) Schematic illustration of the recording setup, showing the patch electrode in contact with a pyramidal neuron in proximal subiculum, and two stimulation electrodes placed on each side, to activate two independent afferent pathways (P1 and P2) impinging onto the same recorded cell. (b) Representative traces showing EPSCs evoked by paired stimulation of the P1 pathway (top trace, P1.1–P1.2), or P1 pathway with intermediate HFS train stimulation of P2 pathway (lower trace, P1.1–(P2)–P1.2) in a rAAV–NPY treated slice. Time points of stimulation for P1 and P2 are shown on bottom line. Note the reduction of EPSC amplitude evoked by P1.2 stimulation after HFS of P2 (inset). (c) Similar traces as shown in (b), but after BIIE0246 application. Note that EPSCs evoked by P1.2 stimulations are of similar amplitude (inset). Scale bar is 100 ms and 100 pA, for both (b) and (c). (d) EPSC amplitude ratios (P1.2–P1.1)/P1.1 of paired EPSCs are plotted for each recorded cell before and after BIIE0246 application in rAAV–NPY treated animals. Dotted lines and open circles show EPSC ratio changes in individual cells. Thick line and filled squares show the mean ± SEM values from all cells (*$p < 0.05$, paired *t*-test). (e) Similar as in D, but for rAAV–empty treated rats.

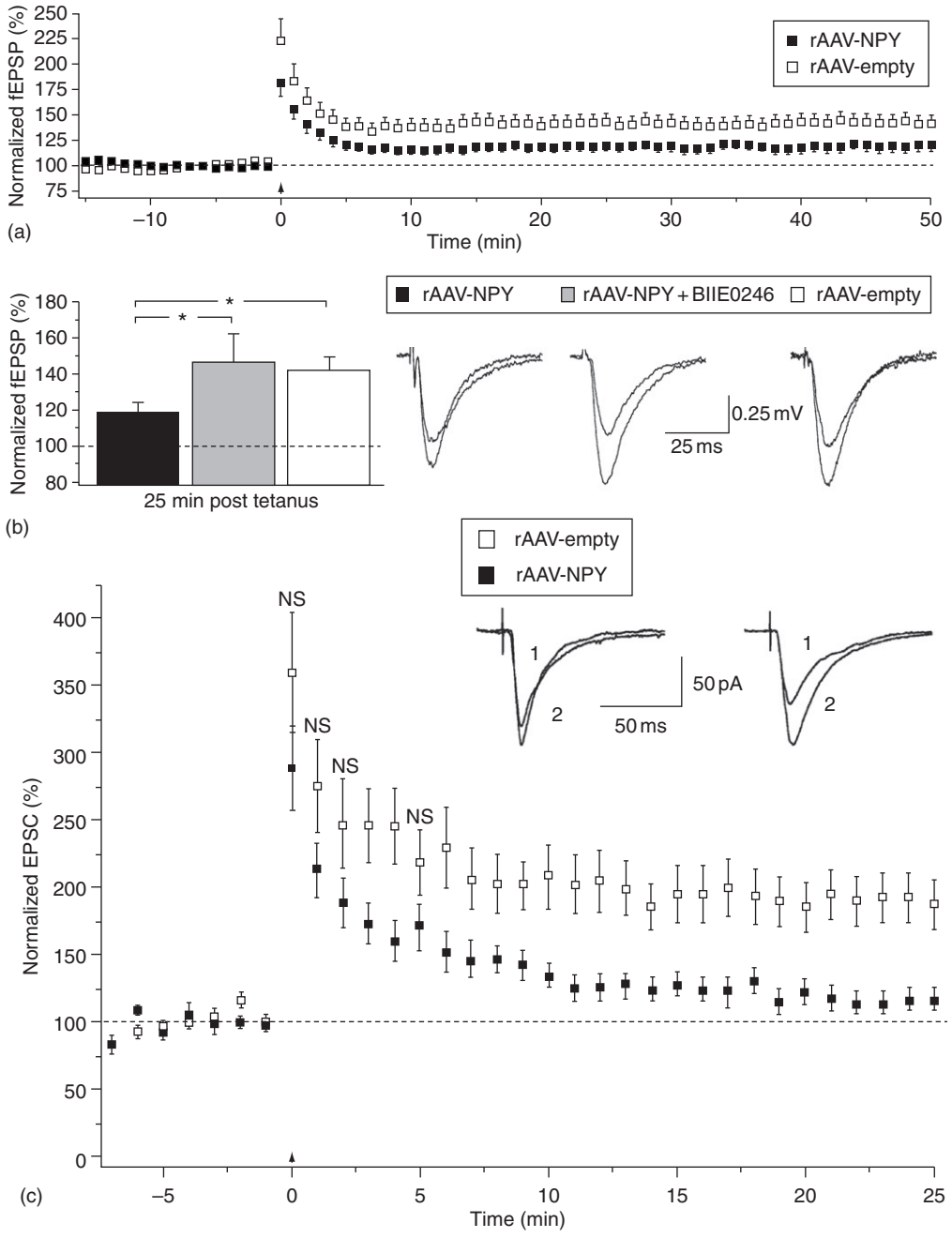


Figure 3 LTP is attenuated by transgene NPY in Schaffer collateral-CA1 and CA1-subicular cell synapses. (a) LTP of fEPSPs in Schaffer collateral-CA1 synapses in rAAV–NPY and rAAV–empty treated slices. LTP is induced by tetanic stimulation (100 Hz, 1 s), marked by arrow at 0 min. Initial slopes of EPSPs are normalized to the average baseline values (−15 to 0 min) and plotted against time. (b) Bar graph showing average values of fEPSP LTP in percent to baseline at 25 min post-tetanus with respective fEPSP traces on the right. Application of Y2 receptor antagonist, BIIE0246, to slices from rAAV–NPY treated animals reversed the attenuation of LTP to similar levels as recorded in rAAV–empty treated slices (*$p < 0.05$, *t*-test). (c) LTP in subicular-CA1 synapses, as recorded by whole-cell patch-clamp from proximal pyramidal cells, is significantly attenuated ($p < 0.05$, *t*-test) at most time points after tetanic stimulation in rAAV–NPY as compared to rAAV–empty treated slices (NS, nonsignificant). *Inset*: Representative EPSCs recorded in subicular cells during baseline (1), and 10 min post tetanus (2), in rAAV–NPY and rAAV–empty treated slices.

of transgene NPY. Our preliminary data indicate that exogenous application of NPY decreases inhibitory drive (as measured by mIPSC frequency) on a basket cell subpopulation of the GABAergic inhibitory interneurons; this influence could possibly contribute to the antiepileptic effect of NPY by disinhibiting inhibitory GABAergic interneurons, and thus increasing inhibitory drive onto the principal cells.

Future Goals

In the future, we will explore whether NPY receptor overexpression using rAAV vectors is possible, and if such treatment alone – or in combination with NPY overexpression – would exert stronger antiepileptic and/or antiepileptogenic effect in the hippocampus. We will also adopt encapsulated cell biodelivery (ECB) technology to deliver gene products via genetically modified and encapsulated cell line transplants into the brain (in this case NPY, or another endogenous antiepileptic neuropeptide, galanin), to evaluate the seizure-controlling capabilities of such approach. The ECB devices provide some additional safety aspects for tentative clinical applications, as they could be easily removed from the brain in case of any adverse effects.

See also: **Neurotrophic Factors**: GDNF: A Neurotrophic Factor Against Epilepsy; **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Gene Therapy in Epilepsy; Viral Vector Approaches for Antiepileptic Gene Therapy.

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Focal Cooling as a Potential Treatment for Neocortical Epilepsies

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Introduction

Scope of the Problem

The end of the twentieth century witnessedremarkable advances in the understanding and treatment of many of the epilepsies. The scientific speculation that some genetic epilepsies were caused by mutations in ion and voltage-gated channels was validated by a series of landmark genetic discoveries. New anticonvulsants, several with highly favorable therapeutic indices, were introduced. Sensitive magnetic resonance and positron-emitting based imaging tests that revealed the focal etiology of many complicated, symptomatic epilepsies emerged and evolved rapidly. Finally, the once radical therapeutic option of surgical resection for both lesional and nonlesional epilepsies became much more accepted as a mainstream therapeutic option for medically refractory patients.

Despite this progress, many patients with epilepsy have been left behind. The focal cortical epilepsies have remained especially problematic. Accounting for up to half of patients with poorly controlled seizures, focal and multifocal seizures arising from the neocortex have proven extremely refractory to both conventional anticonvulsant therapy and the newer surgical approaches. Even with guidance from modern neuroimaging techniques that allow functional anatomic correlation of seizure onset with surface rendering of the neocortex, the surgical treatment of cortical seizures is successful in about 50–60% of cases only. There are at

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least three reasons why surgical management of neocortical epilepsy is so difficult. First, conventional neurophysiological techniques frequently have a difficult time localizing the site of a patient's seizures. Second, identification of the specific neurological function(s) residing in the seizure onset zone, essential for an accurate prediction of adverse effects of resection, can be very difficult. Third, in many instances, the expectation that resection will leave a permanent neurological deficit precludes surgery altogether.

Alternatives to Permanent Resection for Neocortical Epilepsy

A variety of invasive but nondestructive strategies have recently been employed or proposed to reduce the frequency and severity of neocortical seizures. For the past 15 years, vagal nerve stimulation (VNS) has been used for selected children and adults with refractory epilepsy. Although a variety of clinical studies have validated its efficacy, the overall reduction in seizure frequency with VNS approximates 50%. This certainly represents an improvement in seizure control, but for many patients the improvement in quality of life is not substantial. There is some experimental evidence that trigeminal nerve stimulation might provide better seizure control, but as yet there have been no human studies of this modality. Transcranial magnetic stimulation for epilepsy has been described in several publications. The initial reports were optimistic, but subsequent controlled prospective trials have not shown significant reduction in seizure frequency. There are also brain slice studies indicating that the direct application of a constant or DC electrical field can diminish neuronal excitability and experimental seizure discharges.

Although DC fields have not yet been tried clinically, there is limited experience with several variations of intermittent electrical stimulation for human epilepsy. At least five publications have identified subsets of patients who benefited from intermittent hippocampal or thalamic stimulation. These results are still too premature to allow definite conclusions. The most sophisticated intervention is a totally implantable, closed-loop feedback system for seizure detection and cortical stimulation to terminate focal seizures. Using the same electrodes for both recording and stimulation, in conjunction with a custom-designed seizure detection algorithm, this device has been able to abruptly terminate seizures by delivering a 100 Hz burst of pulses lasting about a tenth of a second. At this time, the reliability of this device is being determined in prospective clinical trails at several North American epilepsy centers.

Background

Another appealing, nondestructive strategy for terminating and possibly preventing focal seizures is the application of focal cooling. There is an extensive literature that documents that cooling reduces synaptic transmission in the mammalian brain, and it should be possible to use new engineering technology to deliver very focal cooling. The first descriptions of the central neurological effects of focal cooling come from articles written by Stefani and Deganello at the end of the nineteenth century. A decade later, the German physiologist Trendelenburg began a systematic study of local hypothermia, investigating its effects on both brainstem autonomic reflexes and the neocortex. While lacking any cellular insights, all three investigators concluded that cooling reduced neurological function at the system level. Throughout the rest of the twentieth century, physiologists continued to use local cooling to investigate cortical and subcortical localization of specific brain functions. Information about the neurobiological effects of cooling advanced over the last fifty years. Using intracellular recording at the frog neuromuscular junction, Katz and Miledi showed that cooling reduced end plate potentials, likely by diminishing the probability of acetylcholine release from presynaptic terminals. More recent experiments in mammalian tissue culture and brain slices have shown that cooling alters excitatory transmission by both pre- and postsynaptic mechanisms. Cooling can augment the magnitude of neuronal action potentials and inhibits the sodium–potassium ATPase. Although the initial ATPase inhibition and concomitant cell depolarization may elicit transient hyperexcitability, cooling eventually reduces neuronal excitability. Our own recent observations indicate that hypothermia rapidly reduces transmitter release from presynaptic vesicles and suggest that this may be a dominant effect of rapid cooling on central neuronal excitability.

The potential clinical utility of cooling for neurological disease has been discussed for half a century. Fay began an extensive investigation of brain cooling in 1938. He suggested that either systemic hypothermia or local cooling of the brain with cooled fluid circulating through a sealed metal capsule might be an efficacious treatment for head trauma and intractable pain. He also thought reducing brain temperature might inhibit tumor growth and tried intracranial cooling for inoperable gliomas. More recent controlled studies have reported benefits of cooling patients after acute head trauma or asphyxia. Interestingly, the temperature reduction in these recent clinical reports is generally no more than 4 °C, which would not be expected to have a very large effect on synaptic transmission. This temperature change is also a much smaller drop than the cooling required to terminate experimental seizures.

Physicians have been aware of a causal relationship between elevated temperature and seizures since Hippocrates. Moreover, numerous *in vitro* and *in vivo* experimental epilepsy studies have consistently demonstrated

that cooling diminishes paroxysmal bursting and can reduce or stop seizure activity. There are encouraging clinical reports suggesting the utility of brain cooling for human seizures as well. A quarter century ago, two separate clinical investigations documented the efficacy of cooling in the therapy of a limited number of patients with acute, prolonged seizures or chronic, recurrent seizures. The initial paper showed that externally cooling (31–36 °C) six patients with refractory status epilepticus stopped seizures in five. The second paper described 25 patients, ranging in age from 8 to 46, who had frequent major motor seizures poorly controlled on chronic anticonvulsant therapy. While under general anesthesia, they were placed in an enclosed chamber and chilled to 29 °C with cold air. At that point, bilateral frontal burr holes were opened and the subarachnoid space or ventricles were irrigated with iced saline, which reduced cortical surface temperature below 24 °C in 21 of the patients. A total volume of 500–20 000 cc of saline was infused into the patients who were slowly recovered from anesthesia. Of the 15 patients followed for 1 year, 11 showed a marked reduction in seizure frequency, including 4 who had been seizure free. These long term favorable outcomes suggest that transient cooling can exert a sustained effect, whose mechanism remains to be determined.

Two more recent descriptions of direct cooling of human brain during operative neurosurgical mapping have confirmed that acutely lowering cortical temperature will terminate paroxysmal discharges. In these cases, focal spikes abruptly stopped when iced saline was applied to the neocortex in the operating room. Both sets of studies have encouraged the development of more convenient methods of brain cooling for epilepsy.

Methods and Recent Results

The inconvenience of cooling with circulating cold water or conventional refrigeration devices has precluded the application of cooling for the chronic therapy of epilepsy. However, improvements in thermoelectric devices and the necessary supporting technology make it essential to reevaluate cooling as a viable therapy for some forms of epilepsy. Thermoelectric devices exploit Peltier's observation in 1834 that a temperature gradient develops at the junction between two dissimilar conductors when an electric current is applied across them. The discovery in the 1920s that synthetic semiconductors were superior to metals as thermoelectric elements hastened progress in this field. The development of modern semiconductors, typically alloys of bismuth, tellurium, selenium, and antimony, has made possible the fabrication of small, light thermoelectric modules or Peltier devices. In these modules, pairs of N- and P-type semiconductors are connected electrically in series and thermally in parallel, between two ceramic plates, to form a wafer (**Figure 1a**). When current is passed, one of the plates cools almost instantly and the other heats, depending on direction of current flow. Thermoelectric devices are capable of generating temperature differentials of 70 °C, but for these differentials to result in cooling, heat must be efficiently removed from the hot side. For most of our work, we have attached the hot side of the device to a copper rod, which removes heat and acts as a convenient holder for a manipulator. These devices can be fabricated in mm dimensions.

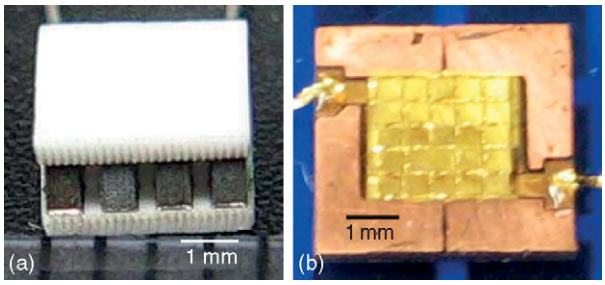


Figure 1 Photographs of thermoelectric or Peltier devices. (a) Commercially available Peltier device showing the individual semiconductors connecting the two ceramic wafers. (b) Thin film Peltier device (Nextreme) composed of 25 smaller units within a 6 mm^2 surface. These devices can be fabricated to a thickness of less than 0.2 mm.

In 1999, we became aware of the attractive features of commercially available thermoelectric devices and began experiments to determine whether they could terminate acute seizures. Initially, we induced seizures in rat hippocampal brain slices by perfusing them with 4-aminopyridine (4-AP), a blocker of voltage-gated potassium channels and a well-established convulsant. We positioned the slices on the surface of a thermoelectric device that had been machined into a plexiglass chamber. In this configuration, we were able to rapidly cool the slices at the onset of electrographic seizures. We found that cooling to 20 °C quickly terminated this paroxysmal activity and also reversibly inhibited evoked field potentials.

These positive *in vitro* results encouraged us to attempt to suppress ictal activity *in vivo*. We found that we could reliably induce focal seizures in halothane-anesthetized rats with 4-AP solution. The 4-AP was inserted 0.5 mm below the pial surface of the motor cortex using a micropipette. The animals developed recurrent focal seizures within 20 min, and continued to have electrographic seizures for approximately 2 h. Untreated seizures lasted between 60 and 80 s. When we allowed a thermoelectric device to directly contact the neocortex immediately above the injection site, and activated cooling to 20 °C at seizure onset, seizure duration was reduced to approximately 7 s (**Figure 2**).

This rapid effect is due to the local cooling, because the thermoelectric device did not influence seizure duration if it was not in direct contact with the cortical surface.

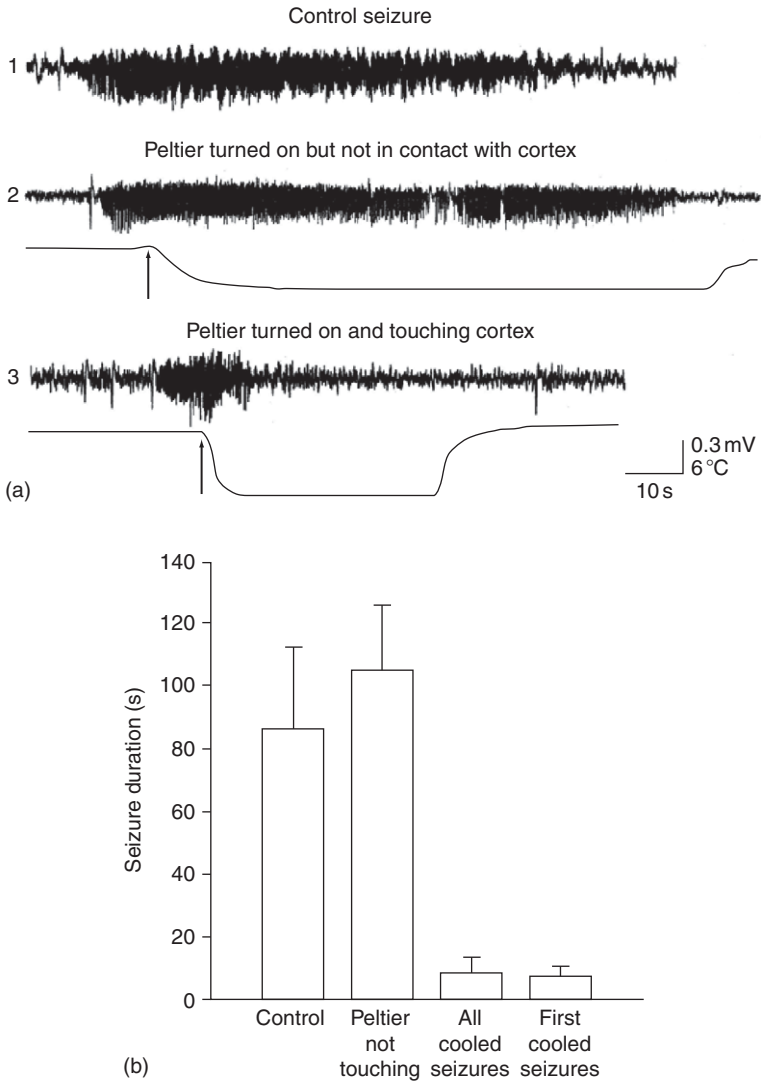


Figure 2 (a) Examples of 4-AP-induced neocortical seizures. (a1) Control seizure lasting 90 s. (a2) Activating the Peltier device did not alter the course of a 110 s seizure when the device was not in direct contact with the cortical surface. (a3) Direct cortical cooling by the Peltier device 8 s after seizure onset terminated the seizure within 9 s. The temperature reductions in a2 and a3 differ because the Peltier surface was cooled from room temperature (about 25 °C) to 20 °C in a2 and from brain surface temperature (about 34 °C) to 20 °C in a3. (b) Cortical cooling significantly reduces the duration of seizures. Cooling shortens the duration of the entire group of cooled seizures as well as the first cooled seizure in each animal. Activation of the Peltier had no effect on seizure duration when the device did not directly contact the cortex. Cooled seizure durations were significantly different from both control groups ($p < 0.001$). This figure was modified and reproduced from Yang X-F and Rothman SM (2001) Focal cooling rapidly terminates experimental neocortical seizures. *Annals of Neurology* 49: 721–726.

In this preparation, there was a progressive decrease in seizure duration below 26 °C, but no difference between 22 and 20 °C. We have not yet tried temperatures below 20 °C. We may even be overestimating the degree of cooling required for seizure termination. Another group has reported that paroxysmal discharges in a slice model of epilepsy were eliminated by rapid temperature reductions of only 1 °C (**Figure 3**).

We went on to develop a frequency-based seizure detection algorithm for the 4-AP seizures, which allowed us to use a closed-loop system to abort seizures. To minimize false positives, we set a relatively high threshold before cooling was activated. In these circumstances, our closed-loop system was as effective in terminating seizures as manual activation. Had we decided to accept more false positives, we could have initiated more rapid cooling and further reduced seizure duration.

Additional experiments have revealed other attractive aspects of cooling for focal seizures. Using a small thermocouple inserted into a 30 g needle, we mapped the cortex below our thermoelectric device and showed that cooling extends only about 4 mm below the surface. Thus, its effect should be localized to just a small region of neocortex below the pia. Recent work has explored the

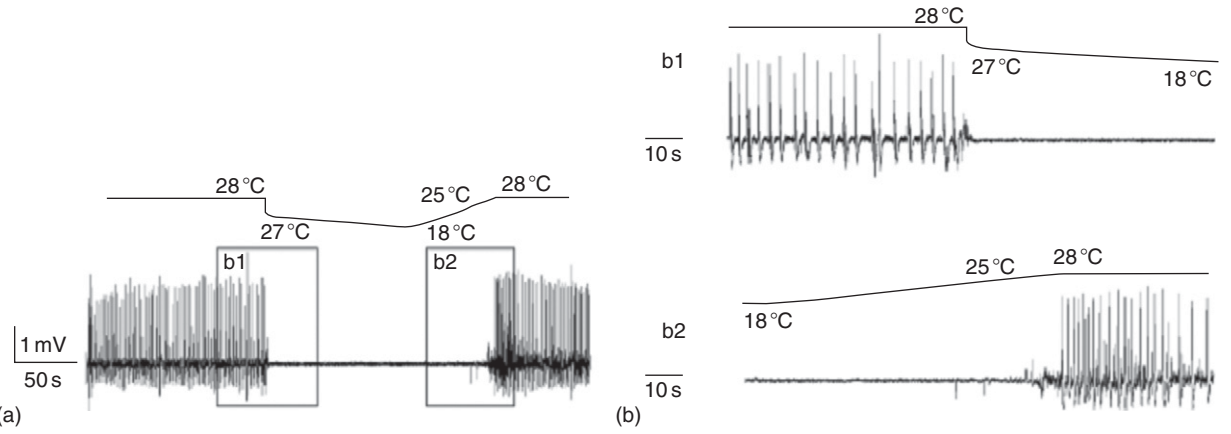


Figure 3 Seizures in hippocampal slice stop after rapid 1 C temperature reduction. (a) Slice seizure induced by 4-AP is characterized by repetitive field potentials, which disappear immediately after rapid reduction in perfusate temperature from 28 C to 27 C. (b) Faster time scale recording of seizure onset and offset. Boxes b1 and b2 in (a) correspond to traces b1 and b2. Although temperature was reduced to 18 C, the seizures disappeared at 27 C. This figure was modified and reproduced from Motamedi GK, *et al*. (2006) Termination of epileptiform activity by cooling in rat hippocampal slice epilepsy models. *Epilepsy Research* 70: 200–210.

possibility of brain damage induced by local cooling. Thus far, we have not seen any evidence that cooling as low as 5 °C for 2 h produces neuronal loss or activates apoptotic pathways. While there is minimal gliosis close to the region of cortical contact with the Peltier, it is similar to the response provoked by any foreign body and cannot be attributed to cooling. Interestingly, when brain slices obtained from transgenic mice expressing the Green Fluorescent Protein were cooled to 5 °C, there was transient, but completely reversible blebbing of dendrite shafts and loss of spines. This is probably an effect of ion pump inhibition, because other investigators have made similar observations after treating brain slices with Na^+/K^+ ATPase inhibitors. We have also failed to observe any neuronal loss in cat neocortex, after 7–10 months of intermittent cooling during neurophysiological localization experiments. These results make us optimistic that the effect of cooling will be exquisitely focal and well tolerated.

A series of more recent experiments demonstrated that cooling is capable of reducing the severity and length of other types of focal seizures. Both the grade and afterdischarge duration of kindled rat hippocampal seizures were reduced by cooling to between 23 and 26 °C with cold saline flowing through thin copper tubing positioned next to dorsal hippocampus. A limited set of observations on a monkey have verified that our thermoelectric device is capable of inhibiting function in the primate neocortex. When our thermoelectric device was placed in direct contact with the pia on the surface of the hand region of the precentral gyrus, there was a consistent, reversible impairment in skilled finger movements associated with a reduction in surface temperature. This indicates that the small thermoelectric devices have sufficient power to cool primate cortex. Of note, even with cooling to 10 °C, the impairment was not instantaneous and did not completely paralyze hand movements.

To move ahead with the design of a fully implantable cooling device for human focal epilepsy, we have begun to address the problem of heat dissipation from the hot side of thermoelectric devices. While a copper rod has worked well for experiments in anesthetized or immobilized animals, a more compact device will be required to transfer heat from a clinical device. There is an extensive literature describing heat pipes, which are hollow, evacuated, wicked tubes that rapidly equilibrate temperature by allowing a liquid to alternately evaporate and recondense under reduced atmospheric pressure. Our engineering colleagues have designed a thin, bendable, laminar heat pipe composed of outer layers of copper foil and an inner layer of sintered copper columns sandwiched between two layers of sintered copper. The charging fluid, water, flows between the copper columns. The mechanical properties of this heat pipe indicate that it should be capable of diffusing sufficient heat from a thermoelectric device in contact with the cortex to maintain a cold side temperature of 20 °C without heating adjacent brain above 38 °C. We envision positioning a similar heat pipe between the hot side of a thermoelectric device and dura, skull, or scalp, so that heat can be efficiently transferred to one of these highly vascular compartments.

Future Plans

At this point there seems to be little reason to pursue further animal work on cooling for focal seizures. The existing data already shows a robust effect of cooling in rodent models and there are no primate focal epilepsy models that have been validated for therapeutic testing.

The most critical question at this time is the degree of cooling required to terminate or prevent human focal seizures, and rodents are unlikely to inform us about this question. There is no rodent model of chronic focal epilepsy that reliably reproduces the human condition; the 4-AP model, which we have used in our studies, is much more severe than even the most refractory human epilepsy. The rats typically have 60–80 s seizures every 2–3 min, which would be unrealistic for almost all human epilepsies. Human seizures would be expected to be much less frequent. It seems likely, therefore, that rodent focal seizures will require a much larger temperature reduction than human seizures. This information is critical because the required degree of cooling will govern the current required to power the thermoelectric device. Moreover, the degree of cooling will determine the amount of heat that has to be dissipated through the heat pipe to the vasculature.

We believe that the most efficient way to sort this out will entail fabricating a cooling device that utilizes cold saline rather than a thermoelectric device. The device would combine a conventional silastic grid used for invasive monitoring and a bladder through which cold saline flows. This device would allow focal cooling of an epileptic focus in appropriate patients during monitoring, prior to surgical resection. It would permit us to determine the degree of cooling required to stop seizures without having to cope with heat dissipation. Once the cooling parameters have been better defined, it will be possible to rationally design an implantable device using thermoelectric technology.

We have recently been encouraged by improvement in the design of thermoelectric devices. It is now possible to use thin film technology developed for the microelectronics industry (Nextreme; **Figure 1b**) to fabricate cooling devices that are less than 200 μm thick and have up to ten times the heat pumping capability of conventional devices. These will be ideal for medical devices.

There are several clear obstacles and objections that need to be addressed before focal cooling can be used to treat human epilepsy. First, the convolutions of the human brain keep approximately two-thirds of the neocortex buried in sulci and inaccessible to surface cooling. However, it should still be possible to cool portions of the substantial area of exposed cortex that are responsible for seizure generation. In addition, cooling the lip of cortical sulci may block the spread of seizures arising within the sulci, a suspected mechanism of wider seizure generalization. It should even be feasible to connect thin, flexible heat pipes to thermoelectric devices to cool structures deeper in the brain. Second, cooling may be as disruptive to normal brain function as some seizures. We are optimistic that there may be a temperature range that separates seizure control from disruption of normal cortical function. The results of Bakken and colleagues that cooling below 10 °C over eloquent cortex did not produce a complete anomia or nonfluent aphasia support this hope.

Third, while we are concerned that the current requirements for present commercial thermoelectric devices exceed an ampere, the short-duty cycle may allow these devices to operate for long periods without recharge or replacement. Moreover, improvements in battery life and thin film thermoelectric design should make it possible to generate more efficient devices soon. Fourth, while there is presently no fully validated closed-loop device for seizure control, several seizure detection and prediction algorithms appear promising. Variations of these algorithms are currently undergoing trials in devices using either an external or implanted detection system linked to electrical stimulation. There is nothing about these systems that would preclude replacing stimulation with cooling as the efferent arm of the device.

Full implementation of cooling as a clinical therapy will still require further applied research. However, given the steady neurobiological progress over the last 5 years and the continuous progress in many phases of mechanical and electrical engineering, it would be surprising if some investigational application of cooling for epilepsy management was not available by the end of the decade.

Acknowledgements

This work was supported by the Alafi Family Foundation and the NIH (R01 NS42936). A part of this work has been discussed in a prior review by Rothman *et al.* (2005).

See also: **Antiepileptic Drugs**: Local Delivery of Antiepileptic Drugs; **Neurophysiology**: Seizure Detection and Prediction; **Seizures**: Seizure Prediction; **Treatments (non-Antiepileptic Drugs)**: Control of Seizure Activity with Electrical Stimulation.

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Relevant Websites

http://www.nextremethermal.com – This site describes the technology for fabricating thin thermoelectric devices that may be used in medical devices.

Gene Therapy in Epilepsy

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Introduction

The application of gene therapy has expanded tremendously in the last two decades. Since the first clinical study almost 20 years ago, gene therapy has been successfully used in very diverse fields, including genetic diseases, metabolic disorders, and more recently neurological diseases. Theoretically, gene therapy can be applied to any disease for which the cascade of pathophysiological events has been identified. Indeed, gene therapy has proved beneficial in simple genetic as well as complex acquired disorders, as this approach enables either overexpression or knockdown (using interfering RNA) of genes within a pathological network. However, some technical constraints remain, and new gene therapy vectors with better efficiency and specificity have been developed to overcome these limitations and widen the field of application. The outcomes of recent clinical trials are very promising and suggest that gene therapy could be beneficially applied for several neurological disorders.

There is a significant unmet need for new therapeutic approaches in epilepsy. About one-third of epileptic patients suffer from pharmacoresistant seizures – despite the development of new antiepileptic drugs. For many of these patients, particularly those with temporal lobe epilepsy, surgical resection is often the only effective therapeutic alternative. Moreover, antiepileptic drugs do not prevent the progression of the disease, and seizure management is often synonymous with lifelong pharmacological treatment, with side-effects that can be debilitating and the risk of increasing refractoriness over time. Some exciting preclinical studies, using experimental animal seizure and epilepsy models, and human data from early stage trials for other neurological disorders, suggest that gene therapy is now ready for translation to the epilepsy clinic.

Background

The majority of epilepsies are complex acquired disorders with both genetic and environmental contributions. Although initial forays into gene therapy targeted simple genetic disorders – for example cystic fibrosis and hemophilia – the early disappointing results suggested that genetic disorders are not necessarily the ideal indications. For the majority of genetic disorders, mutations are found throughout the body, and typically it is not a single cell population or tissue that needs correction in order to reverse the disease phenotype. Moreover, the efficient transduction of both multiple cell types and/or organs is problematic; further, systemic gene therapies, particularly those involving viral vectors, have often elicited significant immune responses, thus limiting the efficacy of the approach.

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In the field of seizure disorders, an increasing number of causative genetic mutations have been identified in familial epilepsies, with the majority resulting from polymorphisms in ion channels. However, pure monogenic epilepsies are relatively rare. Further, it is often not clear which cells (within the CNS) harboring the mutation(s) are most critical in inducing the epilepsy phenotype. The situation is further complicated by compensatory mechanisms that cloud the link between a genetic mutation and the hyperexcitability phenotype. Finally, it is very difficult to design an effective gene therapy intervention for a disease that involves a large area of the brain, simply because of the technical limitations of achieving widespread gene transfer. For these reasons, genetic forms of epilepsy are amongst the most challenging and have not been the initial target for development of gene therapy-based treatments.

Focal epilepsies, and in particular temporal lobe epilepsy, are more ideal candidates for gene therapy. The pathophysiology of temporal lobe epilepsy has been well studied in animal models, as well as in the analysis of surgically resected tissue, and several candidate genes show promise as potential therapeutic targets. Furthermore, the epileptogenic area can be well defined by imaging and physiological recording techniques. Gene therapy allows specific targeting of the epileptogenic region, thus sparing the surrounding healthy tissue and minimizing side effects that often go hand in hand with small molecule antiepileptic drug treatment.

Methods – Gene Delivery to the CNS

Delivery of nucleic acids to the brain is a technical challenge because of the presence of the blood–brain barrier, which limits access to the CNS of peripherally administered drugs or biological agents. Intranasal administration of DNA, using different vectors, has been attempted; however, the resulting level of brain transgene expression obtained with this technique is very limited. Furthermore, any systemic delivery method is not appropriate for therapies in which transduction of only a focal region of the brain is required, since current vectors typically are not able to target selected subpopulations of cells when delivered systemically.

An invasive approach, specifically local injection via stereotactic surgery, is a far more efficient method to deliver a therapeutic gene to a specific area of the brain. Moreover, with the current generation vectors, a relatively low number of virions (vector particles) can be injected and typically do not induce an immune or toxic or inflammatory response. In addition, very high levels of transgene expression in the vicinity of the injection can be achieved by this route. To date, this approach is by far the most commonly used method of gene delivery to the brain, and the only approach used in current neurological gene therapy clinical trials. A major advantage of direct in vivo gene transfer to the brain is the limited immune response induced after intraparenchymal delivery. The CNS is largely devoid of antigen presenting cells, with only a very limited lymphatic system present. However, invasive surgery induces a breach of the blood–brain barrier and the potential penetration of large molecules and even cells, including activated lymphocytes. Therefore, the notion that the brain is immunologically privileged has been somewhat reevaluated; while the immune response observed in the brain is generally less robust or functionally important than in other peripheral organs, it remains an important factor in the choice and design of the technique for gene transfer. Other essential factors to be considered are the efficiency of gene delivery, the level and stability of transgene expression, and the ability to regulate transgene expression. Different techniques have been used to express a gene in a specific region of the brain, including cells transplantation in an ex vivo approach (fetal cells, immortalized cells, fibroblasts), nonviral vector delivery (e.g., liposomes and polymers), and viral vectors. Of the latter, the most commonly used in the CNS include herpes simplex virus, lentivirus, adenovirus, and adeno-associated virus (AAV).

Nonviral, lipid, and/or polymer-based systems of gene delivery are the simplest technique for gene delivery. Their main advantages are their high loading capacity, low immunogenicity, and their ability to transfect nondividing cells. However, gene expression is inefficient and transient, and these systems are not yet sufficiently developed for effective clinical translation in neurology. Similarly, nonviral delivery of naked DNA is not currently feasible because of the low efficiency of transfection and the high level of immune response.

The two main techniques used for gene therapy in clinical applications are ex vivo gene transfer involving transplantation of the transduced cells and direct in vivo cellular transduction by viral vectors. Cell transplantation approaches currently emphasize the use of stem cells, typically embryonic stem cells (ES) or adult stem cells. Their main advantage is the high compatibility of the transplant with the host. Additionally, ES cells are pluripotent and can differentiate into either glia or different neuronal phenotypes. In vitro, these cells can be transfected to express a protein of interest. However, controversy over the use of human ES cells (which requires destruction of the embryo), has limited their use in the clinic. The development of porcine fetal tissue as xenograft material has been proposed to overcome the limitation of stem cells availability. Xenotransplants have been made in patients with neurodegenerative diseases. Studies suggest that the graft may survive and even successfully develop synaptic contacts with host cells, but the use of this approach is still limited because it carries additional risk of infection due to animal pathogens – and the

probability that the graft will be rejected is increased compared to allogeneic grafts.

Viral vectors are currently the most promising tools to directly introduce a gene into the brain, with the current emphasis on herpes simplex virus (HSV), adenovirus, lentivirus, and AAV vectors. Retroviruses are not applicable for the adult brain and differentiated neuronal cells because they require the cell to undergo mitosis for integration and expression. Furthermore, the use of retroviruses in gene therapy has raised safety issues due to the possibility of insertional mutagenesis. HSV, AAV, and lentiviruses (typically HIV, EIAV (equine infectious anemia virus), or FIV (feline immunodeficiency virus)) transduce both dividing and nondividing cells, and are therefore suitable vectors for gene therapy of neurological disorders. Each of these vector systems has distinct advantages and limitations for CNS applications and potential clinical translation. These advantages and limitations have been extensively reviewed elsewhere (see Further Reading).

Our lab has focused on the use of recombinant AAV vectors (rAAV) due to the lack of induction of a cellular immune response and the high level of transgene expression that can now be routinely achieved. AAV is a nonpathogenic single stranded DNA virus that belongs to the parvovirus family. In the absence of a helper virus that provides the genes necessary for its replication, AAV infects the cell; following transport and uncoating, the genome remains stable (as an episome) essentially for the life of that cell. In this form, AAV is nondeleterious for the host cell and gene expression can persist stably (**Figure 1**).

Recombinant AAV (rAAV) are obtained by insertion of a transgene cassette between the inverted terminal repeats sequences (ITR). Further development of the vector is still needed, including improvement of expression cassettes, which have a packaging limit of approximately 4.7 kb, and the characterization of cell-specific promoters to express in a restricted subset of cells. A large number of AAV serotypes have now been isolated from humans and nonhuman primates, and many of those that have been characterized so far display differing tropism for various neuronal types and brain areas. The rAAV vectors that have been characterized to date have strong neuronal tropism, and are therefore not optimal for gene therapy of disorders requiring transduction of glial cells. High throughput functional screening was developed for more recently isolated serotypes, which have been tested for their tissue tropism and gene transfer potency. Several serotypes are raising interest. Amongst them, AAV9 displays a broad tropism and shows a higher transfection efficiency than the other serotypes in the different tissues tested, making it an interesting vector for systemic disorders. AAV2 was the first vector used and remains the vector of choice for most neurological indications. This serotype displays a very good neuronal tropism, negligible diffusion, and no local or diffuse toxicity. AAV1 also has strong neuronal tropism and no toxicity, with the advantage of a higher transduction efficiency. In our hands, these two serotypes (AAV1 and AAV2) remain the most appropriate vectors for current and future therapeutic applications.

Results

Experimental Studies

Experimental animal models of epilepsy have been used to assess several alternative gene therapy approaches.

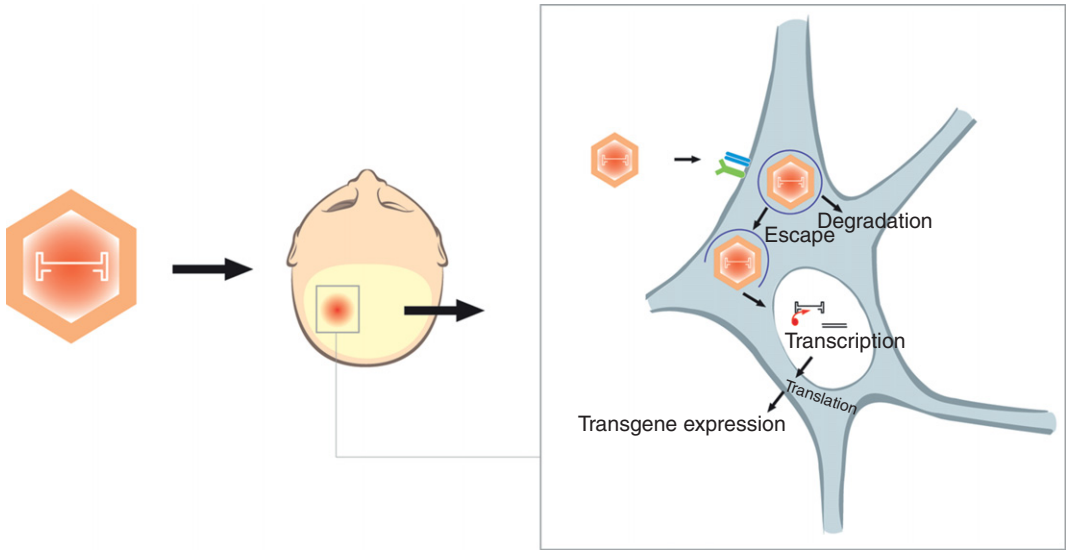


Figure 1 Schematic illustration of rAAV transduction into the host cell in gene therapy. rAAV binds to heparan sulphate proteoglycans (with integrins or fibroblast growth factor as coreceptors), and is endocytosed. A portion of rAAV escapes the endosomal compartment and translocates to the nucleus. Second strand synthesis in the nucleus provides a stable double strand DNA template for transcription and leads to the expression of the transgene.

Genetic strategies to enhance GABA-mediated inhibition has been an early strategy, based on pharmacologically validated data showing that an increase in GABAergic neurotransmission in the epileptogenic area increases the threshold for excessive neuronal excitability, hence decreasing seizure occurrence. Different techniques of in vitro or in vivo transfection of glutamic acid decarboxylase (GAD, the rate limiting enzyme that catalyzes the synthesis of GABA), have been used to increase GABA levels in the tissue of interest. Transplantation of fetal GABAergic neurons into the *substantia nigra* (SN), a structure involved in the propagation of seizures, induces transient decrease in seizure severity in the kindling model. Similarly, transplantation of engineered mouse cortical neurons and glia expressing GAD65 into the SN or piriform cortex shows an anticonvulsant effect. Viral vector-based approaches have also been used to directly express GAD in cultured rat hippocampal neurons. The different techniques used in these studies were able to induce the expression of exogenous GAD in the target cells and thereby increase GABA production and release. However, the expression obtained with ex vivo transduction and subsequent cell transplantation was only transient in some studies. In addition, the effects observed were largely the consequence of a local increase of GABA levels from the transplanted cells – which therefore appear to act simply as biological GABA pumps. A complementary approach has been to express GABA(A) receptor subunits, specifically the alpha1 subunit; this study showed therapeutic efficacy in a rat pilocarpine model. However, the effect of a strategy that targets cell populations non-specifically are difficult to predict. Indeed, in the hippocampus, the hyperexcitability occurring in epilepsy is likely due (at least in part) to the reorganization of neuronal circuits – including the loss of a specific population of interneurons and feedback inhibition. Conversely, some interneurons are preserved under these epileptic conditions, and are believed to underlie network synchrony. Transduction (and auto-inhibition) of these two different systems could have dramatically opposite effects on epileptic seizures. This complexity emphasizes the importance of both promoter choice and vector tropism. More importantly, these considerations emphasize that for conditions like epilepsy, vectors may need to be engineered to transduce a specific cell population.

Over the past decade, the modulatory roles of neuropeptide Y (NPY) and galanin, as well as the action of adenosine, have been well established. The observation that epileptic seizures induce the release of these neuropeptides and nucleoside led to the hypothesis that they play an important role in arresting epileptic activity. Experimental studies further confirmed their anticonvulsant and neuroprotective roles, and suggested that these neuropeptides and adenosine, and their receptors, constitute an endogenous system to control epileptic activity. These systems thus appear a promising target for the development of new therapeutics – and in particular may be excellent targets for gene therapy.

Several experimental studies showed that galanin is released during epileptic seizures and has an inhibitory effect on neuronal activity through presynaptic inhibition of glutamatergic transmission, as well as a strong neuroprotective effect. Administration of galanin or nonpeptide ligands for the galanin receptor also induces a robust anticonvulsant effect in animal models of limbic seizures. In a study from our laboratory (see Further Reading), a recombinant adeno-associated viral (AAV) vector, constitutively overexpressing galanin, was injected into the rat hippocampus. Kainic acid-induced seizure activity was significantly decreased, confirming the antiepileptic effect of galanin in vivo. Interestingly, administration of rAAV-galanin resulted not only in long lasting expression of galanin, but also in the transport of the neuropeptide along the axonal arborization. rAAV-galanin has also been demonstrated to suppress acute seizures activity induced by electrical stimulation in rats and had a neuroprotective effect on hilar cells.

The inhibitory neuromodulator adenosine has also raised interest as an endogenous anticonvulsant. Decreased adenosine levels have been observed in different models of epileptogenesis and epileptic activity. Interestingly, adenosine has also been shown to restrict the site of epileptogenesis. An experimental approach using an ex vivo gene therapy based on transplantation of cells engineered to release adenosine, has shown that secreted adenosine protects from seizures in the kindling model.

In several animal models of epilepsy, seizure-induced increases of NPY mRNA and protein have been observed in the dentate gyrus of the hippocampus, suggesting a modulatory role of the neuropeptide on neuronal excitability. This role was confirmed by in vitro data showing that application of NPY to hippocampal slices reduces glutamatergic synaptic excitation; in vivo studies have also shown a strong anticonvulsant effect of NPY mediated by the Y2 and Y5 receptors. In addition, NPY knockout mice develop spontaneous epileptic seizures, confirming the importance of NPY in controlling neuronal excitability. In human tissue from temporal lobe resection, NPY-mediated neurotransmission is altered by seizures, and the modulatory role of NPY on epileptic activity has also been validated on hippocampal slices. The effect of chronic overexpression of NPY in the hippocampus, mediated by gene therapy was examined in the kainic acid model in rats. rAAV-mediated gene transfer of NPY (injections into the hippocampus) delayed seizure onset and dramatically decreased the occurrence of epileptic seizures. Moreover, in a progressive spontaneous seizure model, the AAV-NPY vector was able to significantly reduce the number of seizures and stop progression, suggesting a potential anti-epileptogenic effect.

Recent Results in Clinical Trials and Future Developments

Clinical experience with gene therapy to date has largely been for rapidly fatal disorders. These studies, for the most part, have not surprisingly shown only limited efficacy, although the tide is shifting and increasingly gene therapy trials are showing significant benefit. Notably, in contrast to its reputation as a high risk intervention, gene therapy has had minimal adverse events compared to other breakthrough technologies, and appears safer than alternative surgical approaches. Published data on neurological gene therapy trials is limited, but appears promising, particularly in Parkinson's disease.

There has been only one clinical genetic intervention study for epilepsy. In this first ex vivo gene therapy trial for epilepsy, surgeons implanted a xenograft of GABA expressing cells in a patient candidate for temporal lobe resection. This procedure failed to show an antiepileptic effect. Several issues arise from the use of transplants in neurological diseases. In addition to the limited availability of some cells and tissues (e.g., embryonic stem cells), the survival of the graft is variable. Experimental results in animal models also tend to show a limited survival time of cell transplants in the epileptic brain. Moreover, transformed cells have oncogenic potential, and large tumors can result. Furthermore, the temporal lobe is a highly heterogeneous structure, organized into complex layers. The type of synaptic connections that a graft would develop in this multisynaptic circuit is unknown. In addition, the transplanted cells would be subject to recurrent hyperexcitability in the epileptogenic area, which may affect the survival of these cells. Data obtained from experimental animal studies suggest that a direct in vivo viral vector approach is a more feasible strategy for clinical trials. As indicated above, the most established vector system for in vivo gene transfer in mammalian brain is that of AAV. Indeed, gene transfer into cells in human hippocampal slices (obtained from temporal lobectomy cases), using an AAV vector, showed an efficient level of cell transduction in epileptic tissue.

The most comprehensive in vivo gene therapy trial for neurological disease to date involved Parkinsonian subjects (see our 2007 *Lancet* study in Further Reading). The infusion of AAV vectors expressing GAD65 and GAD67 in the subthalamic nucleus induced a significant and persistent improvement in motor symptoms. None of the patients developed any immune response against AAV or the transgene GAD, and no adverse events relating to the gene therapy was observed. In addition, biodistribution studies have shown that the transgene expression is limited to the structure injected, with negligible spread into the surrounded structures. A second study has also recently been published (see 2008 *Lancet Neurology* paper in Further Reading) in which the authors again used AAV, but a different transgene, the growth factor neurturin, delivered to the putamen and caudate. This study showed also safety and tolerability, with significant improvements in clinical outcome.

In contrast to neurodegenerative disorders in which attempts to replace or regenerate lost cell populations have been a common theme, in epilepsy the emphasis has been to disrupt the abnormal epileptic activity and restore normal inhibition. The most established approach, and the most consistent experimental data, have been obtained through modulation of endogenous inhibitory peptidergic signaling (i.e., with galanin and NPY), as well as using the nucleoside, adenosine. These strategies are most likely translate to clinical trial in the near future. Indeed, we submitted an AAV-NPY based clinical trial to the Recombinant DNA Advisory Committee of the USA (www4.od.nih.gov/oba/RAC/meetings/Sept2004/RACagenda092304.pdf) and have subsequently filed an IND to the US FDA for a Phase I trial.

Conclusions and Future Directions

In conclusion, the experimental and clinical data obtained from other neurological diseases have shown the feasibility of gene therapy for epilepsy. Subjects who are good candidates for temporal lobectomy are an ideal population, since the gene transfer would occur in the brain region which has been planned for resection, providing a built-in rescue procedure if the gene therapy was ineffective or associated with significant adverse events. The relatively low risk associated with gene therapy, and the promising preclinical data on both NPY and galanin gene transfer in experimental animal models, suggest that temporal lobe epilepsy is an ideal context for clinical testing.

Gene therapy for neurological diseases is still at an early stage. It will require more safety data as well as technical advances to consider gene therapy as a therapy for a larger population of epileptic patients. Viral vectors are undergoing further developments to restrict gene transduction to a cell subpopulation and allow a better control of gene expression. Cell type-specific promoters, conditional expression with inducible promoters, and insertion of a larger expression cassette, are all features of these vectors now under development. The diffusion of the vector (in particular through retrograde transport) is also an important factor to take in account.

In the NPY clinical trial, the approach is that of potentiating an endogenous system of seizure modulation. This approach is worth further analysis and development, inasmuch as it may induce less compensatory effects, and be more efficient than trying to compensate for a loss of a specific neuronal population, in particular, in a sclerotic hippocampus that has encountered multiple plasticity phenomena and reorganization.

The goal of gene therapy for epilepsy is to obtain not only a sustained anticonvulsant effect, but also an antiepileptogenic effect that will block the progression of the disease, maintain focalization of the epileptic zone, and/or stop secondary generalization.

Gene therapy offers a variety of tools to target the disease at different levels, from expression of inhibitory neuropeptides or neuromodulators to antiapoptotic genes, to neurotrophins and receptors channels. With such a comprehensive approach, there is a strong likelihood of achieving a robust therapeutic effect. To conclude, experimental and clinical data suggest that gene therapy is likely to have a significant impact on epilepsy management in the coming decade.

See also: **Inflammation**: Inflammatory Responses and Cytokines; **Neurotrophic Factors**: Fibroblast Growth Factor-2; GDNF: A Neurotrophic Factor Against Epilepsy; **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides; Viral Vector Approaches for Antiepileptic Gene Therapy.

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Ginseng and Other Herbal Treatments for Epilepsy

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Introduction

Although a large number of anticonvulsant drugs are available to treat those with epilepsy, a significant proportion of patients with epilepsy are refractory to treatment. In addition, a significant number of patients suffer from side effects from the drugs used to treat their seizures. Thus, there is a continued need to develop more effective and safer drugs for the treatment of epilepsy.

Plants have provided an important source of medicine. In fact, the availability of plant products, such as quinine for the treatment of malaria, has played an important role in the history of mankind. There are a number of traditional systems of medicine, such as traditional Chinese medicine, Ayurveda (Indian), Tibetan medicine, unani-tibb (Greco-Arabic), and Kampo (Japanese), that have long histories of effective use of herbal preparations. In addition, various native peoples, in the Americas and elsewhere, have a history of using plant material to effectively treat symptoms of disease. Although these traditional medicines are considered to have efficacy, the scientific basis of that efficacy is not understood. As science evolved and the field of medicine emerged, investigations into herbal remedies attempted to demonstrate efficacy in controlled trials and to identify the active components. As more work has been done with these traditional medicines, it has become apparent that there are a number of active ingredients in plants. A number of prescription medications currently in use in the United States are isolated from plants or derived from plant components. In fact, plants are the basis of at least 30% of the drugs available today. However, the use of herbal extracts is not generally accepted by Western medicine.

Our understanding of herbal products has been limited by the fact that there are often multiple active ingredients in one species of plant. Some of the components may have beneficial effects and some may be toxic. There has rarely been standardization of the preparation of extracts

The goal of gene therapy for epilepsy is to obtain not only a sustained anticonvulsant effect, but also an antiepileptogenic effect that will block the progression of the disease, maintain focalization of the epileptic zone, and/or stop secondary generalization.

Gene therapy offers a variety of tools to target the disease at different levels, from expression of inhibitory neuropeptides or neuromodulators to antiapoptotic genes, to neurotrophins and receptors channels. With such a comprehensive approach, there is a strong likelihood of achieving a robust therapeutic effect. To conclude, experimental and clinical data suggest that gene therapy is likely to have a significant impact on epilepsy management in the coming decade.

See also: **Inflammation**: Inflammatory Responses and Cytokines; **Neurotrophic Factors**: Fibroblast Growth Factor-2; GDNF: A Neurotrophic Factor Against Epilepsy; **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides; Viral Vector Approaches for Antiepileptic Gene Therapy.

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from these plants. In addition, the concentrations of the active components in these plants may vary, depending on the growing conditions (including weather and soil type) and which part of the plant is used (roots compared with leaves and stems or seeds or berries) to prepare the extract. Finally, clinical or animal studies to demonstrate effectiveness have not always utilized the same preparation. Thus, herbal products have struggled to gain acceptance in modern medical practice.

In 2004, the Food and Drug Administration (FDA) in the United States developed specific guidelines for the development of botanical products. These guidelines take into account the fact that there is often a long history of human use of a particular plant. For legally available products with no known safety issues, there is a reduced requirement for preclinical safety data and reduced documentation of the chemistry, manufacturing, and controls needed to support an Investigational New Drug (IND) application for initial clinical studies. In fact, the FDA does not require identification of all of the constituents in a botanical product for an IND application. There is, however, a requirement for controls that will ensure the identity, purity, quality, potency, and consistency of the botanical product.

For the purposes of the FDA guidelines, a botanical product is a labeled product that contains vegetable matter as ingredients. There are specific definitions for whether a botanical product is considered a food, a dietary supplement, and/or a drug. A botanical product is considered a drug if it is intended for use in diagnosing, mitigating, treating, curing, or preventing disease – as it would be if it were marketed as an anticonvulsant or antiepileptic agent. As a drug, it is subject to different regulations than if the botanical product were to be considered a dietary supplement.

Background

Over 50 plants, or plant products, have been reported to have anticonvulsant activity. Most of these products have been reported to have efficacy in in vivo animal models, but a few have been shown to have activity only in in vitro systems/models. Of these products, about one-third has been analyzed to determine the active components. The structure of the active component has been determined in 80% of those plants in which the active compound has been isolated. As can be seen in **Figure 1**, a wide variety of these structures have activity in the central nervous system, from fairly simple compounds to steroidal saponins to quite complex compounds. Thus, more than a single class of compounds produced in plants has activity, and research on the activity of these compounds needs to be comprehensive.

To date, none of the currently available anticonvulsant drugs was derived from a plant product or derived from a traditional medical system. Huperzine A, a cholinesterase inhibitor used in traditional Chinese medicine for dementia, is moving towards clinical trials as an anticonvulsant.

Methods

Any of the currently available models of seizures or epilepsy could be used to test the efficacy of herbal products. However, if one wants to evaluate the antiepileptic effects of a plant product that is used in traditional medicine somewhere in the world, then one would like it to be effective in an in vivo model. Although an in vitro model (such as induced epileptiform activity in a hippocampal slice) might be a faster way to screen a large number of extracts or isolated components, these preparations have significant limitations with respect to interpreting the in vitro effects. If an extract or herbal product has activity in an in vivo model, then it must be able to cross the blood–brain barrier (not the case for an in vitro model); if an active metabolite is important for the activity of the product/extract, then it may only have efficacy in the whole animal. An in vivo model will not distinguish between an effect on seizure spread or focal seizure initiation, but drugs acting on either will have antiepileptic efficacy.

Within the in vivo models of seizures and epilepsy, one could use either an acute or chronic model of seizures. A chronic model of spontaneous seizures most closely mimics the clinical situation, but requires long-term monitoring. In contrast, an acute model has the advantage that the seizures will occur at a predetermined time, as elicited with electrical stimulation or with chemical convulsants. Normally, with electrical stimulation, the animal receives a stimulus train to determine the seizure threshold and then a suprathreshold stimulation is used to repeatedly elicit seizures for drug testing. With chemical convulsants (pilocarpine, kainic acid, etc.), the seizure onset is more gradual and it is possible to determine whether a drug alters the early processes involved in seizure initiation. Using electrical stimulation or infusion of pentylenetetrazol, it is possible to test the effect of drugs on the seizure threshold.

The National Institutes of Health (NIH) has an anticonvulsant screening program that was designed to encourage and facilitate the discovery of new therapeutic agents for the treatment of seizure disorders. Evaluation of a submitted compound begins with a comparison of the compound's structure with stru ctures of other compounds in the NIH database (over 21 000 compounds). Compounds are then screened in a variety of accepted animal models of seizures. Herbal products can be submitted to this program for testing in these animal models. The program will test compounds that consist of complex mixtures of constituents, as well as isolated/purified

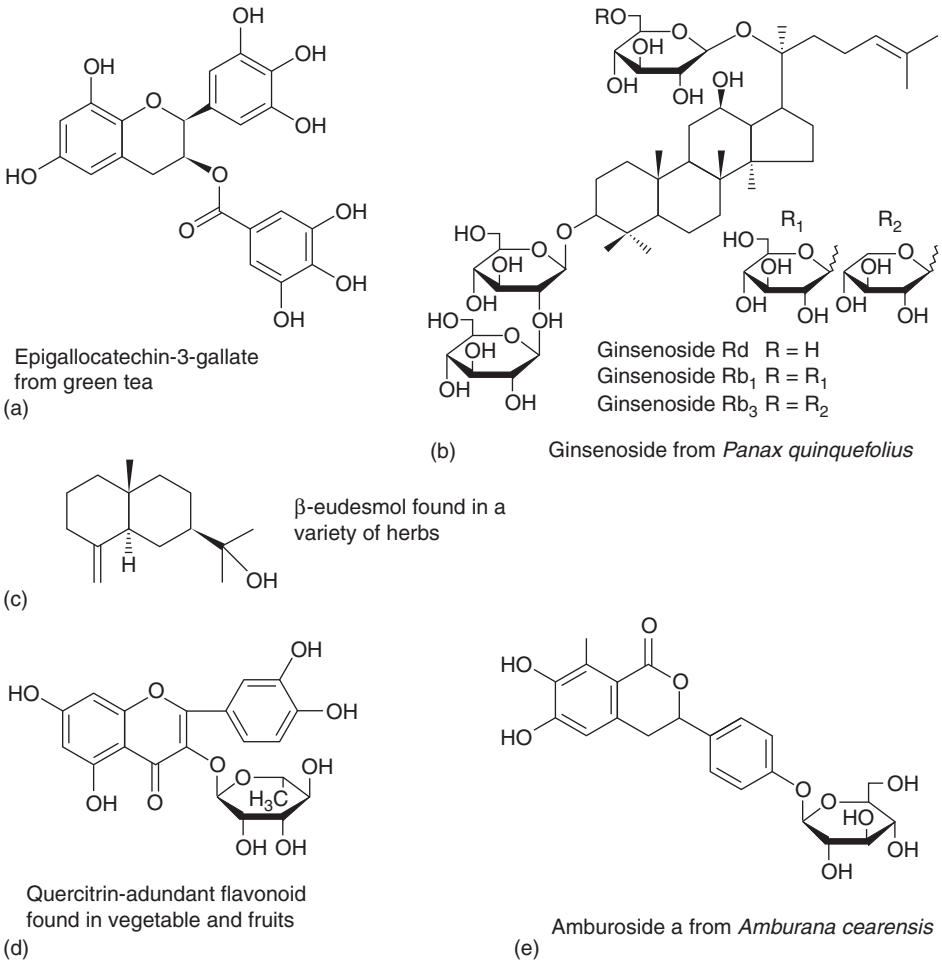


Figure 1 (a) The structure of EGCG, a polyphenol found in Green Tea. EGCG has been shown to have anxiolytic activity, antioxidant activity and an interaction with $GABA_A$ receptors. (b) The structure of three of the active components (ginsenosides) in ginseng that have been shown to have anticonvulsant activity. (c) A sesquiterpenol found in a variety of Chinese herbs. β-Eudesmol has been shown to reduce convulsions induced in mice. (d) The structure of the glycoside form of quercetin, a flavonoid from vegetables and fruits that has been shown to have antioxidant properties. (e) The structure of a compound isolated from the trunk-bark of *Amburana cearensis*, a tree common to the Brazilian northeastern savannah, which has been shown to have neuroprotective activity.

constituents. The results are returned to the investigator and the investigator can make a decision about whether to proceed further with drug development.

Recent Results

One herbal product that has been reported to have anticonvulsant activity is ginseng. The results from animal testing of ginseng will be used here as an example of the process involved in testing an herbal product. There are seven major species of ginseng, but the three most commonly used in traditional medicines are *Panax ginseng* (Asian), *P. quinquefolius* (American), and *P. japonicus* (Japanese). In traditional Chinese medicine, Asian ginseng is used for shock, syncope, shortness of breath, and weak pulse; American ginseng is used for mild depression, chronic stress, age-related memory loss, and cloudy thinking. Native Americans have used American ginseng as a bitter tonic and for nervous afflictions. The biological activity in ginseng is thought to be due to the presence of saponins (**Figure 1**) called ginsenosides. Numerous pharmacological effects have been attributed to ginsenosides, both in the central nervous system and peripherally. Few clinical trials have been carried out for any species of ginseng.

Over 28 ginsenosides have been isolated from all major species of ginseng. These ginsenosides have been divided into two groups, based on structure: the panaxadiols (Rb_1, Rb_2, Rb_3, Rc, Rd, Rg_3, Rh_2, and Rh_3) and the panaxatriols (Re, Rf, Rg_1, Rg_2, and Rh_1). Although the root is most commonly used, the leaves and stems of the ginseng plant also contain ginsenosides. According to traditional Chinese medicine, Asian ginseng nourishes the *yin* and *qi* and

is energetically 'hot,' while American ginseng is believed to be 'cooler.' Interestingly, American ginseng generally contains a lower Rg_1/Rb_1 ratio than does Asian ginseng, and this difference is thought to be the basis of the difference in the 'hotter' and 'cooler' nature of the two species. That ginseng may have anticonvulsant activity was suggested in 2002. It was reported that the kainic acid-induced seizures in rats were shorter when the animals were pretreated with a mixture of ginsenosides from Asian ginseng. In addition, some of the ginsenosides have been reported to have neuroprotective activity.

Although knowledge about a product's mechanism of action in the central nervous system is not needed to develop a drug for clinical use, understanding the action would enhance development of related drugs. However, relatively little is known about how the ginsenosides might work as either neuroprotective or anticonvulsant agents. Ginsenosides are amphiphilic in nature and have the ability to intercalate into the plasma membrane, leading to changes in membrane fluidity. This property could alter the function of the membrane itself and also alter the function of receptors and other proteins within that membrane. There is also some evidence of direct interactions of ginsenosides with membrane bound steroid receptors. However, the most accepted theory is that at least some of the ginsenosides have antioxidant activity and the ability to scavenge free radicals. How an antioxidant would be anticonvulsant is not known. There could also be actions of the ginsenosides that have not yet been discovered.

For the initial study of the anticonvulsant properties of ginseng, both American and Asian ginseng varieties were considered. However, the relatively higher concentration of the panaxadiols (Rb_1 and relatives) in American ginseng suggested that this species might contain more of the anticonvulsant compound(s). An analysis determined that an extract from the root of Asian or American ginsengs contained 30.2% ginsenosides; extract from the leaves and stems contained 85% ginsenosides. These preparations were tested in animals given either pilocarpine (300 mg kg^{-1} i.p., preceded by methylscopolamine 1 mg kg^{-1} subcutaneous) or pentylenetetrazol (50 mg kg^{-1} i.p.). When status epilepticus was induced with pilocarpine, administration of these extracts actually increased mortality; it appeared that the root extract (30% ginsenosides) was more toxic than the extract prepared from the leaves and stems (85% ginsenosides). This observation suggested that some of the non-ginsenosides in the root extract are toxic. The study also led to the recommendation that persons with epilepsy should not take any of the available ginseng products available as food supplements in the United States, since they are all prepared from ginseng root.

Although the results with the extract from the leaves and stems were somewhat variable, it appeared that there might be some anticonvulsant activity in this preparation. These results, plus some data in the literature suggesting a neuroprotective action of the panaxadiols, led to the hypothesis that the panaxadiols (Rb_1 and relatives) might contain the anticonvulsant activity. Therefore, a partial purification of ginseng, to concentrate the Rb ginsenosides, was carried out using leaves/stems from American ginseng. The product of this partial purification, called the Rb extract, was then compared with the root preparation and leaves/stems preparation (also from American ginseng). Doses of the whole root and leaves/stems preparations were calculated to provide equivalent doses of the ginsenosides in the Rb extract. The Rb extract had a dose-dependent anticonvulsant effect – increasing the latency to the seizures, decreasing the seizure score, and shortening the seizure duration. In addition, it reduced weight loss and subsequent neuronal damage after pilocarpine, and reduced mortality after pentylenetetrazol. In contrast, the root extract worsened seizures (as indicated by the decrease in latency to seizure onset and mortality), while the extract from the leaves and stems had relatively little effect (did not worsen seizures, but also did not reduce their severity). The Rb extract was also tested for effectiveness against kainic acid-induced seizures. The extract significantly increased the latency to seizures and decreased the seizure score in a dose-dependent fashion. These results suggest that some ginsenosides are anticonvulsant, but that other components of the whole extract from roots or leaves/stems are either proconvulsant or contain components that neutralize the anticonvulsant activity (**Table 1**).

The obvious next step was to determine which of the components in the Rb extract has anticonvulsant activity. The Rb extract contains 24.8% Rb_1, 46.4% Rb_3, and 13.1% Rd. The anticonvulsant activity of these individual ginsenosides was tested in the same animal models and using the same measured parameters. Doses of individual ginsenosides were calculated to provide an equivalent dose to the 20 mg kg^{-1} of the Rb extract. Using purified ginsenosides, a mixture was also prepared that matched the relative concentrations of the ginsenosides in the Rb extract. This mixture was used to determine that these three ginsenosides are the active components in the Rb extract. Pretreatment with Rb_1, Rb_3, Rd, or the mixture significantly increased the latency to the first wet dog shake induced by kainic acid. All the ginseng products appeared to reduce the seizure score after administration of kainic acid and pilocarpine, but this decrease did not reach statistical significance. Only the mixture of purified ginsenosides and the Rb fraction significantly reduced the seizure score. The same trend held for the seizures induced by pentylenetetrazol; the purified ginsenosides caused a modest increase in the latency to the seizures (which did not reach statistical significance). Only the mixture and the Rb extract produced a significant increase in the latency and duration of the seizure activity.

Table 1 Activity of various preparation from American ginseng

	Pilocarpine		*PTZ*		*Kainic acid*	
	Latency	*Severity*	*Latency*	*Duration*	*Latency*	*Severity*
Root	–	–	–	–	n.t.	n.t.
Leaves/stems	–	+	+	+	n.t.	n.t.
Rb extract	++	++	++	++	++	++
Rb_1	–	+	–	+	++	+
Rb_3	–	+	+	+	++	+
Rd	–	+	+	+	++	+

– = no effect; + = suggestion of anticonvulsant activity, but not statistically significant; ++ = statistically significant anticonvulsant effect; n.t. = not tested.

These data suggest that the individual ginsenosides have some anticonvulsant activity, but since a dose–response curve was not done with each of the individual ginsenosides, it is not known whether a single ginsenoside has equivalent efficacy to the Rb extract. The mixture of ginsenosides suggests that the activity of the individual ginsenosides is additive – producing an effective product.

The Rb extract was also submitted to the NIH anticonvulsant screening program for testing. It was tested in three animal models of seizures – subcutaneous pentylenetetrazol (50 mg kg^{-1}), 6 Hz stimulation at two stimulus amplitudes, and kindling. The Rb extract was not considered effective in these models. However, the animals were only evaluated for the presence or absence of seizures; no delay in seizure onset or changes in seizure severity were specifically measured.

Because of the long history of safe use of ginseng in humans, a preparation of root extract would need little additional safety data before a clinical trial could be started. However, the data suggest that the root extract might actually be harmful. The animal studies show that ginseng is a mixture of active compounds, include one group with anticonvulsant activity and one group that appears unsafe when the animal has seizures. When a partial purification is done to remove the potentially harmful components and concentrate the anticonvulsant components, the anticonvulsant potential looks quite promising. Either a defined extract, such as the Rb extract, or purified individual ginsenosides could be developed for clinical testing as anticonvulsant medication. However, because there is no history of use of these specific preparations in humans, for FDA approval they are considered new drugs. In this instance, a complete battery of preclinical data would need to be collected and submitted for review – making the development of such compounds, even for preliminary testing, quite time-consuming and expensive.

Future Goals

Given the number of plants that have been shown to have some anticonvulsant activity in either animal experiments or in humans, it is highly likely that useful medications for the treatment of epilepsy could be developed from herbal products. Successful development of new drugs from plant products will require careful consideration of the needs for animal testing, including plans for future development and clinical testing. The most efficient route is to demonstrate activity with an existing botanical product with clear evidence of safety with human use. However, even in this instance there is a need to determine the component(s) in the botanical product that has anticonvulsant activity. This determination would allow for standardization of the product with respect to its clinical utility, and would result in greater acceptance of such a product for use by patients with epilepsy and by the physicians who care for them.

The identification of a single (or multiple) component with anticonvulsant activity from a botanical product would be useful from a number of standpoints. If, as in the case of ginseng, the whole extract is less effective (or possibly harmful) than the partially-purified compound, identification of the active ingredients would direct the partial purification or concentration of the active components while simultaneously removing the harmful ingredients. Alternatively, the active ingredient could be purified and developed as a single agent. Finally, the structure of the active component could be used as a lead compound for the development of a new class of anticonvulsant compounds through the development of structural analogs – a strategy that might be more efficacious than tedious purification processes.

Studies of botanical products, particularly those most likely to be ingested by patients with epilepsy, must also provide evidence of safety for patient use. It is possible that some readily available herbal products, such as ginseng root extract, are potentially harmful in patients with epilepsy. These products may lower the seizure threshold or have interactions with medication to either increase the side effects or reduce the effectiveness of that medication. Changes in pharmacokinetics due to addition of an herbal product, and changes in efficacy or changes in seizure threshold, would need to be systematically examined.

While providing evidence of the mechanism by which a botanical product produces its anticonvulsant effect is not necessary for clinical trials (or for obtaining a patent on a compound), investigations into the underlying mechanism of a candidate substance could further our understanding of epilepsy. In neurology and psychiatry, much of our current understanding of disease is based on laboratory studies of drugs that are clinically effective. Investigations into the effects of novel anticonvulsants could provide important insights into the mechanisms of seizure onset and termination in the brain, and/or to understanding the abnormalities present in the epileptic brain.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy; **Ketogenic Diet**: Dietary Therapies for Epilepsy; **Seizures**: Seizure Initiation and Termination; **Treatments (non-Antiepileptic Drugs)**: Herbal Therapies for Epilepsy.

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Relevant Websites

http://www.ninds.nih.gov/funding/research/asp/index.htm – Information about the Anticonvulsant Screening Program.

http://nccam.nih.gov/health/bydisease.htm – Listing of herbal and alternative approaches to various diseases. There is nothing listed for epilepsy.

http://www.fda.gov/cder/guidance/index.htm – Specific FDA guidelines for the development of botanical products.

Herbal Therapies for Epilepsy

S C Schachter, Beth Israel Deaconess Medical Center, Boston, MA, USA

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Introduction

Despite unprecedented growth in the number of FDA-approved epilepsy therapies, seizures in approximately 25% of patients with epilepsy remain refractory to pharmacological treatment. Only a small fraction of these patients qualify for, and have access to, therapeutic brain surgery. Further, worldwide most people with epilepsy have no access to modern treatments. Therefore, there continues to be a pressing need for new and effective treatments for epilepsy, especially for those that are accessible and affordable to patients everywhere.

Herbal medicines, a subgroup of natural products, have provided several small molecules used to understand biological processes as well as the pharmaceutical agents that treat human disease. Herbal medicines display remarkable specificity and potency. Structural characteristics of natural products have been used to develop modern synthetic compound libraries. Over half of the best selling prescription drugs contain natural product derivatives.

In Asian countries, herbal medicines have an extensive history and continue to be used widely. For example, in China, there are at least 2500 hospitals devoted to Traditional Chinese Medicine (TCM), and the majority of patients treated at these hospitals receive herbal medicines. In addition, 95% of general (western style) hospitals in China have TCM departments. In China at least 13 000 farms specialize in the production of TCM herbal medicines, and more than 800 TCM pharmaceutical factories were found to produce an annual output of 400 000 tons of 500 kinds of herbal medicines.

In the Republic of Korea (South Korea), 151 hospitals and 8734 clinics of Korean Medicine have been devoted to providing medical care in the tradition of Korean Medicine. Most patients in those hospitals and clinics receive herbal medicines as the primary form of

While providing evidence of the mechanism by which a botanical product produces its anticonvulsant effect is not necessary for clinical trials (or for obtaining a patent on a compound), investigations into the underlying mechanism of a candidate substance could further our understanding of epilepsy. In neurology and psychiatry, much of our current understanding of disease is based on laboratory studies of drugs that are clinically effective. Investigations into the effects of novel anticonvulsants could provide important insights into the mechanisms of seizure onset and termination in the brain, and/or to understanding the abnormalities present in the epileptic brain.

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http://www.fda.gov/cder/guidance/index.htm – Specific FDA guidelines for the development of botanical products.

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treatment. Herb farms constitute one of the main agricultural industries of the country. Approximately 100 species are farmed, and nearly 300 species are collected from the wild and marketed. Similarly, Taiwan, Africa, and South America have rich traditions in herbal medicines.

People with epilepsy have used diverse therapies for thousands of years. Some of these therapies persist even today, especially where access to medical care is poor. Indeed, antiepileptic drugs (AEDs) and epilepsy surgery are relatively new in the history of epilepsy treatment. For example, bromides were introduced in 1857, phenobarbital in 1912, and epilepsy surgery in the 1940s.

Despite a lack of convincing evidence of efficacy and safety, surveys suggest that up to one-third of patients with epilepsy in the United States and the United Kingdom take herbs and/or dietary supplements, and that the majority of these patients do not discuss this intake with their physicians. Most of these patients do not take alternative medicines for seizure control, but rather for general health maintenance or for other symptoms – for example, valerian for difficulty sleeping, St. John's wort for depression, and *ginkgo biloba* for memory disturbance. Thus, the alternative medicines taken by a patient with epilepsy may be a clue to that patient's side effects from their anticonvulsants or comorbid disorders.

Although botanicals have historically been an important source of therapeutic compounds in general, they have been relatively understudied as epilepsy therapies. Therefore, our research has focused on botanicals as potential anticonvulsants. The overarching goal of our research program at Harvard Medical School is to (1) develop a pipeline of herbal medicine extracts and their constituent compounds that have promising activity in animal epilepsy models and relevant in vitro assays, (2) conduct the necessary preclinical studies to proceed with early-stage clinical studies, (3) perform these clinical studies, and then (4) partner to develop the compounds as FDA-approved therapies for epilepsy.

Background

Over the centuries, many Asian as well as African and South American herbal medicines have traditionally been used to treat convulsive diseases. A partial list includes the followings:

Gastrodia elata;

Uncaria rhynchophylla;

Menispermum dauricum;

Shitei-To, a mixture of extracts from three medicinal herbs – *Shitei* (Kaki Calyx; calyx of *Diospyros kaki* L. f.), *Shokyo* (Zingiberis Rhizoma; rhizome of *Zingiber officinale* Roscoe) and *Choji* (Caryophylli flos; flowerbud of *Syzygium aromaticum* [L.]);

a mixture of radish and pepper;

Qingyangshen;

Kanbaku-taiso-to, a mixture of three herbal drugs – *Glycyrrhizae Radix*, *Tritici Semen* and *Zizyphi Fructus*;

Paeoniae Radix; and

Zheng Tai Instant Powder (a complex prescription of traditional Chinese medicine indicated for grand mal epilepsy).

Published reports suggest that several of these herbs may be neuroprotective, while others show efficacy in animal models of epilepsy and hippocampal slice models.

Published clinical studies have reported on over 135 different herbs used singly or in 80 different combination formulas for the treatment of seizures. The ten most frequently used herbs in published reports are:

Pinella ternate (Ban Xia),

Arisaemi japonicum (Tian Nan Xing),

Acorus calamus (Shi Chang Pu),

Gastrodia elata (Tian Ma),

Buthus martensii (Quan Xie),

Poria cocos (Fu Ling),

Bombyx bartryticatus (Jiang Qiang),

Citrus reticulate (Chen Pi),

Uncaria rhynchophylla (Gou Teng),

Glycyrrhiza glaba (Gan Cao), and

Salivae miltiorrhizae (Dan Shen).

Most of these studies are observational, including case reports.

Despite the large number of pre-clinical and clinical publications, scientific studies of herbal medicines, including those with potential use in epilepsy, have been limited by inconsistent descriptions of herbal extraction methods, lack of characterization and further in vitro and in vivo testing of the active constituent compounds, and poorly described clinical study designs and patient populations.

Methodological Approaches

Our botanical epilepsy research program has systematically addressed these limitations and begun to develop a pipeline of compounds isolated from herbal medicine extracts that have promising activity in animal epilepsy models and relevant in vitro assays, conduct the necessary pre-clinical studies to proceed with early-stage clinical studies, perform these clinical studies, and then partner to develop and commercialize the compounds as FDA-approved drugs for epilepsy.

Our group, which includes collaborators in India, the Middle East, and the Far East, has now identified and tested nearly 30 herbal medicine extracts and pure, extract-derived compounds isolated from Chinese, Japanese, and Indian herbal medicines in animal models of epilepsy. We have also carried out vitro assays to characterize membrane currents mediated by AMPA, NMDA,

and $GABA_A$ receptors; and voltage-dependent sodium, potassium, and calcium ion channels. The specific methods and processes involved are further detailed in the upcoming sections.

Identification of Botanicals

Botanicals are selected based on careful consideration of many factors, such as knowledge and previous experience of the investigators, promising results from preliminary work by the investigators, published results of in vivo or in vitro studies from other laboratories, electronic database searches, published clinical recommendations of senior herbal and epilepsy experts in China and Korea, review of original (traditional) textbook references, and previous clinical studies.

Compound Isolation

Herbal extracts, and compounds derived from those extracts, are obtained using standard methods, and the purity of the compounds is assessed by thin layer chromatography or high pressure liquid chromatography. Molecular structure and weights are determined using various well-validated techniques. Sufficient quantities of extracts and compounds are produced for all the in vitro and in vivo studies, and samples are saved for future reference.

In Vitro Assays

Compounds and their parent extracts are analyzed using in vitro assays that focus on currents mediated by AMPA, NMDA, and $GABA_A$ receptors, and voltage-dependent sodium, potassium, and calcium ion channels. Techniques used include (1) whole-cell patch-clamp recording of ligand- and voltage-gated currents in cultured rodent cortical and hippocampal neurons, (2) whole-cell patch-clamp recording of recombinant NMDA receptors in human embryonic kidney (HEK293) cells, (3) characterization of NMDAR-mediated excitotoxicity in primary neuronal cultures from mouse cortex, and (4) determination of NMDA-induced nitric oxide (NO) production in cultured primary neurons from mouse hippocampus.

Epilepsy Animal Models

In parallel to the in vitro experiments, compounds as well as their parent extracts are tested in the screening epilepsy animal models of the NINDS Anticonvulsant Screening Program (ASP), including maximal electroshock seizure (MES) and subcutaneous pentylenetetrazol (sc PTZ) tests following oral and intraperitoneal (i.p.) administration; compounds are also examined in the levetiracetam-sensitive 6-Hz psychomotor seizure test following i.p. administration. Extracts are tested, in addition to the pure compounds, because they may contain active compounds that were too low in concentration to be isolated. When quantities allow, extracts and compounds are also evaluated for their ability to prevent the development of kindling.

In addition to these efficacy studies, effects on motor behavior are assessed by their propensity for causing behavioral impairment. For mice, behavioral impairment is determined by the rotarod test; behavioral impairment in rats is estimated from a battery of behavioral assessments including gait, stance, placing response, and muscle tone. The dose producing behavioral impairment in 50% of the population (i.e., TD_{50}) is calculated, and where possible, the protective index (TD_{50}/ED_{50}) is then calculated. In rats, minimal motor impairment (MMI) is determined by overt evidence of ataxia, abnormal gait, and stance.

Collectively, the results from these studies provide a reasonable estimate of a candidate substance's anticonvulsant profile and form the basis for selecting lead substances for further preclinical and subsequent clinical evaluation.

Results to Date

Of the nearly 30 extracts and compounds isolated from those extracts that have been procured or isolated and have undergone partial or complete in vivo and in vitro testing, nearly three-quarters of the materials show efficacy in one or more animal models. A common underlying mechanism of action of these materials appears to be protection against NMDA- and glutamate-mediated neuronal death, including Huperzine A, our lead compound. This finding is encouraging because glutamate-mediated hyperexcitability is believed to underlie the seizures and the neuronal degeneration that is often associated with chronic epilepsy, especially mesial temporal lobe epilepsy involving the hippocampus. Ionotropic glutamate receptor antagonists have been viewed as an important class of compounds for new epilepsy therapies, both to inhibit seizures and to block glutamate-mediated neuronal degeneration. However, previous NMDA receptor antagonists developed for epilepsy have been associated with histopathological and neurochemical changes in the brains of adult rats, and cognitive and behavioral side effects, including psychosis, in adult humans, thereby limiting their clinical usefulness.

Huperzine A

The first compound studied by our group was Huperzine A, a sesquiterpene Lycopodium alkaloid isolated from Chinese club moss (*Huperzia serrata*), also known as the Chinese

folk medicine *Qian Ceng Ta* (Figure 1). Huperzine A is also found in related plants, such as *Phlegmariurus carinatus*.

(-)-Huperzine A was first isolated from *Huperzia serrata* in 1986, and the average content of Huperzine A by weight is 0.011%. Its chemical nomenclature is [5*R*-(5a,9b,11*E*)]-5-amino-11-ethlidene-5,6,9,10-tetrahydro-7-methyl-5,9-methanocycloocta [*b*] pyridin-2(1*H*)-one. The CAS No. for Huperzine A is 120786-18-7; its molecular formula is $C_{15}H_{18}N_2O$ and molecular weight 242.32. Huperzine A is stable when stored at room temperature.

Huperzine A has been shown to be neuroprotective in several models, likely by acting as a non-competitive antagonist of NMDA receptors at one of the polyamine binding sites at or near the PCP and MK-801 ligand sites, but without the psychotomimetic side-effects of PCP or MK-801. Pretreatment of primary neuronal cells with Huperzine A reduced glutamate-induced toxicity. Pretreatment of cultured rat primary cortical neurons with 1 μM Huperzine A significantly attenuated apoptosis induced by serum deprivation, possibly by effects on intrinsic caspase-3 pathways. Two-week oral Huperzine A (0.1 mg kg^{-1} twice daily) following transient global ischemia in gerbils reduced memory impairment and neuronal degeneration in the CA1 region compared to sham controls, and partially restored hippocampal choline acetyltransferase activity. Similarly, Huperzine A (0.01–0.1 mg kg^{-1}, i.m.) improved spatial working memory in aged monkeys and young adult monkeys with scopolamine-induced cognitive impairment.

Huperzine A is a potent, highly specific and reversible inhibitor of acetylcholinesterase, with comparable potency to physostigmine, galantamine, donepezil, and tacrine. It produces dose-dependent increases of norepinephrine and dopamine in rat cortex when administered i.p. or locally through a microdialysis probe, and has many other actions, including the ability to protect cells against hydrogen peroxide, beta-amyloid protein (or peptide), and staurosporine-induced cytotoxicity and apoptosis. These protective effects are related to its ability to attenuate oxidative stress, regulate the expression of apoptotic proteins Bcl-2, Bax, P53, and caspase-3, protect mitochondria, upregulate nerve growth factor and its receptors, and interfere with amyloid precursor protein metabolism.

Huperzine A has been administered to healthy volunteers and patients in numerous trials, many in China, demonstrating acceptable safety and tolerability as well as efficacy in Alzheimer's disease, benign senescent forgetfulness, vascular dementia, and myasthenia gravis. It has traditionally been used in China for the treatment of swelling, fever and inflammation, blood disorders and schizophrenia, and is approved and used in China for the treatment of Alzheimer's disease. Huperzine A was classified as a dietary supplement by the FDA in 1997. As a nutriceutical, it is available in health food stores or via the internet, labeled as a memory aid, and is being studied in a multi-center NIH-sponsored trial for Alzheimer's disease at dosages up to 400 μg BID. There are no available published data of efficacy or tolerability in patients with epilepsy in the medical literature.

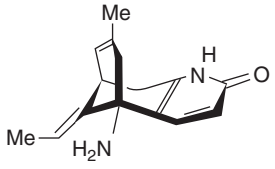


Figure 1 Huperzine A.

Huperzine A was selected for study in animal models of epilepsy based on its known neuroprotective properties. Under the auspices of the ASP, Huperzine A (ADD 357133) was found to be potently active against s.c. PTZ-induced seizures and less so against maximal electroshock-induced seizures following oral administration to Swiss-Webster mice, with peak anticonvulsant activity at one hour. At doses of 1, 2, and 4 mg kg^{-1}, a maximum of 62.5% protection was observed. Impairment on the rotarod test was observed in 75 and 100% of mice tested at doses of 2 and 4 mg kg^{-1}, respectively. The TD_{50} was 0.83 mg kg^{-1}.

In further work using the 6-Hz model, ED_{50} values for i.p. Huperzine A were 0.28, 0.34 and 0.78 mg kg^{-1} for 22, 32, and 44 mA, respectively, suggesting marked potency as well as a possible advantage over phenytoin, carbamazepine, lamotrigine and topiramate, each of which display limited efficacy in this model at doses devoid of behavioral toxicity (Table 1). The less than 2-fold ratio of dosages effective across the range of stimulation currents suggests a further possible advantage of Huperzine A over other drugs that are active in this model, such as levetiracetam.

We have also studied Huperzine A with the mouse formalin pain model. One mg/kg i.p. produced complete inhibition of pain behavior in all treated animals at all time points, and 0.5 mg kg^{-1} i.p. (60% of the TD_{50}) produced near complete inhibition. Both sets of results demonstrated statistically significant differences from controls ($P < 0.01$), and suggest potential efficacy in somatic (e.g., inflammatory, traumatic) as well as neuropathic pain syndromes.

Table 1 ED_{50} (mg/kg, i.p.) for AEDs in the 6-Hz model (data courtesy of H. Steve White, PhD)

AED	*22 mA*	*32 mA*	*44 mA*
Phenytoin	9.4	>60	>60
Lamotrigine	4.4	>60	>60
Levetiracetam	4.6	19.4	1089
Valproic acid	41.5	126	310
Huperzine A	0.28 (0.16–0.48)*	0.34 (0.28–0.40)*	0.78 (0.56–1.01)*

ED_{50} – dose at which seizures are blocked in half of the treated animals; i.p. – intraperitoneal; *– 95% confidence interval.

Challenges for the Future

The study of herbal medicines and botanicals for the identification of possible new epilepsy therapies, using a plant to bench to clinic strategy that is inspired by centuries-old tradition and that utilizes modern methods, appears very promising. This approach, and others underway around the world, may also yield neuroprotective agents. Theoretically, such agents could protect against the onset of epilepsy, against seizure worsening, or against progressive behavioral changes (e.g., memory loss). Further, some of the herbal medicines and their derived compounds that are active in the animal models of epilepsy may be found to have novel neuronal mechanisms of action that could lead to the development of additional pharmacological interventions for epilepsy.

Huperzine A may represent a new class of anticonvulsant compounds with a novel combination of mechanisms of action. Plans are now underway to conduct a proof-of-principle assessment of the compound's safety and tolerability and to study early-stage pharmacokinetics of Huperzine A (up to 800 $\mu g\,day^{-1}$ (0.8 $mg\,day^{-1}$)) as add-on, open-label therapy in patients with medically-refractory partial seizures, with or without other seizure types. A secondary aim is to provide preliminary data on the efficacy of Huperzine A and its effects on mood and memory function in this cohort of patients.

The pre-clinical study of herbal medicines poses several challenges. Chief among these are developing the best and most cost-effective methods for (1) identification, authentication, extraction and fractionation of botanical materials to study, (2) characterization of the active compounds, (3) isolation of sufficient quantities of active compounds for pre-clinical and clinical studies, and (4) establishing analytic methods for assessing purity of compounds and serum concentrations.

See also: **Antiepileptic Drugs**: Antiepileptic Drug Mechanisms; **Inflammation**: Neurodegeneration, Neuroprotection and Inflammation in the Brain; **Ketogenic Diet**: Dietary Management of Epilepsy: Role of Glucose and Ketone Bodies; Dietary Therapies for Epilepsy; **Treatments (non-Antiepileptic Drugs)**: Ginseng and Other Herbal Treatments for Epilepsy.

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Immunoliposome Gene Therapy and Its Potential Use in Treatment of the Epilepsies

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Introduction

Despite the advances during the decade of the brain, there is still no effective treatment for Alzheimer's disease, Parkinson's disease, ALS, Huntington's disease (HD), and many monogenic diseases such as Rett's syndrome, fragile x syndrome, Canavan's disease, Tay-Sachs disease, and the mucopolysaccharidoses including Lafora's disease. In all the cases, the mutated gene is known and cloned. However, copies of the potentially life-saving genes sit dormant in research laboratories because of the problems in expressing an exogenous gene in the brain. There is a perception that delivery across the BBB is an insurmountable problem.

In recent years, attempts to cure monogenic diseases using viral vector delivery methods – and particularly using adeno-associated virus (AAV) vectors – have been widely heralded. However, the tragedies associated with gene therapy have been widely publicized, damping enthusiasm for this approach. The first person publicly identified as having died in a clinical trial for gene therapy was injected with an adenovirus-vectored gene therapeutic and died four days later, apparently having suffered a massive immune response triggered by the use of the viral vector used to transport the gene into his cells. Adeno-associated virus vectors were assumed to provide an increased safety margin, but another patient died recently during a clinical trial using AAV-gene therapy to treat inflammatory rheumatoid arthritis. Her death halted the clinical trial and further heightened the concerns surrounding viral vector-mediated gene therapy.

Demyelination is a problem associated with viral vectors. And unpredictable responses have also been seen in other viral-vector studies. For example, a gene-therapy trial for severe combined immune deficiency (SCID) caused leukemia in three children. This incident underscores the value of laboratory testing. For example, in animal model studies of mucopolysaccharidosis, an adeno-associated virus (AAV) vector was used to deliver the GUSB gene to knockout mice and 33% of these treated mice were found to have developed hepatocellular carcinoma. This finding raised safety concerns over the clinical use of AAV vectors. The cessation of gene-therapy trials using retroviral vectors, and the apparent failure of cystatin B-HIV-TAT viral protein as a potential new therapy for Unverricht–Lundborg epilepsy emphasize a need to develop alternative delivery methods.

Ideally, gene therapy should use a nonviral carrier formulation that can be administered intravenously for ease of clinical use. The method should be available for multiple administrations and should provide widespread expression throughout the brain. The gene being replaced should be targeted to the brain and shielded during its transit to the brain from the ubiquitous exonucleases and endonucleases present in blood circulation. The nonviral vector should protect the replacement gene from immune recognition, and prevent uptake by the reticuloendothelial system. Finally the carrier vector must transport the replacement gene through the blood–brain barrier (BBB) capillaries and must then be released to the cells of the neuropil without untoward side effects. Development of such a method is the topic of this article. We describe the effectiveness of pegylated immunoliposomes (PILs) in delivering an exogenous gene to the brain.

Background

Inherited forms of epilepsy have been recognized for years, and recently the specific loci and genes involved in many epilepsy syndromes have been identified. Causative mutations have been found for several single-gene conditions, and this information has guided the identification of candidate genes for multifactorial epilepsy disorders. Monogenic epilepsies would also naturally be the model of choice for initial studies of gene therapy because of their simplicity, even though multifactorial epilepsies are more common.

Lafora's Progressive Myoclonic Epilepsy as a Candidate for Gene Therapy

In 1911 Gonzalo Lafora first described a fatal progressive myoclonus epilepsy with periodic acid Schiff (PAS+) stain intracellular inclusion bodies in an adolescent boy.

Lafora's disease was later shown to be a generalized metabolic disorder involving glycogen and mucopolysaccharides. It is now recognized as an autosomal recessive, adolescent onset, progressive and fatal generalized polyglucosan storage disorder with predominant central nervous system manifestations. Eighty percent of patients die by 18 years of age, and afflicted family members rarely live beyond 30 years. Although rare in the USA, the gene for Lafora's disease has been enriched in segments of society in Southern Europe (Italy, France, Spain), Northern Africa, India, Pakistan, and the Middle East. It is also seen in sectors of the population in the Appalachian Mountains and in villages of Quebec, Canada, where cultural practices lead to a high rate of consanguinity.

Delgado-Escueta and colleagues, in their studies of the 6q24 locus of Lafora's disease, have refined the map position, constructed a physical map of 6q24, isolated genes, and defined the mutations. They also isolated the full-length mouse cDNA clones as well as mouse genomic clones of laforin (i.e., epm2a) and mapped it to mouse chromosome 10, the syntenic region of human 6q24. Consequently, because the gene locus of this disease has been fully characterized and its clinical (and pathological) characteristics faithfully reproduced in the knockout mouse model, gene therapy approaches for Lafora's disease can be rigorously tested in this mouse model prior to adapting laboratory methods to the clinical situation.

Gene Therapy in Lafora's Disease may Forecast the Treatment of Other Single-Gene Disorders

There are many inherited diseases of the CNS which are candidates for treatment by gene therapy. Lysosomal storage disorders encompass about 50 metabolic diseases that include neuronal ceroid lipofucinoses, mucopolysaccharidoses (MPS), mucolipidoses IV, sphingolipidoses, sphingomyelinoses (Niemann–Pick disease), gangliosidoses (e.g., Tay-Sachs and similar diseases), glycoproteinoses, and other monogenic inborn errors of metabolism that collectively affect about 1 in 5000 live births. In each of these diseases, there is a deficient activity of enzymes responsible for catabolizing substrates in the lumen of lysosomes. All these deficiencies lead to a characteristic pathological accumulation and intracellular storage of the substrate. The accumulation of undigested metabolites in lysosomes leads to multisystemic dysfunction, including progressive neurologic deterioration, mental retardation, hepatosplenomegaly, blindness, and early death. Even though all of these diseases are inherited as single-gene defects, the development of new therapies has been slowed because tissue delivery of the (single) primary enzyme or protein defect is difficult, but bone marrow transplants and gene therapy offer promise.

The Rationale for Immunoliposome-Based Brain Delivery

Most drugs cross the BBB as a result of their lipid solubility profile; however, permeability is a function of molecular size, and their molecular mass must be <400–600 Da to cross the BBB. Exceptions to this rule (e.g., cyclosporine (1203 Da), vinblastine (814 Da), and vincristine (825 Da)) also include drugs which cross via transporter-mediated mechanisms, such as fluoro-deoxyglucose, vigabratin, and dihydroxy phenylalanine (L-DOPA). Gene therapeutics are invariably large molecules, which do not cross the intact BBB. This difficulty in efficient delivery and expression of therapeutic DNA inhibits the potential of gene therapy. Possible methods for delivery of genes to the human brain include capillary disruption, invasive craniotomy, cationic-liposome DNA complexes, or viral vectors. Each technique has significant limitations, and the efficiency of viral vectors for delivering genes across the BBB has yet to be shown. Intracerebral implants require craniotomy and deliver therapeutic agents to only a localized region. The efficiency of osmotic disruption of the brain capillaries is unproven; further, like those treatments requiring craniotomy, the method is unsuitable for multiple treatments. Gene delivery whereby DNA is complexed with cationic liposomes has also been tested, but these formulations aggregate in saline solutions. Consequently, when administered i.v., the aggregation results in selective gene expression in the lung, with no expression in the brain. Demyelination is a problem associated with viral vectors, and the safe use of even modified human viruses requires further development.

Gene therapy would ideally use a nonviral formulation; would be intravenously given for ease of clinical use and multiple administrations; and would provide widespread expression throughout the brain. Recent work indicates that this therapeutic approach is possible when pegylated immunoliposomes are used to target the gene to the brain. The liposome capsule shields the therapeutic DNA from the ubiquitous exonucleases and endonucleases present in the circulation. Polyethylene glycol strands attached to the liposome (pegylation) allow the liposome to escape immune recognition, and prevent uptake by the reticuloendothelial system. Monoclonal antibody (MAb) to the transferrin receptor permits the liposome to undergo receptor-mediated transport through the BBB capillaries and the cells of the neuropil. The liposome capsule undergoes lipolytic degradation to release its contents, without untoward side effects.

Studies on Pegylated Immunoliposome Delivery to Brain

The attachment of immunoreactive moieties to liposomes, and the formation of immunoliposomes designed

to target specific tissues, have been known for two decades. However the cellular barrier formed by closely situated brain endothelial cells was assumed to exclude liposomes, even small (50–80 nm) unilamellar vesicles. The most dramatic new advance in BBB transit methodology has been the demonstration that immunoliposomes (antibody-directed liposomes) can be used for brain delivery of drugs and other neuropharmaceuticals (such as those based on antisense nucleic acid targeting). Investigators showed that this approach for the delivery of ^{3}H-daunomycin in pegylated immunoliposomes (PILs) increased brain drug delivery by 4 orders of magnitude. Subsequent studies showed that i.v.-administered PILs bound to the luminal and abluminal rat (or pig) brain capillary membranes, undergo endocytosis, and release more than 90% of the liposome cargo within CNS or glioma cells.

A related approach utilizes the fact that proliferation of high grade astrocytomas is associated with overexpression of the epidermal growth factor receptor (EGFR). In a ground-breaking study, Pardridge and colleagues applied their 'nonviral' PIL-targeted gene delivery system to suppress EGFR in an experimental human brain tumor model (Figure 1). These investigators had demonstrated that PILs were able to deliver an exogenous gene (β-galactosidase) uniformly into the rat brain, where its expression could be imaged. In applying this method to tumor therapy, the targeted gene delivery system used an 'artificial virus' with the EGFR antisense gene inserted into a nonviral expression plasmid, packed within an 85 nm PIL.

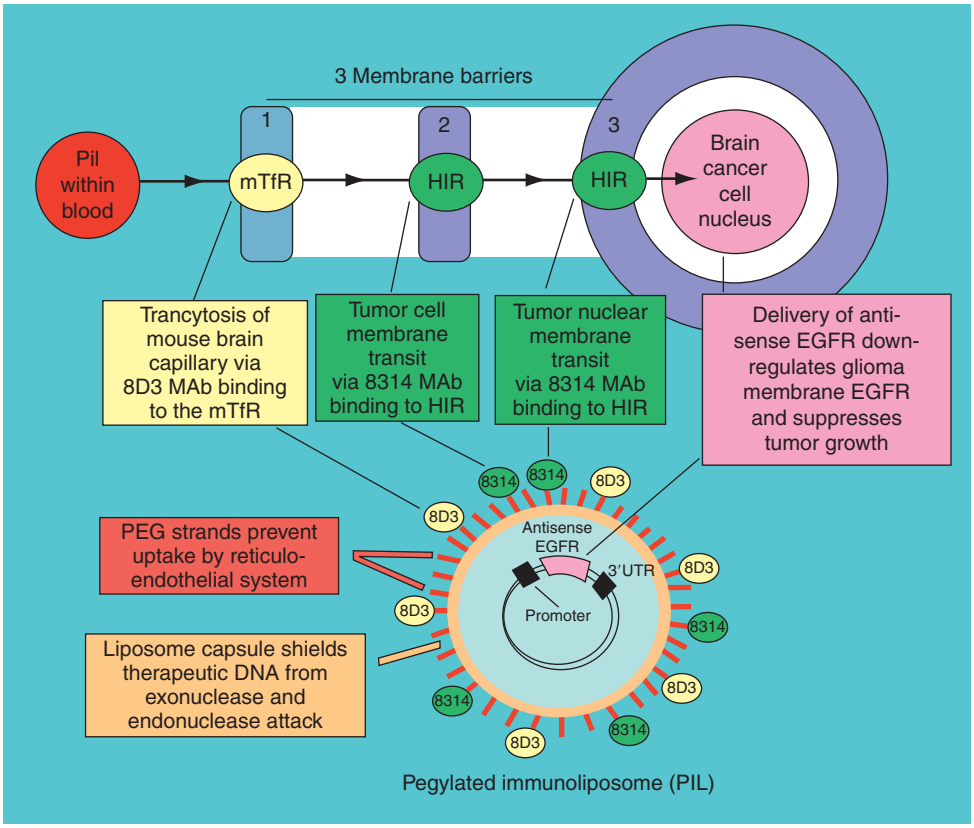


Figure 1 CNS gene therapy in brain cancer using an 'artificial virus' pegylated immunoliposome (PIL) delivery system (from Cornford and Cornford, 2002). Lower panel: Encapsulation of the therapeutic EGFR antisense plasmid DNA within a 'nanocontainer' liposome, prevents degradation of the therapeutic DNA by the ubiquitous endonucleases present in vivo. Strands of polyethylene glycol (PEG^{2000}) extend from the surface of the pegylated liposome, and the targeting MAb (about 35–50 MAb molecules per individual liposome) is attached to the tip of about 2% of these PEG strands. 'Peglyation' of the liposome prevents uptake of the PIL by cells lining the reticuloendothelial system (RES). The expression plasmid encoding the EGFR antisense gene is encapsulated within the interior of the PIL for brain delivery. Upper panel: In order to reach the nuclear compartment of the glioma cell, the intravenously administered PIL must traverse three biological barriers. (1) The BBB at the tumor microvasculature, (2) the tumor cell plasma membrane (TCM), and (3) the nuclear membrane of the tumor cell. The first barrier, the luminal and abluminal membranes of the BBB, is traversed by the 8D3 MAb to the transferrin receptor (TfR), which is abundant on the brain capillary. The second barrier, the human TCM, is traversed by the murine 8314 MAb to the human insulin receptor (HIR), and the HIR MAb also traverses the third barrier, the tumor cell nuclear membrane. Delivery of the antisense EGFR oligonucleotide to glioma nuclei down-regulates epidermal growth factor receptors and suppressed tumor growth. This work also predicts that gene delivery across sequential biological barriers (i.e., placenta and fetal BBB) in utero is feasible with PIL methods.

The surface of the PIL was supplied with two different peptidomimetic MAbs for transport across the multiple biological membranes separating the blood from the cancer cell nucleus. The 8D3 MAb to mouse transferrin receptor facilitated transport of the PIL across the murine BBB. The second barrier, the human glioma cell membrane, was traversed by the murine 8314 MAb to the human insulin receptor (HIR). The HIR MAb also traversed the tumor cell nuclear membrane, due to the usual intracellular trafficking of insulin to the nucleus, and this characteristic makes insulin-receptor targeting useful in widespread PIL delivery of genes to the primate brain.

Mice bearing intracranial U87 human gliomas were treated with this expression plasmid encoding antisense mRNA against the EGFR. The life-span of the mice treated once weekly (for 4 weeks, with i.v. EGFR antisense gene therapy) increased 100% relative to controls. Small brain tumors were seen in surviving mice (which had received gene therapy), while massive tumor growth was confirmed (postmortem) in the control mice. The unusual success of this animal model study, and its successful subsequent application to gene delivery in primates, provide hope that the human counterpart will soon be forthcoming. The work also suggests that the replacement of the missing or mutated genes within the CNS can be achieved using the PIL technology. Enhanced gene expression is seen with HIR-targeted PILs, and insulin-receptor based PILs efficiently deliver genes to the primate brain.

More recently, the same molecular 'Trojan horse' methods have been used in conjunction with RNA interference (RNAi) gene therapy using the EGFR (instead of antisense mRNA) as the liposomal cargo. Short hairpin RNA encoding nucleotides 2529–2557 of the human EGFR was encapsulated within immunoliposomes and administered to SCID mice bearing U87 glioma implants. The feasibility of this RNAi approach was evaluated by showing that it was possible to achieve a 90% knockdown of brain tumor-specific gene expression with a single intravenous injection in adult rats or mice with intracranial brain cancer. The survival of mice with intracranial human brain cancer was extended by nearly 90% with weekly intravenous injections of PILs carrying this plasmid DNA It has been proposed that RNAi-based gene therapy can be coupled with gene therapy that replaces or restores mutated tumor-suppressor genes, to build a polygenic approach to the gene therapy of brain cancer.

Experimental Parkinsonism has also been successfully treated using PIL-delivered gene therapy. In these studies, the unilateral intracranial administration of 6-hydroxydopamine was used to cause a loss of dopamine hydroxylase in the striatum, and apomorphine injections result in a contralateral rotation behavior of these rats. Three days after Transferrin-receptor targeted PILs were employed to deliver a tyrosine hydroxlase (TH) expression plasmid, investigators measured the normalized TH enzyme activity, along with a 70% reduction in rotational behavior. The pharmacological effect was both time- and dose-dependent. In contrast, this reversal of rotational behavior has not been seen in studies when the lesioned rat is treated with TH genes which are targeted with a viral vector; effective treatment may require transduction of a significant volume of the striatum with the TH gene, which may not be possible with a single intrastriatal injection of a viral vector. These investigations (which compare two alternate methods of gene delivery) seem to support the conclusion that in treating CNS disorders, PIL-delivered gene therapy appears to be more effective than therapy based on viral vectors.

In summary, the liposome functions as a biological nanocantainer (sometimes called an 'artificial virus') and shields its cargo from nuclease attack. The 8D3 monoclonal antibody allows the immunoliposome to function as a molecular Trojan horse, and deliver the desired gene product across the mouse BBB via receptor-mediated transcytosis. If immunoliposome nonviral delivery methods can treat the disease in animal models (see **Figure 1**), an immunoliposome-based cure for diseases such as Lafora's epilepsy may be developed for widespread clinical use.

Methodological Approaches and Results to Date

How is CNS Gene Delivery Accomplished? Preparation of Pegylated Immunoliposomes

When the expression of the exogenous luciferase gene is studied, the commercially available PGL3 luciferase vector (Promega; Madison, WI) may be used to provide a source of plasmid nucleic acid. The same vector may be modified and a PGL3-laforin plasmid is prepared to study gene replacement in Lafora null mutant knockout mice. EcoR1 can be used to cut the luciferase cDNA from the PGL3 plasmid at the 5″ flanking end, between the SV40 promoter and the 3′UTR. The PGL3-laforin expression plasmid is then formed by annealing Laforin cDNA in place of the luciferase cDNA.

Immunoliposomes are synthesized in a series of stepwise reactions beginning with plasmid nucleic acid preparation. The expression of plasmid may be driven by the broadly expressed simian virus 40 (SV-40) promoter, or alternatively by the brain-specific promoter taken from the 5′ flanking sequence of the human glial fibrillary acidic protein (GFAP) gene. The rationale driving this decision is that the transgene is expressed in both brain and peripheral tissues when the SV-40 promoter is used, whereas its expression is confined to the brain when under the influence of the brain-specific GFAP promoter. Thus, the SV-40 promoter, used in combination with

transferrin-receptor targeting, will deliver and express the gene construct to brain and to tissues such as the liver, spleen, and muscle in situations where the EPM2a gene is to be restored in Lafora knockout mice. (In these animals, peripheral tissues as well as the brain are affected, and also contain Lafora inclusion bodies.) In contrast, if the treatment situation requires expression of the gene only in CNS tissues (as might be the case for studies of hereditary childhood absence epilepsy), a GFAP promoter would be the logical choice because this promoter has been shown to selectively drive brain specific expression.

The lipids are mixed to form pegylated liposomes, mixed with plasmid DNA, and then extruded to make liposomes of specific size dimensions. Excess external nucleic acids are removed enzymatically. Finally the monoclonal antibody, which targets the finished product to a brain capillary receptor, is added to the PEG-strands and the immunoliposomes are purified by gel filtration chromatography for systemic intravenous administration.

Receptor-mediated transcytosis of the PIL and its cargo is dependent upon the selectivity and affinity of the targeting monoclonal antibody for the mouse transferrin receptor. If the 8D3 MAb proves to be unsuited, or proves to be an inefficient means of delivering (pPGL3-luciferase or pGL3-laforin) DNA in the knockout mouse, alternative methods may be considered. The RI7–217 MAb is another rat monoclonal antibody which undergoes receptor mediated transcytosis via the mouse transferrin receptor, and its brain uptake is comparable to that of the 8D3 MAb. The 8D3 MAb is the vector of first choice simply because it affords better delivery to renal and hepatic tissues, and slightly better delivery to the brain.

Milligram quantities of the peptidomimetic 8D3 MAb which is used as the (TfR) targeting ligand are required. This 8D3 hybridoma cell line grows efficiently in the peritoneal cavity of nude mice. Nude mice generate ascitic fluid containing high specific activity MAb. The MAb must be thiolated in order for it to be attached to the liposome. Using Traut's reagent, it can be attached to the tips of the polyethylene glycol strands, converting the liposome to an immunoliposome. Gel filtration chromatography is then used to separate the PILs from unreacted material, and the eluted PILs are then concentrated and filter-sterilized prior to injection.

Immunoliposomes are injected intravenously, and the cargo is delivered into the CNS. Studies suggest that the gene therapeutic is delivered to an episomal location within the cells, so there is no concern over insertional mutagenesis. In rodent models, the effect of the gene therapeutic persists for about 2 weeks. While multiple injections would be necessary over an extended treatment period, the advantage is that untoward effects due to overexpression of the administered gene can be controlled by simply ceasing to treat for the desired period.

Electron Microscopic Confirmation of Laforin-PIL Preparations

The electron microscopic appearance of an immunostained PIL is shown in the inset of Figure 2. A small volume of the PIL preparation was deposited directly onto a formvar-coated grid, and dehydrated. The presence of immunoreactive IgG attached to the PEG strands was confirmed by staining the grids with goat antirodent 20 nm gold particles. Here we counted 7 ± 7 ($X \pm$ SD; $n = 24$) gold particles per PIL, a measure not inconsistent with a density of 31 MAb/liposome estimated from PIL-isotope incorporation. Other studies have shown that large 20-nm gold particles underestimate epitope density by at least 50%, compared to analyses utilizing either 5- or 10-nm gold particles. Furthermore, because 50% of the epitopes are situated under the liposome, and not exposed to the MAb, there is a further 50% underestimation of MAb density. Thus, the observation of seven 20-nm gold particles per liposome would represent ~28 MAb per PIL.

This illustration also compares the dimensions of this receptor-mediated drug delivery system with that of the biological barrier to the CNS. The PIL (depicted in the inset) is 70–100 nm in diameter, and its path of transcytosis through the capillary endothelial cell varies from

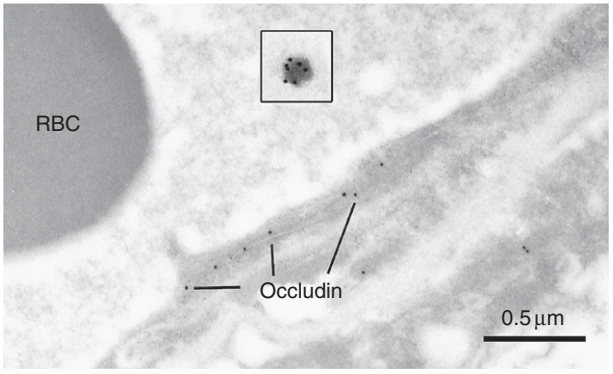


Figure 2 Comparative illustration of normal rodent brain capillary, and a pegylated immunoliposome (PIL) containing DNA from the Laforin plasmid. Tight-junctional proteins, such as occludin, are highly expressed at endothelial cell junctions in the cortical BBB, preventing the blood-to-brain passage via interendothelial clefts. The PIL utilizes receptor-mediated transport to undergo a transcellular passage from blood to brain. The inset depicts a pegylated immunoliposome, illustrated at the same magnification as the capillary profile. 10 nm gold particles are tagged to the polyclonal rabbit antiserum to occludin (Zymed Laboratories Inc, San Francisco, CA). Occludin-immuno-reactive epitopes are expressed intermittently along the length of the interendothelial junction, and no reactivity is apparent at any other blood–brain barrier domain. A targeting antibody (e.g., rat antiserum to the mouse BBB transferrin receptor, TfR; or in control immunoliposomes, normal IgG) is bound to the tips of the polyethylene glycol strands covering the immunoliposome, and is identified with a primary antibody followed by a gold-conjugated secondary antibody (e.g., goat anti-mouse 20 nm gold particles; Ted Pella Inc., Redding, CA).

200 to 450 nm. A typical CNS drug MW is 357 Da (smaller than a 1 nm gold particle). **Figure 2** compares the relative sizes of a 10 nm-gold labeled occludin epitope to that of the PIL nanocontainer; this illustration emphasizes the huge increment in BBB large molecule drug delivery that can be achieved with molecular Trojan horse technology.

Fusion Proteins

If a large molecule biopharmaceutical does not have to be protected within a liposomal container, it is now possible to reengineer the drug as a fusion protein. These IgG fusion proteins are new chemical entities that are dual- or triple-function molecules. They enter the brain by binding to an endogenous BBB receptor, and induce the desired pharmacologic effect in brain following passage across the BBB. A recent review (see Pardridge in Further Reading section) details how one end of the engineered molecule recognizes a receptor (such as the human insulin receptor) to permit BBB transit. The other end of the molecule may be a recombinant neurotrophin, or a therapeutic enzyme, or a single chain Fv antibody; in each separate situation, the alternate end is able to produce a unique therapeutic effect within the CNS. This type of therapeutic would be useful in treating a single gene defect where enzyme replacement therapy is effective, but (because of the ubiquitous endo- and exonucleases) not suited to delivering a gene therapeutic via intravenous injection.

Challenges for the Future use of the Trojan Horse Liposome Method

Even though more than 80 million individuals in the United States have some disorder of the brain, and more than 98% of all new drugs do not cross the BBB, less than 1% of the drug companies in the world today have a BBB drug-targeting program. There is currently a critical need for new neurotherapeutics designed for the treatment of neurological diseases. Despite the advances during the decade of the brain, there is still no effective treatment for Alzheimer's disease, Parkinson's disease, ALS, Huntington's disease (HD), or many other neurodegenerative conditions. For example, the gene for HD was identified a decade ago, yet an approved new therapy has not yet emerged. Antisense or RNAi therapy designed to selectively block the CAG repeats in the huntingtin transcript is theoretically possible, but developing this strategy was not pursued because the large molecule drugs do not cross the BBB.

There are numerous single-gene defect diseases that have devastating effects on the children of adult carriers, such as Rett's syndrome, fragile x syndrome, Canavan's disease, Tay-Sachs disease, and the mucopolysaccharidoses including Lafora's disease. In all the cases, the mutated gene is known and cloned. However, gene-based therapies have not been developed in most cases, because of problems in expressing an exogenous gene throughout the brain. There is a perception that delivery across the BBB is an insurmountable problem – or new treatments for CNS disorders are discussed within a context that fails to mention the problem of BBB transit However, neonatal and childhood onset diseases may be treated with brain-target gene-based therapies; available information suggests that pharmaceuticals such as molecular Trojan horses – which successfully undergo BBB transit – can also bind to endogenous receptor mediated transporters, and may be able to ferry gene products in the placental barrier, delivering selected nucleic acid to replace a deleted or mutated gene into the fetal brain.

There is now a real opportunity to translate recent advances in gene localization of inherited epilepsies and BBB gene delivery into the development of a new therapy. Available information suggests that Lafora's disease is candidate for gene therapy. Given the adverse events seen in studies employing viral vectors, the development of alternative methodologies for brain gene delivery has to become a high priority. Furthermore, we believe that continued and increasing support for gene therapeutic studies involving the administration of receptor-specific Trojan horse liposomes can produce results which will translate into therapies for a variety of genetically based disorders. The power and promise of this method have been shown in a variety of animal models of CNS diseases, where gene delivery has been achieved, and amelioration of the disease is apparent.

See also: **Antiepileptic Drugs**: Local Delivery of Antiepileptic Drugs; **Genetics**: Genetics of Lafora and Juvenile Myoclonic Epilepsies; Large-Scale Mapping and Epilepsy Gene Discovery; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Resculpturing Synaptic Transmission with Neuropeptides; Gene Therapy in Epilepsy; Viral Vector Approaches for Antiepileptic Gene Therapy.

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New Research on Biofeedback for Seizure Control

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Introduction

Over the last three decades, the behavioral treatment of epilepsy using different kinds of biofeedback has become increasingly considered; for the most part, results from such trials have been encouraging. In biofeedback, the subject is provided with on-line visual or auditory feedback based on a covert physiological signal, such as heart rate, skin conductivity, or brain wave pattern. This protocol can enable a subject to learn to control – actively – the physiological signal. A number of biofeedback signaling approaches have been suggested for the management of epilepsy, including different electroencephalographic (EEG) frequencies, EEG cortical potentials, and peripheral physiological responses such as respiration.

The galvanic skin response (GSR) is an accessible and sensitive index of centrally generated autonomic activity. It reflects changes in peripheral physiology through the sympathetic nervous system. Skin conductance increases with enhanced arousal level. The peripheral GSR activity is centrally regulated by a matrix of brain regions which are involved in interoceptive representations and homeostatic control. The manipulation of peripheral arousal using biofeedback has direct and indirect consequences upon central brain states. In contrast to other technically demanding biofeedback protocols for seizure control, such as EEG or respiratory control, GSR biofeedback training represents an accessible and easy-to-implement behavioral procedure.

Background

Slow Cortical Potential: Indicator of Cortical Excitation

Among biofeedback treatments, EEG-biofeedback using slow cortical potentials (SCPs –negative DC shifts recorded using surface EEG) has been shown to produce the most successful reduction of seizures in patients with drug resistant epilepsy. In healthy subjects, SCPs index specific cognitive processes, including alertness, attention, information processing, motor anticipation, and response preparation. However, in epilepsy, spontaneous SCPs are viewed as pathological signatures, related to interictal states and reduced seizure threshold. In an experimental model of epilepsy, chemical generation of an ictal focus elicits a corresponding cortical negative potential (SCP), and in patients with epilepsy, negative DC shifts on EEG are often observed clinically in anticipation of, and during, epileptic seizures. In both cases, increased cortical negative potentials reflect an overexcitability of neural pathways, predisposing to seizure generation. A mechanistic explanation argues that SCPs are related to depolarization of apical dendrites in cortical pyramidal cells, reflecting cortical excitation. Pharmacological treatment of seizures is frequently associated with a reduction in SCPs. Correspondingly, decreasing negative potential shifts through biofeedback may, therefore, attenuate cortical excitation to successfully reduce seizure frequency.

A slow cortical potential can be recorded during a simple behavioral procedure – the forewarned reaction time task. The subject is presented with a warning stimulus that indicates that, after a fixed interval, he/she should make a rapid motor response to a second (imperative) stimulus. In the time period between the warning and imperative stimulus, there is a slow negative shift in the EEG, the contingent negative variation (CNV) signal. The CNV has two components: An early wave appears immediately after the warning stimulus and reflects a cholinergically mediated orienting response. The later wave is related more closely to motor preparation and anticipation of the imperative stimulus, and reflects dopaminergic activity.

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Relationship Between Central and Peripheral Arousals

The relationship between peripheral and central arousals was investigated using the GSR and SCP/CNV as physiological parameters of interest. The CNV was measured at different physiological states during GSR biofeedback-induced aroused and relaxed conditions; GSR biofeedback was used to increase or decrease the subject's arousal level. It was found that there was an inverse relationship between GSR and CNV amplitudes – increases in peripheral sympathetic activity were associated with reductions in this EEG index of cortical neural excitation. That is, an increase in peripheral GSR activity was associated with less cortical excitation (**Figure 1**).

Neural Mechanisms Related to Central and Peripheral Arousals

A recent fMRI study identified brain areas involved in generation of the CNV in healthy subjects. Activity in thalamus and within limbic cortices, and motor and supplementary motor cortices (SMA) was correlated with CNV amplitude. CNV activity in some of these areas (cingulate cortex, SMA, and insula) was also correlated with peripheral GSR change. The results were consistent with the theory that amplitude of the CNV is regulated by a specific thalamocortical circuit, with a higher CNV amplitude related to increased information flow between thalamus and cortex. Together with the observation from the EEG study, these data indicate that modulation of peripheral GSR may influence thalomocorical circuits, and thereby change the epileptic seizure threshold. In the EEG studies, the reduction in CNV amplitude associated with GSR arousal was attributed to the regulation of sensory input to the cortex via the thalamic reticular nucleus (RN). Convergent evidence from animal studies

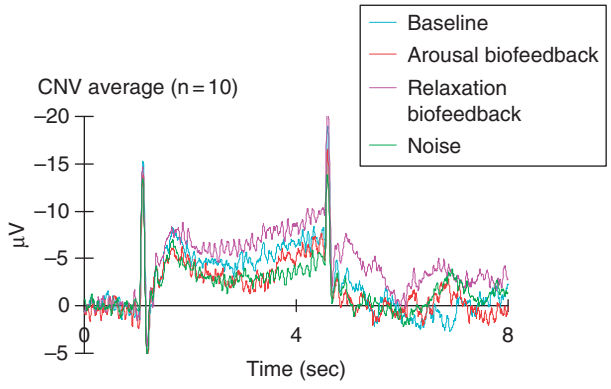


Figure 1 CNV amplitude during different physiological states. CNV amplitude increased with relaxation biofeedback. Arousal biofeedback and noise have a similar effect on CNV and reduced amplitude.

indicate that RN neurons, which are GABAergic (inhibitory), regulate sensory information in thalamocortical neural circuits, and therefore probably modulate seizure threshold by altering excitatory input to the cortex.

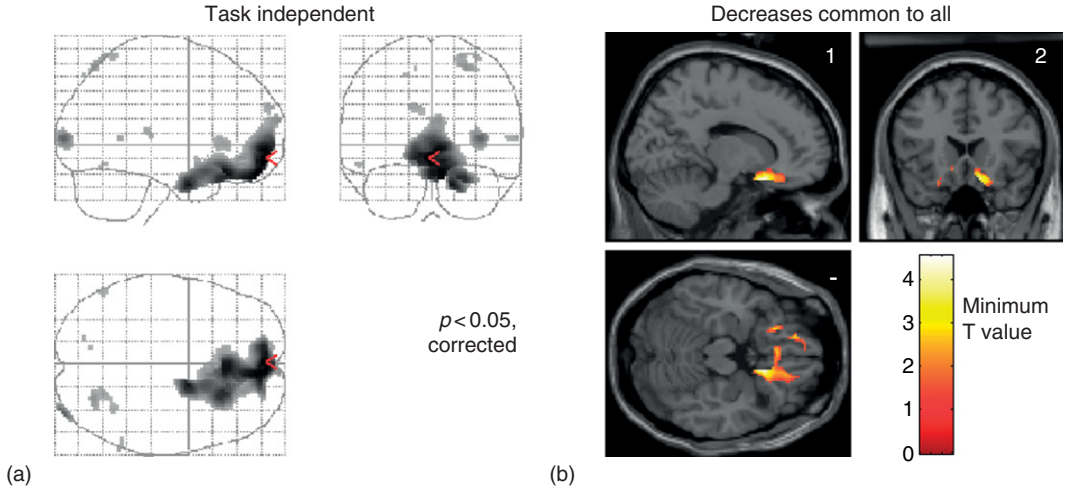
In other studies using fMRI, investigators identified brain structures involved in the regulation of peripheral GSR activity (**Figure 2**). There was a clear negative correlation between the level of sympathetic tone (GSR level) and activity within subgenual cingulate, and orbitofrontal and ventromedial prefrontal cortices. These brain regions are typically more active when individuals are in a restful and disengaged state, and are deactivated with attention and arousal. For therapeutic purposes, GSR biofeedback is given to patients to increase arousal level, and thus should enhance deactivation of these "default-state" brain regions.

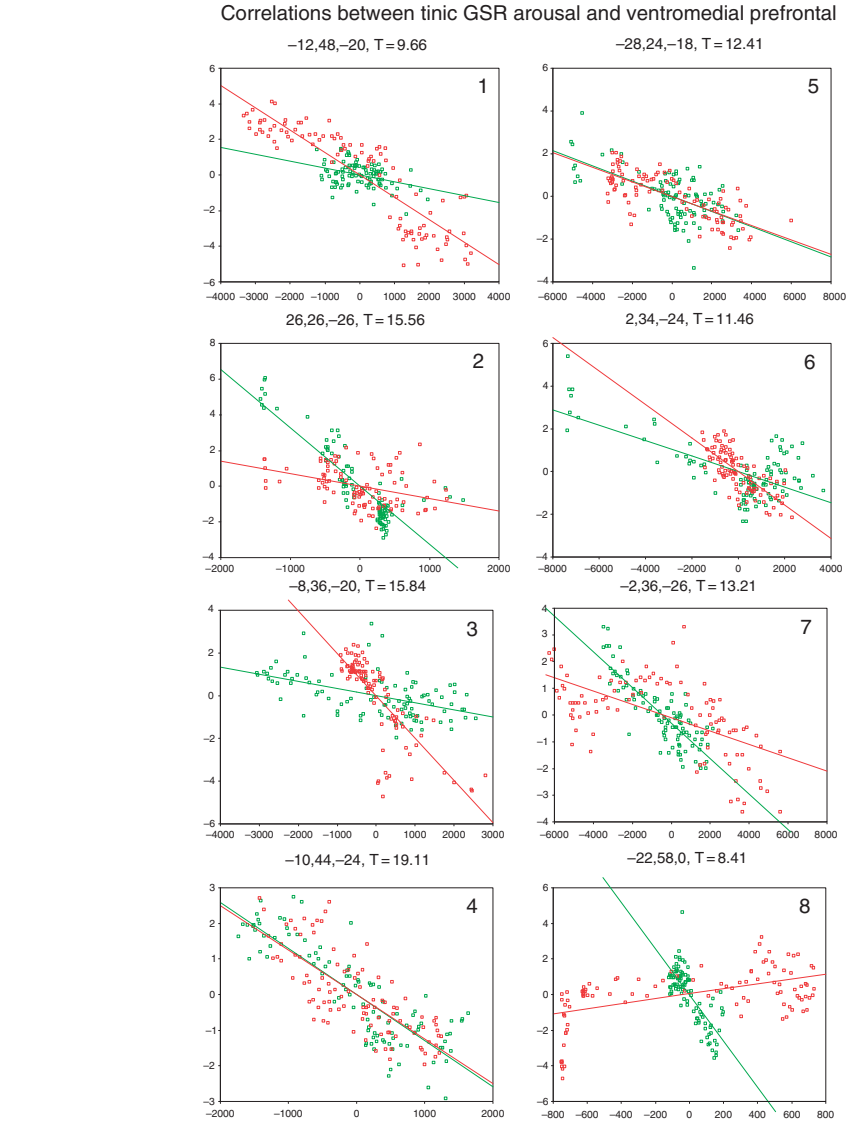
In summary, the peripheral state of arousal measured with the GSR was found to be inversely related to cortical arousal measured with the slow cortical potential (SCP) using a contingent negative variation (CNV) paradigm. This observation suggests that increased sympathetic arousal attenuates cortical excitability. We, therefore, hypothesized that training patients to increase GSR arousal through biofeedback training will lead to improved seizure control (**Figure 3**).

Methods

We conducted a preliminary, randomized, controlled study with 18 patients with drug resistant epilepsy. Patients were randomly assigned to either the active biofeedback group or the noncontingent biofeedback (control) group. Measurement of GSR and the implementation of biofeedback were undertaken using a biofeedback system (the Inner Tuner: Professional, Ultrasis Plc., London). Biofeedback took the form of the same computer-generated graphics in both control and biofeedback conditions, presented visually on a computer monitor in front of the patient at eye level. In the biofeedback active group, a reduction in the participant's skin resistance (i.e., GSR arousal) resulted in a series of animations (progressing from a picture of a fish, to a mermaid, to a woman, to an angel, and to a star – with rightward movement through the sequence) when the participant's GSR changed in the intended direction (**Figure 4**). Should the patients' GSR reverse its resistance, the display would return to an earlier animation form. Thus, in the biofeedback session, the subject watched the 'evolution' of the graphics as GSR sympathetic activity increased (skin resistance decreased).

In the control (sham biofeedback) group, patients were shown a video of the same computer-generated animation as was used in the active biofeedback condition. In the control group, the patients also tried to alter their GSR level using the sequence of video events on the computer





Pearson correlations between adjusted BOLD response in both tasks and GSR level
S1 −0.84, S2 −0.65, S3 −0.52, S4 −0.82, S5 −0.68, S6 −0.66, S7 −0.64, S8 0.28

(c)

Figure 2 (Continued)

screen. However, in contrast to the active treatment condition, in each sham control condition, the patient unknowingly watched a video animation which was prerecorded. The progress of the animation change was gradually increased, session by session, so that the patients in the control group would also have the impression of progress in each session. However, the animation changes were unrelated to the patient's own GSR change, as the GSR monitor was not connected to the computer generated animation.

The study was composed of three phases: a three month baseline period, a one-month treatment period in which the respective groups of patients were given either active biofeedback treatment or sham control biofeedback, and a 3-month follow-up period. In all phases, patients were asked to keep a careful record of their number of seizures (Table 1).

During the treatment phase, participants attended a total of 12 sessions (3 sessions per week). Each session lasted thirty minutes, during which patients assigned to the active treatment group received true biofeedback, and patients assigned to the control group received sham biofeedback. They were not informed as to whether they were given sham or contingent GSR biofeedback, but were all instructed to actively attend to and change the animation on the computer screen by increasing their level of alertness. After the completion of the treatment, each patient was asked to continue to keep careful seizure records for another three months and to practice the skill they learned in biofeedback session at home, without the biofeedback machines, preferably on a daily basis. They were also asked to use this same skill as a countermeasure when they were aware that a seizure was about to start.

The primary outcome measure of this study was the change in seizure frequency after a month of GSR biofeedback treatment, as a proportion of baseline seizure frequency. The secondary outcome measure was an increase in percentage GSR reduction over each treatment session which, in the biofeedback active group, provided an index of behavioral learning of GSR biofeedback skills over the 12 treatment sessions.

We hypothesized that the therapeutic effect of GSR biofeedback treatment would be physiologically based, and not merely a placebo effect of the behavioral intervention. We also predicted a significant reduction

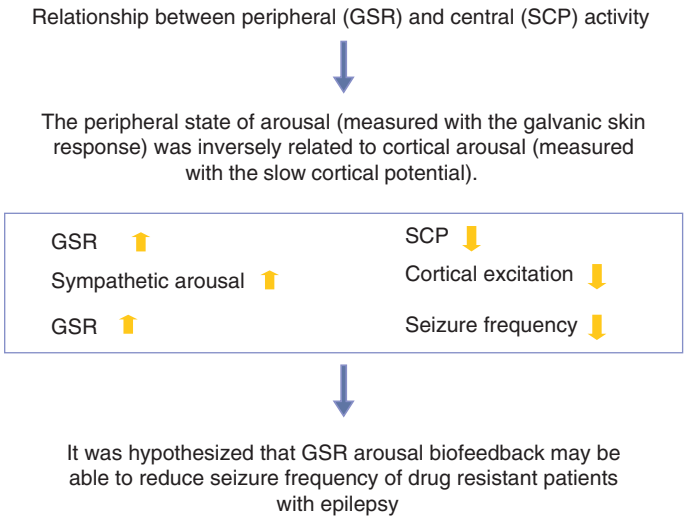


Figure 3 The figure illustrates the summary of background for the GSR biofeedback treatment for epilepsy.

Figure 2 Regional brain activity associated with decreases in skin conductance level. (a) Regional brain activity related to task-independent decreases in skin conductance level (SCL). Decreases in SCL were associated with increased activity in ventromedial prefrontal cortexVMPFC and OFC. A conjunction analysis was used to identify regional activity negatively correlating with across both biofeedback relaxation and arousal tasks ($p < 0.05$, corrected). (b) Regional brain activity associated with decreases in tonic skin conductance level common across all subjects (conjunction analysis associated with decreases in skin conductance), presented on a normalized template brain scan at ($p < 0.05$, corrected) for whole brain. The color scale represents the minimum *t*-value shared by all eight subjects contributing to the conjunction analysis. The common brain activity was found in ventromedial prefrontal VMPFC and orbitofrontal cortex OFC. (c) Plots of individual subjects data showing correlations between skin conductance and ventromedial and orbitofrontal BOLD activity during biofeedback relaxation (green dots) and arousal tasks (red dots). Data are plotted for the peak voxels of activity identified in first-level individual subject analyses. Pearson correlations showed significant negative correlation between adjusted orbitofrontal VMPFC and OFC BOLD responses and task-independent skin conductance SCL activity in the eight subjects.

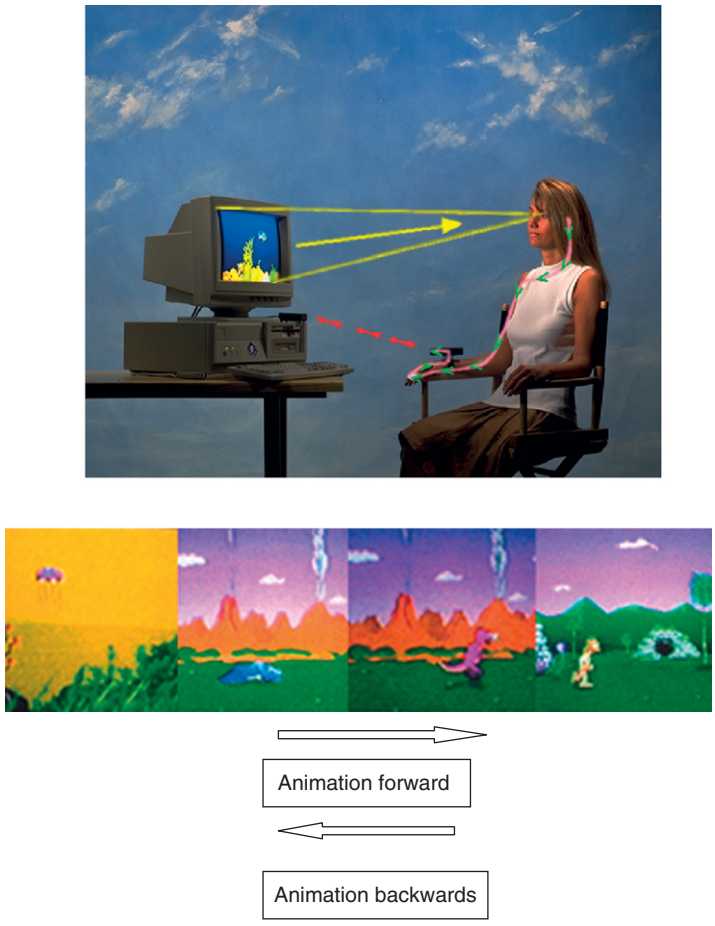


Figure 4 Setting-up of biofeedback and the animation change on the screen. An animation moves forward with decrease in skin resistance and backward with increase in skin resistance.

Table 1 Experimental condition

Before treatment	*Treatment*	*After treatment*
3 month, recording baseline seizure frequencies	1 month GSR biofeedback treatment 30 min session, 3 times/week for 4 weeks biofeedback and control	3 month, recording posttreatment seizure frequency + countermeasures

in seizure frequency after a month of treatment in the active biofeedback group – in contrast to no significant benefit observed in the control group.

Recent Results

Changes in Seizure Frequency

Patients who experienced the active GSR biofeedback intervention experienced a significant reduction in seizure frequency (anecdotally, also seizure intensity) that was not experienced by the control patients who received sham biofeedback ($p = 0.017$). In the biofeedback active group patients, a month of GSR biofeedback training was associated with a significant decrease in seizure frequency ($p = 0.01$). Out of 10 patients in the biofeedback group, six patients showed more than a 50% seizure reduction compared to their seizure frequency before the treatment. The mean percentage change of seizure frequency was −49.26% (SD = ±41.64, median = −56.4%) for the biofeedback group. One patient became seizure free. In contrast, there was no significant reduction seen in the control patients who received sham biofeedback training (mean percentage reduction of seizure +24.59% (SD = ±45.64, median = +9.53%)).

The change in the seizure frequency of six individuals showing a good response to treatment (response rate >50%) over 28 weeks (pretreatment for 12 weeks, treatment for 4 weeks, and posttreatment for 12 weeks) is shown in Figure 5. The response rate is calculated as the percentage of patients who showed more than a 50% reduction in their seizure frequency compared to their pretreatment frequency.

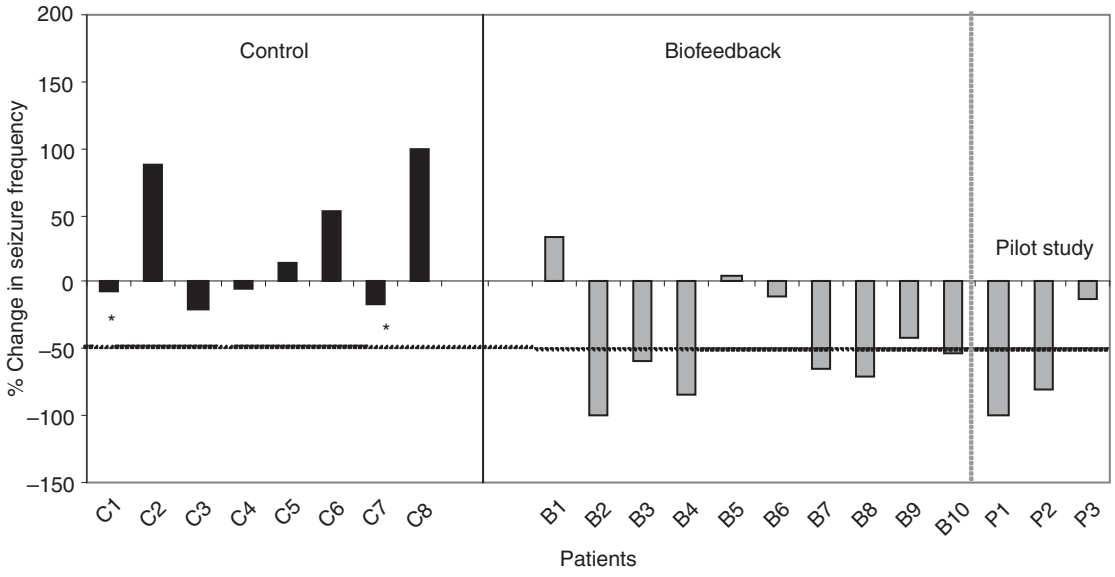


Figure 5 Percentage seizure frequency change after GSR biofeedback treatment in control and biofeedback groups. * Shows patients who dropped out in the middle of the training.

The following graph shows individual data of patients who received active biofeedback treatment (Figure 6).

Relationship Between Seizure Reduction and GSR Change

Patients in both the biofeedback active and control groups were given the same instructions to attend to the visual feedback display and try to influence the animation by increasing their alertness level. As a result, both groups of patients were able to change their GSR (by demonstrating an increase in sympathetic arousal). In fact, change in GSR reached significant levels both in the control and in biofeedback groups: biofeedback group ($p = 0.001$) and control group ($p = 0.028$). The mean percentage change in GSR was greater in the biofeedback group (mean% $\pm$ SD $= 42.58 \pm 30.16$) compared to the control group (mean% $\pm$ SD $= 25.27 \pm 26.9$).

The main difference between the patient groups was therefore the extent to which they learned to control GSR level through biofeedback, indicated by the degree of improvement of patients in GSR biofeedback performance. This control was broadly indexed by the average percentage GSR change over last three sessions compared to the first three treatment sessions. Patients in the active biofeedback group showed a significant improvement in performance of GSR biofeedback ($p = 0.007$), whereas patients in the control group demonstrated no significant improvement in GSR control with time (group difference $p = 0.004$). This observation is interesting as it means that developing the ability to modulate GSR arousal state through biofeedback training is the crucial predictive factor for seizure reduction. This relationship was further reflected in a significant correlation between reduction in seizure frequency and the degree of patients' improvement in GSR biofeedback performance, in both the biofeedback and control groups ($\rho = 0.736$, $p = 0.001$) (see Figure 7). Across individuals, the correlation between the magnitude of GSR reduction and seizure frequency did not reach significance, reinforcing the notion that the learning and implementation of the biofeedback technique were a greater therapeutic factor in reducing epileptic seizures than perhaps the overall change in GSR arousal itself.

In summary, the efficacy of GSR biofeedback compares extremely favorably with other treatments of refractory epilepsy where a 21–47% response rate is reported for new anti-epileptic drugs, and a 27% response rate is reported for vagus nerve stimulation. GSR biofeedback training has potential as a potent adjunctive nonpharmacologic means of reducing seizure-frequency in drug resistant epilepsy without obvious side-effects.

Future Goals

Finally, there are a number of important limitations to generalizing the findings of this initial patient study. First, the high demands of the study protocol made it difficult to recruit a large sample size. In addition, only one investigator was available to undertake the biofeedback/control sessions, and thus was not blind to the interventions being used for each patient. The patient group was not homogenous in terms of seizure types, and the study did not actively monitor the patients' compliance with their anti-epileptic drugs (e.g., using blood serum samples taken at regular

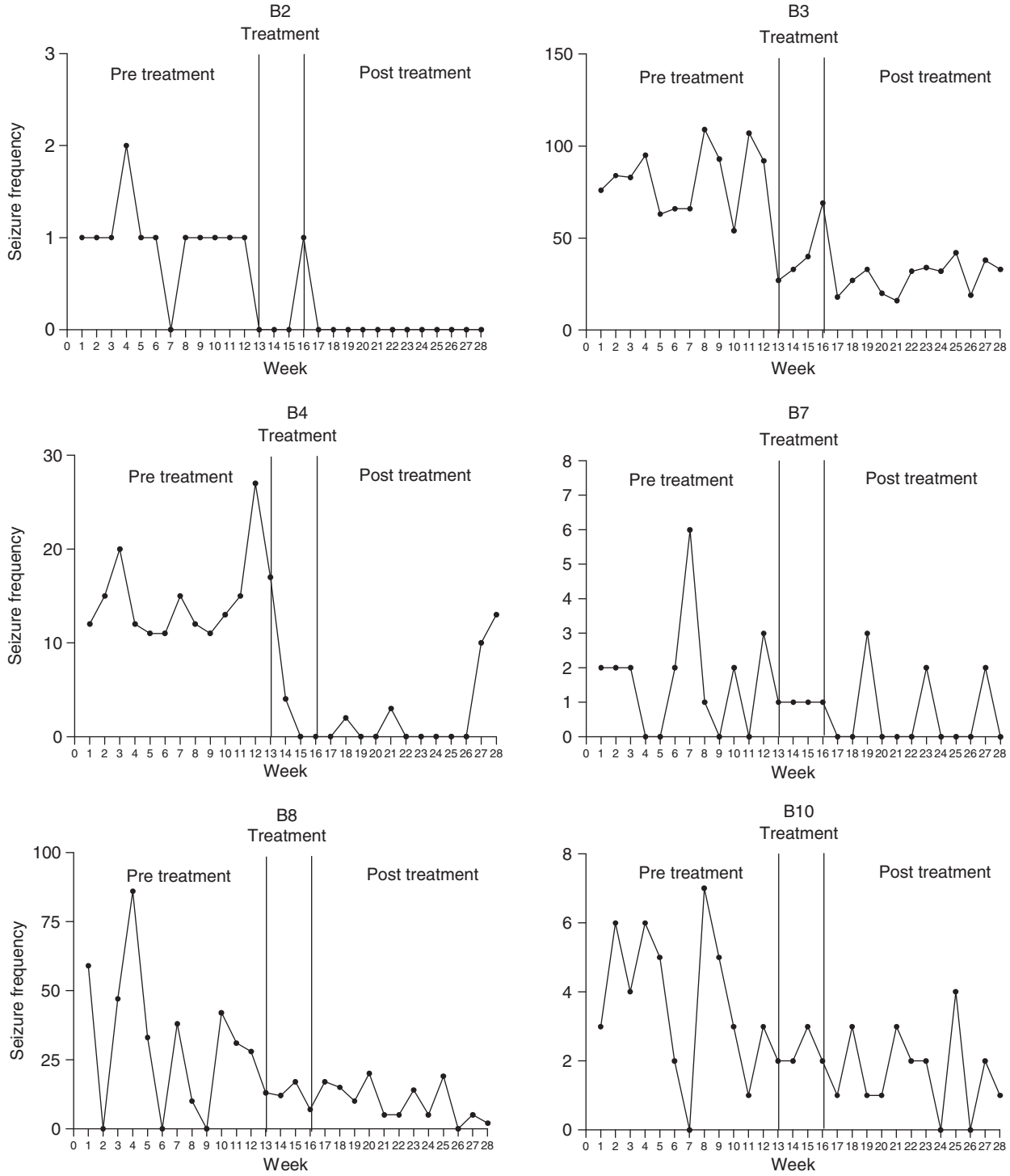


Figure 6 The figure illustrates the changes in individual seizure frequency change in patients over 28 weeks covering the period of GSR biofeedback treatment. The figure shows data only from those patients who showed a response rate >50%. The vertical axis indicates the actual number of patients' seizures.

intervals); this measure would have ensured that differences in compliance between the two groups did not account for the findings. While these limitations mean that caution is needed in data interpretation, the current study provides the basis for wider investigations applying this type of biofeedback to achieve significant seizure reduction. Certainly, the robustness of the findings, even in this small clinical group, is encouraging. Further work is needed to explore the effects of

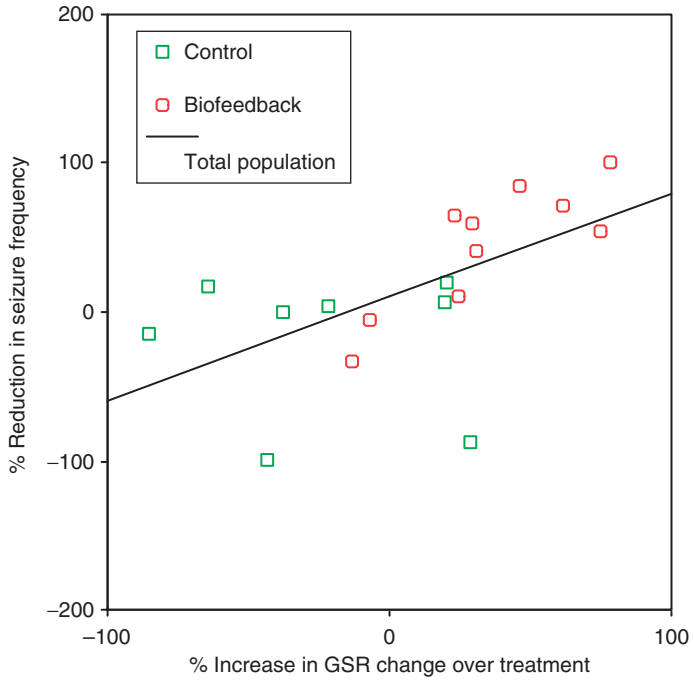


Figure 7 Correlation between seizure frequency change and performance improvement in GSR biofeedback.

biofeedback in a larger group of patients with refractory epilepsy, and in patients suffering from different seizure types, so that the therapy may be better targeted to those who are most likely to benefit. Our group continues to pursue a comprehensive understanding of the neural mechanisms, as revealed in EEG and fMRI analysis, through which GSR biofeedback reduces epileptic seizures.

See also: **Neurophysiology**: Seizure Detection and Prediction; **Psychiatric Symptoms**: Behavioral Hyperactivity and Psychiatric Symptoms Induced by Seizures; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; Autonomic Consequences of Seizures, Including Sudden Unexpected Death in Epilepsy; Seizure Prediction.

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Viral Vector Approaches for Antiepileptic Gene Therapy

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Introduction

The Rationale for Epilepsy Gene Therapy

Epilepsy afflicts about 2.5 million people in the United States, and now, anti-epileptic medication effectively controls seizures in about 70% of this population. For the remaining 30%, however, medications do not adequately control the seizures. In these intractable cases, surgical resection provides a final treatment option, but only if a circumscribed site of seizure genesis can be defined and does not impinge upon brain sites associated with critical functions, such as speech. With the emergence of gene therapy techniques, viral vector gene transfer and expression offers – in theory – an attractive, alternative treatment option for focal epilepsies. By definition, focal epilepsy arises from a circumscribed site of seizure genesis, so the localized nature of in vivo viral vector transduction should prove sufficient to influence excitability in those brain areas critical to the initiation of seizure activity. In addition, adeno-associated virus (AAV) vectors and lentivirus vectors exhibit a dominant neuronal tropism in vivo. Because it is generally believed that the excessive synchronous discharge by large populations of neurons arises from a decrease in inhibitory function, an increase in excitatory function, or both, the neuronal tropism of these viral vectors provides an opportunity to directly influence neuronal excitability. Certainly, the ability to transduce neurons provides a wide range of potential therapeutic targets.

Background

The Untoward Consequences of Variable In Vivo Tropism

Excitatory and inhibitory neurotransmitter receptors, as well as various ion channels regulate the excitability of neurons, providing what appear to be ideal targets for anti-epileptic gene therapy. However, our first attempts to regulate excitability using viral vector gene transfer uncovered an important liability inherent to in vivo viral vector gene transfer and expression. N-methyl-D-aspartic acid (NMDA) receptors influence seizure sensitivity, and a fully functional receptor requires the NMDA receptor 1 (NMDAR1) protein. Therefore, we reasoned that if one impaired expression of the NMDAR1 subunit protein, seizure sensitivity would be significantly reduced. Indeed using recombinant AAV vectors, we did find that transfer and expression of a 729 base pair NMDAR1 antisense reduced NMDA receptor function in vitro and significantly reduced NMDAR1 protein in vivo. Furthermore, using a CMV promoter to drive gene expression, in vivo transduction significantly attenuated focal seizure sensitivity. However, when expression of the same NMDAR1 antisense construct was driven by a tetracycline-regulated minimal CMV promoter, the seizure sensitivity actually increased, such that the animals were more seizure sensitive. The observation that NMDA receptor mediated excitation drives endogenous GABA inhibition provided a tenable explanation for these diametrically opposed results. In the case of the CMV promoter, primary seizure output neurons likely comprised the preponderance of transduced cells; thus, removal of NMDA receptor excitation blunted the seizure sensitivity. However, in the case of the regulated minimal CMV promoter, the preponderance of gene expression probably occurred in inhibitory GABA interneurons, so removal of the endogenous excitatory drive to these inhibitory interneurons increase seizure sensitivity. The likelihood of such an occurrence was validated when equal amounts of each AAV vector were administered together. Some neurons supported expression from both promoters, but significant numbers of transduced neurons exhibited transduction from only one or the other of the promoters. This difference could not be attributed to a virus-specific tropism, because all of the vectors were recombinant AAV type 2 viruses. Thus, a slight change in only the promoter evoked a substantial shift in the final outcome of the gene expression. These surprising findings markedly emphasize how differential vector or promoter tropism might markedly alter the ultimate therapeutic outcome. Certainly, a promoter's tropism could easily differ between experimental animals and humans. Likewise, it is possible that the tropism inherent to specific viral vectors may differ between experimental animals and humans. Thus, without some detailed knowledge of the transduction pattern in humans, any manipulation of neurotransmitter receptor proteins or ion channels could easily lead to heightened seizure sensitivity instead of the desired reduction of seizure sensitivity. Given the paucity of knowledge regarding viral vector tropism in the human CNS, such a therapeutic liability poses an unacceptable clinical risk.

The Focus upon Neuroactive Peptides

Neuroactive peptides provided another potential therapeutic target, particularly since several neuropeptides have been shown to exert anti-seizure activity in several

animal models. Numerous studies have shown that local infusion of the endogenous neuropeptides, galanin or neuropeptide Y (NPY), significantly suppresses seizure activity. Therefore, we reasoned that if one could express and constitutively secrete a neuroactive peptide, then as long as the appropriate receptor was present in the area of transduction, differential promoter or vector tropism would not influence the outcome. In general laminar proteins are constitutively secreted from a wide range of cells. In the case of the laminar protein, fibronectin, a secretory signal sequence traffics the fibronectin into a constitutive secretory pathway. Therefore, we constructed an AAV vector in which the coding sequence for the active galanin peptide was preceded by the secretory signal sequence of the laminar protein, fibronectin. Thus, the active peptide should be secreted from the transduced cell in a nonregulated fashion. Because it had already been shown that focal infusion of galanin significantly attenuated local seizure activity, the primary question was whether this approach could secrete enough vector-derived galanin to influence local seizure activity. Indeed, our studies demonstrated that the fibronection secretion signal sequence did cause constitutive secretion of the gene product from the transduced cells in vitro, and upon transduction in vivo, exerted significant attenuation of focal seizure sensitivity and blockade of seizure-induced cell damage. Furthermore, our studies demonstrated that effective seizure suppression could be obtained regardless of the promoter. Thus, as predicted, constitutive secretion of a neuroactive peptide circumvented the liability of differential promoter tropism. Because intractable mesial temporal lobe epilepsy presents the most likely gene therapy application, we subsequently showed that following AAV transduction, enough vector-derived galanin was secreted in the piriform cortex to essentially block both behavioral and electrographic seizures produced by systemic kainic acid administration. Clearly, this novel gene therapy approach proved quite effective in attenuating seizure activity in vivo.

Although these studies with galanin provided a proof-of-principle, we wished to explore how this approach might generalize to other neuropeptides, as well as receptor-specific neuropeptide fragments. Vezzani's laboratory previously showed that AAV mediated expression of preproNPY significantly attenuated seizure activity, but this effect was only robust when extensive, high levels of gene expression were obtained. Furthermore, such an approach assumes that the transduced cell is capable of processing the preproNPY and appropriately trafficking the NPY, such that vector-derived NPY is released upon neuronal stimulation. When we utilized the constitutive secretion construct with NPY, like galanin, transduction of the piriform cortex markedly attenuated kainic acid-induced seizures. In addition, expression and constitutive secretion of the NPY 2 receptor-specific fragment, NPY (13–36), also significantly attenuated kainic acid-induced seizures. Thus, these findings show that the constitutive secretion approach also can be used with receptor-specific neuropeptide fragments. Although other anti-seizure gene therapy approaches have been reported, at present the results with galanin and NPY provide the most promising gene therapy approach for the treatment of epilepsy. Several issues, however, remain to be addressed to optimize the chances for a successful clinical trial.

The Potential Impact of Pre-Existing Immunity and Seizure Pathology on Anti-Seizure Gene Therapy

Although neuropeptide gene therapy appears effective in animal seizure models, two major issues remain: pre-existing AAV immunity and pre-existing seizure pathology. Approximately 80% of the human population tests positive for antibodies to wild type AAV serotype 2 (AAV2) capsid proteins, and in 30–70%, antibodies are present that neutralize the AAV2 capsid. Although the blood-brain barrier normally prevents peripheral immune responses from gaining access to the CNS, site infusion of a vector would likely disrupt the blood-brain barrier, at least locally. Furthermore, it has been established that seizures can cause transient leakiness in the blood-brain barrier. If indeed the antibodies to AAV2 gain access to the CNS, then vector efficacy could be seriously compromised. For example, Mandel's laboratory has shown that when rats are immunized with AAV2, high levels of inactivating antibodies are found in the circulation, and subsequent infusion of AAV2 vectors into the striatum do not support expression of the vector-derived gene product. One means to avoid such an inactivation would be to use a different AAV serotype, and indeed the administration of a recombinant AAV serotype 5 (AAV5) vector circumvented this antibody inactivation. Given the range of different AAV serotypes, numerous options exist to avoid pre-existing immunity to AAV 2 – though one must insure that the tropism of the alternate serotype proves adequate for the particular gene therapy application.

A second issue involves seizure-induced neuropathology. In most studies, viral vector transduction precedes the seizure manipulation, a temporal sequence made necessary by the lag time between viral vector infusion and subsequent gene expression. In marked contrast, any clinical trial will involve viral vector treatment in brain tissue that has experienced chronic seizure activity. We have partially addressed this issue by showing that AAV mediated expression and constitutive secretion can still attenuate seizure sensitivity in animals that had been previously kindled to class V seizure activity. Electrical kindling, however, does not produce the degenerative neuropathology typically observed in chronic temporal lobe epileptics. Thus, it is crucial to demonstrate the

efficacy of viral vector transduction and gene expression in chronically seizure-exposed tissue.

To achieve immune evasion and optimal transduction of pathological brain tissue, studies must focus upon properties of the AAV vector, in particular properties conferred by the capsid proteins. As seen with the immunity studies, different AAV serotypes can evade immunity to AAV2 capsids, and to date, eleven different serotypes (AAV1 through AAV11) and over one hundred variants have been isolated from human and nonprimate tissue samples. A permutation in this serotype approach involves rational mutagenesis of the capsid proteins. By introducing specific point mutations in certain AAV capsid proteins, the tropism can be significantly altered. An example involves removing or introducing the ability to bind heparin. In the present context, though, the success of rational manipulation of the AAV capsid depends upon prior knowledge pertaining to the phenotypic traits of the capsid and/or structural information derived from previous crystallography studies. Unfortunately, we presently do not have *a priori* knowledge of the properties necessary to evade immune responses or optimally transduce neuropathological brain tissue. One means to circumvent this weakness is through the combination of DNA shuffling and directed evolution, where phenotypic traits can be selected in the total absence of any *a priori* knowledge.

Methodological Considerations

Vector Optimization Using DNA Shuffling and Directed Evolution

The rapidly developing fields of DNA shuffling and directed evolution provide an excellent means to select mutant AAV vectors that exhibit a range of specific properties. For example, Schaffer's laboratory recently introduced random changes in a single AAV2 capsid protein using error prone PCR and then selected those mutants that exhibited specific properties. By combining error prone PCR with directed evolution, they were able to identify unique AAV2 variants that exhibited marked reduction in heparin binding or evaded AAV2 immunity in vivo. These studies provided the proof-of-principle that directed evolution could be successfully applied to AAV.

Another means to introduce substantial diversity into AAV capsid proteins utilizes the rapidly advancing technique of DNA shuffling. Through this method, enormous diversity can be generated across a wide range of different AAV capsid proteins. Essentially, libraries of hybrid genes can be generated by random fragmentation of a pool of related genes, followed by reassembly of the fragments in a self-priming polymerase reaction. When this technique is combined with directed evolution, both single and multigene traits can be evolved rapidly even though they might require many mutations to achieve the improved phenotypes. As mentioned previously, we have no *a priori* knowledge of those properties that would optimize transduction of chronic seizure-exposed tissue. Thus, any attempt at rational mutagenesis would proceed in the absence of a guiding rationale. In contrast, DNA shuffling combined with directed evolution allows selection of those mutants that actually fulfill the desired phenotypic properties. We currently are pursuing this approach in order to identify the optimal AAV vector for anti-seizure gene therapy.

Recent Results

Recently, Samulski's laboratory has developed a combinatorial strategy involving DNA shuffling of AAV serotype capsid sequences to generate a library of AAV mutants,

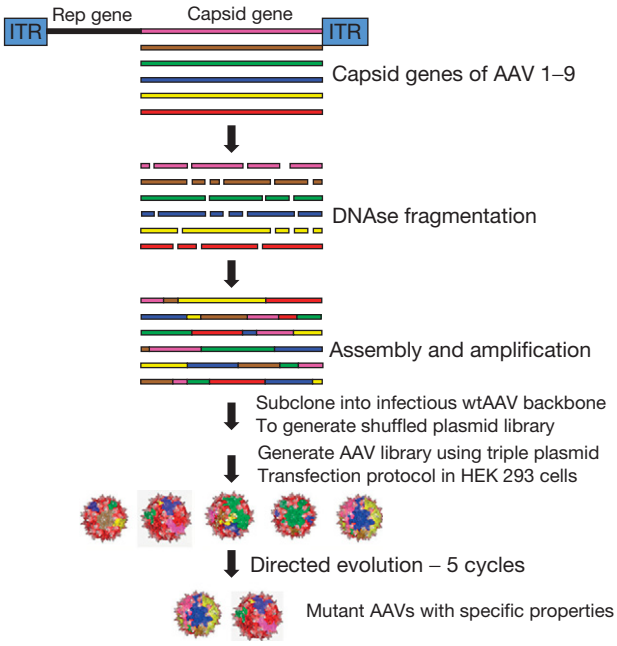


Figure 1 A flow chart illustrating the DNA shuffling/directed evolution procedure. Initially, the capsid protein sequences from AAV serotypes 1–9 are partially digested with DNAse creating fragments ranging in size from 400 to 1200 base pairs. The fragments are then assembled through a two stage PCR procedure and subsequently subcloned into an infectious wild type AAV backbone. The resulting plasmid library is packaged as virus using the standard triple plasmid protocol, and then the target tissue is infected with this AAV library. For in vitro applications, the infectious mutants are rescued by coinfection with adenovirus, but for in vivo applications, rescue is accomplished by PCR amplification, because wild type adenovirus is toxic and would only rescue those mutants in adenoviral permissive cells. This selection process is repeated for five cycles, leading to the identification of those mutants that most effectively infect the target tissue. Thus, by combining DNA shuffling and directed evolution, one can develop mutant AAVs that exhibit highly selective properties. (ITR-AAV inverted terminal repeats).

which provides the starting materials for directed evolution of cell type/receptor-specific AAV variants. With limited DNase digestion, followed by extraction of 400–1200 bp fragments, capsid regions of AAV serotypes 1–9 were successfully shuffled (see **Figure 1**). Then a mixture of fragments was subjected to PCR with Taq DNA polymerase, first without primers and then using a long template PCR system with forward and reverse primers. The purified PCR products were cloned into a wild type AAV2 plasmid backbone to generate a plasmid library with chimeric AAV capsid genes. Subsequently, a mutant AAV library was generated using established plasmid transfection protocols. The initial in vitro studies focused upon the CS1 cell line, derived from chinese hamster melanoma. The cells were infected with the mutant AAV library and then wild type adenovirus was added so as to rescue and replicate those AAV mutants that infected the cells. By repeating this process five times, an AAV mutant was identified that proved very efficient for CS-1 cell transduction. Sequence analysis and alignment with parental serotype sequences revealed a chimeric capsid subunit protein (VP1) derived from AAV1, AAV8, AAV2, and AAV9, so this mutant was termed AAV-1829. Obviously, this DNA shuffling, directed evolution technique proved capable of producing a unique AAV serotype.

When the tropism was explored in the rat CNS, quite surprising results emerged particularly in comparison with the established transduction patterns of the parental AAV serotypes (Li, Samulski and McCown, unpublished results). A recombinant AAV-1829 vector was produced in which a CMV promoter drives expression of the green

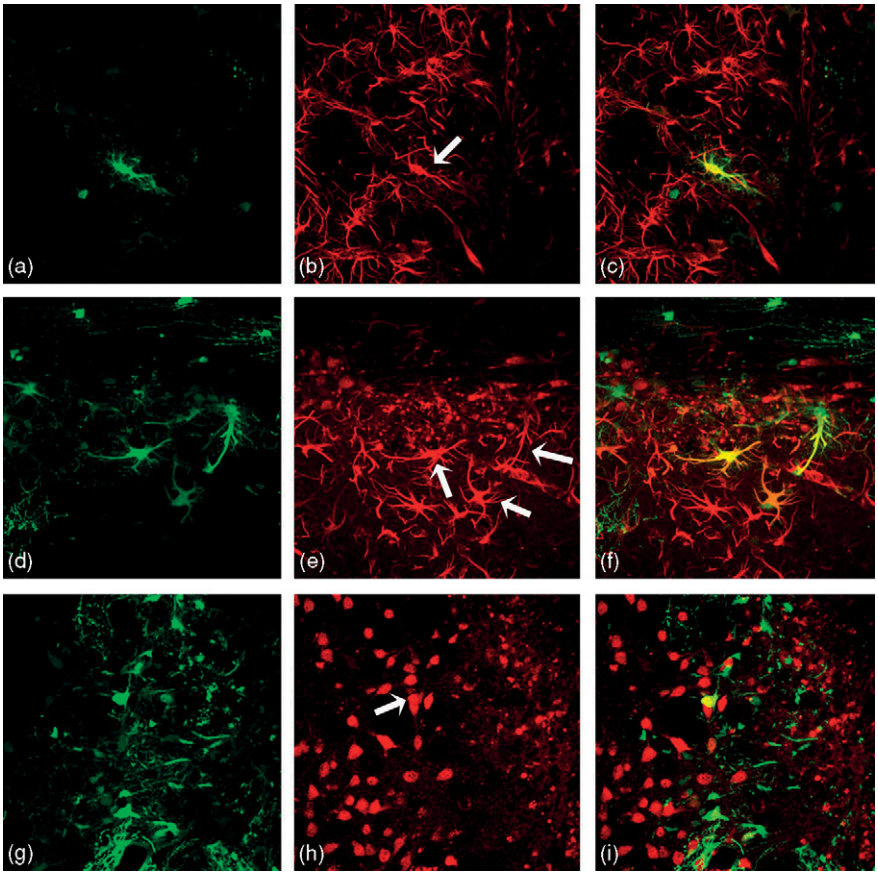


Figure 2 The CNS tropism of the mutant AAV vector, AAV-1829. Rats received a 2 μl infusion of AAV-1829-GFP vectors into the striatum, and 7 days later the brains were processed for immunohistochemistry. When immunohistochemistry was conducted for glial fibrillary acidic protein (GFAP), confocal microscopy identified numerous GFP positive cells that colocalized with GFAP-like immunoreactivity. Panel a shows a 2 μm slice through a GFP positive cell that also proved positive for GFAP-like immunoreactivity (panel b, arrow). Panel c shows the merge of a and b. More surprisingly, numerous GFP positive cells exhibited colocalization with nestin-like immunoreactivity, a marker of neuronally fated progenitor cells, reactive astrocytes or type I astrocytes. Panel d shows a 2 μm slice through GFP positive cells that also proved positive for nestin-like immunoreactivity (panel e, arrows). Panel f shows the merge of a and b. Finally, Panels g–I show the colocaliztion of a GFP positive cell with NeuN-like immunoreactivity, a marker for neurons. However, the number of GFP positive neurons were quite small in comparison with the total number of GFP positive cells. Thus, in direct contrast to the predominant neuronal tropism of AAV serotypes 1, 2, 8, or 9, AAV1829 exhibited a nonneuronal tropism in vivo. These findings demonstrate the ability of DNA shuffling/directed evolution to generate novel AAV mutants.

fluorescent protein (GFP) marker gene. One week after infusion of this AAV-1829-GFP vector into the rat striatum, the level of transduction was low and not particularly neurotrophic. However, many of the GFP positive cells colocalized with GFAP, a marker for astrocytes (**Figure 2 (a–c)**). More surprisingly, a large number of the GFP positive cells exhibited colocalization with nestin, indicative of either neuronally fated progenitor cells, reactive astrocytes, or type I astrocytes (**Figure 2(d–f)**). There were some GFP positive cells that colocalized with the neuronal marker, NeuN (**Figure 2(h–g)**), but these cells were in the clear minority of AAV-1829 transduced cells. These in vivo findings indicate that the DNA shuffling and directed evolution technique allowed the selection of a mutant AAV that exhibited a markedly altered the CNS tropism. Furthermore, the altered pattern of tropism appears quite unique in comparison with the CNS tropism of the parental AAV serotypes from which AAV-1829 was derived. Thus, this approach will allow future research to identify unique AAV mutants that fulfill specific properties within the CNS, such as optimal transduction of seizure-induced pathological tissue and evasion of AAV2 neutralizing antibodies.

Future Directions and Conclusions

Viral vector gene therapy provides a promising alternative therapy for intractable focal epilepsies. Our studies have established a novel gene therapy platform that not only has proven quite effective against seizure activity, but also circumvents several therapeutic liabilities. The expression and constitutive secretion of galanin, NPY, or NPY (13–36) markedly suppresses limbic seizure activity. However, further advances are necessary to optimize this therapy. To this end, we are using the techniques of DNA shuffling and directed evolution to identify optimal AAV vectors for anti-seizure gene therapy.

See also: **Peptides**: Galanin Modulation of Epileptogenicity; **Treatments (non-Antiepileptic Drugs)**: Epilepsy and Gene Therapy: Re-sculpturing Synaptic Transmission with Neuropeptides; Gene Therapy in Epilepsy; Immunoliposome Gene Therapy and its Potential use in Treatment of the Epilepsies.

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ANTIEPILEPTIC DRUGS

Contents

Clinical Drug Development in Epilepsy
Gender-Specific Side-Effects of Antiepileptic Drugs

Clinical Drug Development in Epilepsy

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Introduction

After rigorous, lengthy, and expensive preclinical testing, identification of appropriate drug formulations, and the development of a reliable manufacturing process, a fledgling antiepileptic drug (AED) is finally ready for clinical testing. Its further journey toward the marketplace will follow a well-circumscribed path, which is largely dictated by the demands of regulatory authorities, such as the Food & Drug Administration (FDA) in the United States and the European Medicines Agency (EMEA). For the most part, clinical trials are performed out of necessity; they may provide important information about efficacy and tolerability, but are predominantly focused on obtaining a license for the sponsor (pharmaceutical company). Regulatory trials are performed in series – from Phase I to Phase III – with little or no overlap, and with the results of one study informing the design of the next. A minimum number of trials is required to achieve regulatory approval but additional studies are often requested, particularly to clarify unexpected results. Such studies can add substantially to the duration of the development program, which currently costs in the region of $800 million for each new AED. Getting the development program right the first time is, therefore, extremely important to the sponsor. Phase IV studies and monotherapy trials are typically undertaken after the initial license (usually as adjunctive treatment for refractory localization-related epilepsy) has been obtained.

Basic Principles

There are a number of issues that need to be considered at the outset of all clinical development programs. The most significant are discussed in detail below.

Sample Size

One of the first considerations is sample size calculation or power. Bigger is invariably better. It would be unethical to conduct a trial with insufficient numbers of patients to demonstrate a statistically significant drug effect. However, it would be similarly unethical to expose large numbers of patients to an experimental treatment that is unlikely ever to reach the marketplace. In any power calculation, the investigator must first select the primary outcome measure and then estimate the size of the treatment effect. A power of 90% is typically employed in regulatory studies, with the cut-off for statistical significance usually set at $p < 0.01$. Where the required recruitment levels are regarded as unrealistic, it may be necessary to engage more investigators, to reduce the number of planned comparisons, or – if all else fails – to relax the statistical stringency.

Inclusion/Exclusion Criteria

Phase II and Phase III studies have strict inclusion/exclusion criteria. These criteria are selected to minimize unnecessary variability and potentially confounding factors in the study population, and to reduce the likelihood of adverse events being unfairly ascribed to treatment. Typical inclusion criteria for Phase III trials include: age between 16 and 65 years, uncontrolled partial seizures with or without secondary generalization, minimum of 4 seizures per calendar month with no seizure-free months, and ability to provide informed consent. Exclusion criteria are usually wide-ranging – for example, more than two or three concomitant AEDs, history of alcohol or drug abuse, psychiatric illness, pregnancy or possibility of pregnancy, single method of contraception (females only), nonepileptic attack disorder, previous status epilepticus, abnormal liver function tests, abnormal

electrocardiogram, progressive neurological disorder, etc. Strict inclusion and exclusion criteria create an artificial environment in which the efficacy and tolerability of a new AED is assessed in a cohort of patients that does not always accurately reflect the epilepsy population for which the drug will be licensed. So-called special populations, such as the elderly and females of child-bearing potential, are often excluded from regulatory trials. Individuals with infrequent seizures or concomitant comorbidities are similarly under-represented. Regulatory approval for use of the drug in children and for idiopathic generalized epilepsies requires additional, dedicated trials that are typically performed after initial marketing.

Randomization

Random allocation of participants to individual treatment groups is an important part of most clinical studies, regulatory or otherwise; the process minimizes bias and increases the probability that observed differences can be ascribed to treatment. In open-label studies, randomization eliminates any temptation to assign a specific treatment to a specific patient. In its simplest form, effective randomization can be achieved by coin-tossing (heads or tails), rolling dice (odd or even), assigning patients according to sequential order of enrolment, or using pre-sealed envelopes containing study group assignments. Automated telephone randomization is increasingly common, particularly in double-blind, placebo-controlled regulatory studies, and often employs a system of random permuted blocks, with stratification as necessary.

Blinding

Preconceptions or prior knowledge regarding efficacy and/or tolerability necessitate blinding of trial participants and/or investigators to minimize bias. Single-blind studies are those in which the patient is unaware of the treatment – whether drug or placebo – whereas the doctor has full knowledge. There is a significant risk of physician bias, and so single-blind studies are rare in the epilepsy field. Most regulatory trials are double-blind, so neither the participant nor investigator knows what treatment has been assigned. Double-blind studies allow objective assessment of treatment and accommodate the placebo-effect, but can be problematic when serious adverse events arise. On rare occasions, the blind may be broken to determine whether a life-threatening adverse event was likely to be treatment-related.

Control

Choice of an appropriate control is important in the design of clinical trials and can influence the outcome and the validity of the resulting observations. In studies employing a historical control, all patients receive the active treatment and pharmacological effects are compared to a baseline period. This is a common approach in open-label Phase IV studies and has the advantage that patients act as their own control. Placebo control is the gold standard for regulatory studies and is preferred for all Phase II and Phase III trials in epilepsy; patients are randomized to add-on treatment with either study drug or an inert tablet (placebo). A dose control design dispenses with the placebo arm and instead compares multiple doses of an individual drug in discrete groups of patients. Active control studies are typically reserved for monotherapy trials in newly diagnosed patients, where the study drug is compared head-to-head with an established agent.

Dose Selection

Selecting appropriate drug doses and titration rates in clinical trials is fundamental to their success or failure. Doses known to be effective in preclinical studies will inform the design of Phase I trials, which in turn inform Phase II, and then Phase III investigations. Thus, dose selection is refined throughout the development program. Interestingly, many modern AEDs are used clinically at doses and titration rates that are lower and slower, respectively, than were originally employed in pivotal trials. High doses achieved via fast titration rates tend to be more efficient in providing persuasive efficacy data for regulatory authorities.

Clinical Pharmacokinetics

Preliminary information regarding the pharmacokinetics of new compounds can be derived from preclinical studies and is often utilized in designing the Phase I program. Pharmacokinetic analysis continues through all stages of clinical development, with protocols being refined to accommodate new data regarding elimination half-life, time to peak concentration, food effects, presence of active metabolites, and routes of elimination. Short half-life drugs may need to be administered several times daily in order to maintain steady-state serum concentrations, whereas long half-life agents will require less frequent dosing. Once- or twice-daily dosing is preferred for all AEDs. Drugs that require more frequent administration may be associated with compliance issues and peak-concentration related side effects. Developing an extended-release formulation should be considered early with such molecules in order to optimize tolerability and ease of use. Separate pharmacokinetic studies in children are usually requested if a license for the drug is subsequently sought in this patient population.

Drug Interactions

Another important pharmacological consideration in clinical drug development is the potential for drug–drug interactions. These interactions can be a substantial impediment to the successful outcome of add-on trials unless

completely understood from the outset. Trials that include patients established on enzyme-inducing AEDs (particularly phenobarbital, phenytoin or carbamazepine) may underreport efficacy due to enhanced metabolism and lower serum concentrations of the study drug. In contrast, those performed in patients concomitantly treated with enzyme inhibitors (such as sodium valproate) may overreport side effects due to reduced metabolism and higher serum concentrations of the new AED. Most development programs include specific Phase II interaction studies so that potential problems are identified at an early stage. A detailed pharmacokinetic and metabolic profile is a statutory requirement for regulatory approval.

Analysis

Data generated from regulatory clinical trials are analyzed by the study sponsor. There are two principal approaches: intention-to-treat and per-protocol analysis. Intention-to-treat is the conservative approach preferred by regulators. It expresses efficacy and tolerability as a percentage of all randomized patients, or more often, of all patients who took at least one drug dose. Protocol violators are regarded as having not responded to the drug. In contrast, per-protocol analysis focuses on patients who reach a recognized end-point. For example, if ten patients start a clinical trial and six return for their first follow-up visit, four of whom are seizure-free, an intention-to-treat analysis will report a seizure-free rate of 40% (4 of 10), whereas the per-protocol figure will be 67% (4 of 6). Thus, per-protocol analyses tend to provide more favorable data and, arguably, provide a more realistic and clinically relevant view of the efficacy and tolerability of a new AED.

Trial Designs

There are two principal designs that can be employed in Phase II and Phase III trials in epilepsy; cross-over and parallel-group. Parallel-group trials are more commonly employed, predominantly because they permit the evaluation of multiple doses versus placebo in a single investigation and allow amalgamation of results from similarly designed protocols.

Cross-Over Studies

Cross-over studies (Figure 1) recruit a cohort of epilepsy patients, who are followed-up for a baseline period of approximately 8 weeks prior to randomization. Participants are generally randomized in 1:1 ratio to active drug or placebo, with titration over the subsequent 2–6 weeks. There then follows an evaluation period (usually 8–12 weeks), after which patients are tapered off their initial treatment. After a washout period of around 2–4 weeks, they are titrated onto the alternative treatment (active drug or placebo). There is then a second evaluation period, before the participants are either withdrawn from the study or given the opportunity to enter a long-term extension phase. The advantages of the cross-over design are that each subject acts as his or her own control, and that a smaller number of patients are required in comparison to parallel-group studies. The disadvantages are numerous. Cross-over studies are often of longer duration than parallel-group studies. There may be difficulty in incorporating multiple dosage arms and in dealing with drop-outs; patients who only complete the first evaluation phase contribute little to the analysis. There is also potential for unblinding, when the effects of the

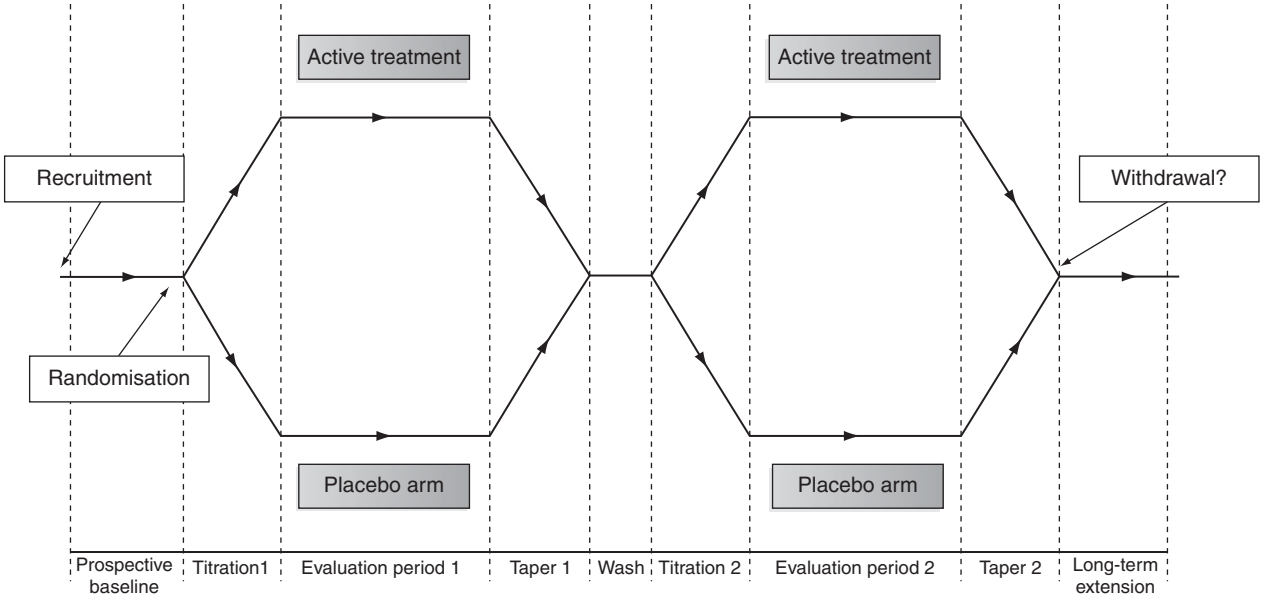


Figure 1 Schematic of simple cross-over design trial.

active drug are more obvious to the participants than those of placebo, and for carry-over effects between evaluation phases, particularly for drugs with long elimination half-lives.

Parallel-Group Studies

Parallel-group studies (**Figure 2**) recruit a cohort of patients who are followed-up for a baseline period of usually 8–12 weeks prior to randomization. These studies permit the inclusion of multiple active drug arms (allowing the assessment of multiple doses) in comparison with a single placebo arm (**Figure 3**). Titration is performed over approximately 2–6 weeks, depending on the pharmacokinetic and pharmacodynamic characteristics of the trial drug. The titration period is followed by a relatively short evaluation period (8–16 weeks), after which patients are tapered off their allotted medication and either withdrawn from the study or entered into a long-term extension phase. The advantages of parallel-group

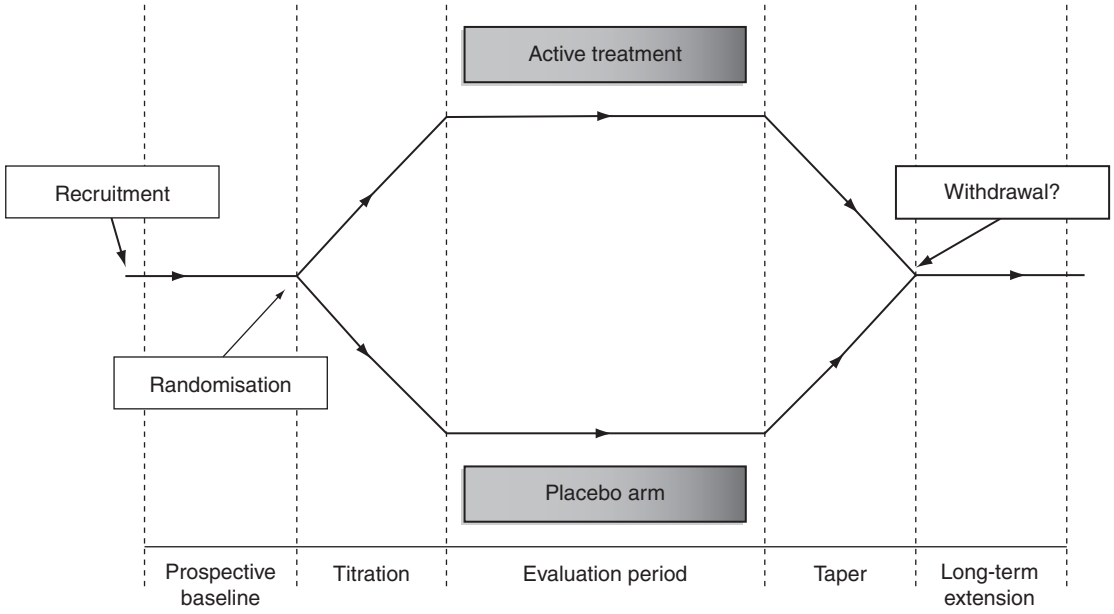


Figure 2 Schematic of simple parallel-group design trial.

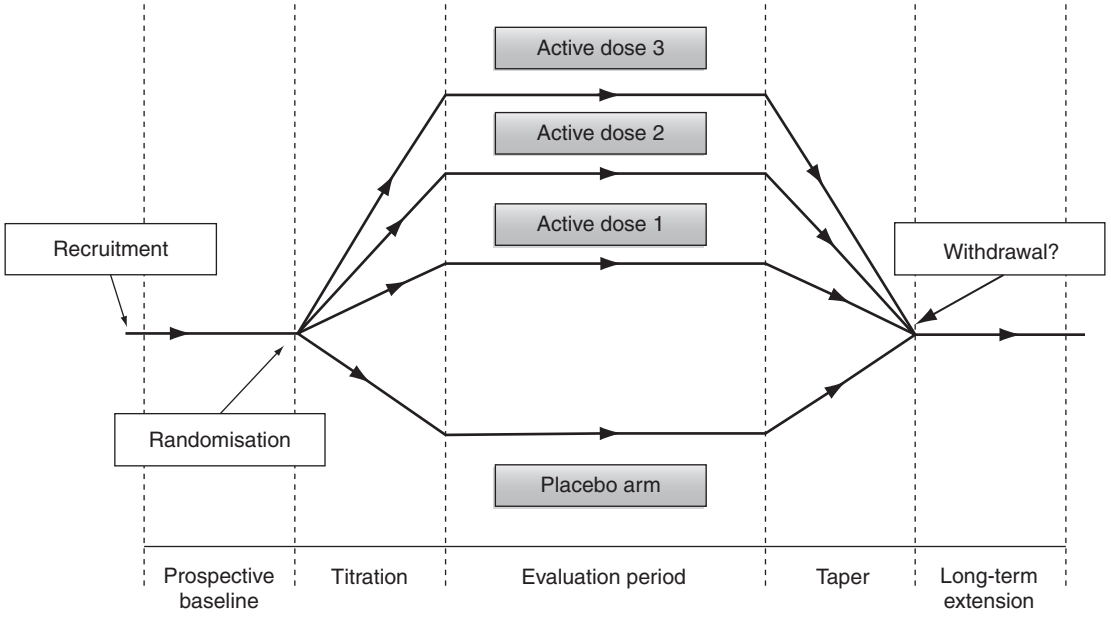


Figure 3 Schematic of multiple dose parallel-group design trial.

studies include relatively short duration, straightforward blinding, and the ability to incorporate multiple dosage arms. On the downside, these trials require a large number of participants and place an emphasis on equitable randomization.

Long-Term Extension Studies

Long-term extension studies follow Phase II and Phase III trials and allow patients to continue treatment with the study medication over a longer period, often several years. Participants who experienced genuine (or perceived) benefit from inclusion in the trial may opt to enter the long-term extension phase and will be tapered/titrated (depending on whether they were receiving active drug or placebo in the evaluation phase) to a predetermined dose of the active drug. This dose is then administered on an open-label basis, with dosage adjustments allowed according to clinical need. Those who failed to benefit from the trial are tapered off their allotted treatment (active drug or placebo) and either withdrawn from the study or given the opportunity to enter the long-term extension phase, on the basis that prior lack of efficacy may have been the result of receiving placebo in the double-blind evaluation period. Long-term extension studies provide the sponsor with valuable safety data which might not be readily apparent from the short evaluation period employed in most Phase II and Phase III studies. They also satisfy the demands of regulatory authorities who require efficacy and safety data on several hundred patients, with multiple years of drug exposure, when considering approval for marketing.

Phase I Trials

Phase I trials represent the first human exposure to any potential new AED and are largely performed in small groups of healthy volunteers. Some Phase I trials recruit a handful of individuals with refractory epilepsy, as patients will often have a different sensitivity to side effects than volunteers. Phase I trials are typically randomized, single- or double-blind, and placebo-controlled. They are predominantly concerned with tolerability and safety, the principal objectives being to establish the likely effective dose range, the maximum tolerated dose, and the pharmacokinetic profile (including early indications of pharmacokinetic interactions). They are often performed in dedicated clinical trial units where volunteers can be observed closely during early drug exposure.

Phase I trials can be subdivided into Phase Ia and Phase Ib. Phase Ia studies involve the single administration of multiple drug doses in a placebo-controlled, dose-escalation design in order to explore the limit of tolerability. Care is taken to avoid the simultaneous exposure of multiple volunteers to a high dose of any novel compound. Phase Ib studies are informed by the results of Phase Ia and typically involve daily drug administration over several weeks. The results of Phase I studies play a significant role in the design of subsequent trials, particularly in terms of dose selection and dosing interval. Where Phase I results deviate from expectation, there may be a requirement to reconsider the dose range (particularly where adverse effects occur at the lowest dose or where they are absent at the highest dose), a need to reformulate the drug (if significant peak concentration toxicity occurs), or even an obligation to abandon development altogether (where significant and insurmountable concerns about the bioavailability, pharmacokinetics or safety profile become apparent).

Phase II Trials

Phase II trials are randomized, double-blind, placebo-controlled, and represent the first use of the new compound in significant numbers of people with refractory epilepsy. The objectives are to establish tolerability and relative safety, to explore the pharmacokinetic profile in epilepsy patients, and to exclude any significant drug–drug interactions. Phase II trials can also provide preliminary evidence of efficacy – and while not often powered to do so reliably, this evidence may be sufficient for the sponsor to make a decision on whether to proceed further with clinical development. Phase II trials can be subdivided into Phase IIa and Phase IIb; the former is essentially a pilot study involving a limited number (~50) of participants, whereas the latter will often recruit several hundred epilepsy patients. This subdivision allows for protocol amendment and optimization.

Phase II trials typically take the form of multicentre, add-on studies, employing a cross-over (rarely) or parallel-group (usually) design with a long-term extension phase. These trials involve recruitment of adults with refractory localization-related epilepsies, experiencing four or more seizures per month, and taking one, two, or occasionally three other AEDs. Phase II studies in epilepsy are far removed from clinical practice. They employ fixed doses and fast titration rates, which allows for little interindividual flexibility and increases the likelihood of withdrawal due to poor tolerability. The short evaluation period does not provide any clinically meaningful information on efficacy and safety.

Phase III Trials

Phase III trials are scaled-up versions of those performed in Phase II, tailored according to the results of previous investigations and powered to confirm efficacy. They are known

as pivotal studies. A minimum of two such studies with broad geographical representation are required for regulatory approval. Each Phase III trial typically recruits around 1000 patients and is routinely performed as a multicentre, randomized, double-blind, placebo-controlled, add-on study in adults with refractory partial seizures, with or without secondary generalization. The principal objective is to demonstrate that the study medication is significantly more efficacious than placebo in reducing seizure frequency. Phase III trials also provide important information regarding tolerability in this setting and allow further investigation of safety, pharmacokinetics, and drug interactions.

Phase III studies generally have a multiple dosing parallel-group design, with a single placebo arm and two or three active treatment arms (which permit investigation of different doses and/or dosing intervals) and a long-term extension phase (**Figure 3**). The advantages of this design include the ability to demonstrate a dose–response relationship and the ability to identify the optimum effective dosage range. Problems include the fixed titration period (which invariably results in a greater number of drop-outs at the highest dose), the need for a large number of patients, and the dependence on good dose selection. Like Phase II trials, pivotal studies are an artificial paradigm; they recruit a highly selected patient population (often with severely refractory epilepsy), employ fixed doses and fast titration rates, and usually only provide evidence of efficacy for partial seizures with or without secondary generalization.

Submission and Approval

After completion of successful Phase III pivotal trials, the sponsor will prepare a detailed new drug application (NDA) for submission to regulatory authorities. This application includes information obtained at all stages of the drug development program, from basic physicochemical properties through preclinical testing, investigation of toxicology, details of manufacturing, and finally the results of all clinical trials, from Phase Ia to Phase III. There is an obligation to include all data (positive and negative) and to make results of individual laboratory studies and case record forms available for scrutiny. Sponsors are encouraged to seek publication of all Phase II and Phase III data in peer-reviewed journals, irrespective of the results, and investigators are required, by law, to retain individual patient records for a 15-year period. Scrutiny of the NDA and any additional material requested by the regulators can take upwards of 12 months.

Should the compound be approved for marketing, what has been learned by whom at each stage of the process? Preclinical studies suggest to the sponsor that the drug is likely to have efficacy and that there is a reasonable separation between effective and toxic doses. Phase I trials convince the sponsor that the new chemical entity is suitable for development as a potential AED. Phase II trials convince the sponsor that the drug is well-tolerated and that it may be effective clinically. Phase III trials convince the regulators that the drug is effective and relatively safe.

As far as prescribing physicians are concerned, once a new AED has been approved, they can be sure that it is better than nothing at all (i.e., better than placebo) in the treatment of refractory focal epilepsies, that it has good tolerability when used over short-periods at doses not exceeding those employed in the trial program, and that there were no significant safety concerns in the 1500–3000 patients exposed to date. Missing information at the time of approval includes: evidence of efficacy for specific epilepsy syndromes; efficacy in infants, children, the elderly and in patients with infrequent seizures; efficacy as monotherapy; comparative efficacy and tolerability versus established agents; optimum target doses and titration rates; the incidence of rare and occasionally life-threatening side effects (c.f. felbamate); and the full spectrum of clinically relevant drug–drug interactions. Much of this missing information will be accumulated subsequently through Phase IV studies and with clinical experience.

Phase IV Studies

Phase IV studies are typically performed in the postmarketing period, once the new drug has received its license as adjunctive therapy for partial seizures with or without secondary generalization in adults. They focus on issues that are not addressed during regulatory trials, such as efficacy for specific syndromes and special populations. Their design is highly variable; they are not constrained by any regulatory requirement and so can range from clinical audit to randomized head-to-head comparisons. There is little use of blinding or placebo-control in Phase IV because efficacy has already been established. These studies are pragmatic and may be considerably more informative in terms of everyday clinical relevance. Investigation of efficacy in other indications (most commonly neurogenic pain, migraine, anxiety, and bipolar disorder) can be undertaken at this stage, but nowadays these studies often overlap with the formal epilepsy development program.

Monotherapy Trials

Monotherapy is the gold standard of epilepsy treatment, and the vast majority of people with well-controlled epilepsy take a single AED. In contrast, polypharmacy has traditionally been associated with poor tolerability,

complicated drug interactions, and a greater degree of cognitive impairment. Although most new AEDs are initially licensed as add-on therapy in refractory localization-related epilepsy, many manufacturers seek a monotherapy license to increase market-share and encourage first-line use of their drug. Formal regulatory approval is required before such a license can be granted.

The regulatory requirements for a monotherapy license differ between Europe and the United States. The EMEA expects that the study drug will show equivalence to (or at least noninferiority to) an established AED at comparable dosage. This equivalence is usually investigated in a head-to-head comparison (active control study). The FDA takes the opinion that equivalence can mean equally ineffective as well as equally effective, and so insists that the new drug proves superior to placebo or to an established AED, irrespective of dose. A series of monotherapy designs has emerged in the United States that aim to prove that monotherapy with the new drug is better than nothing (placebo) or almost nothing (pseudoplacebo or low dose control).

Active Control

An active control monotherapy study compares the efficacy and tolerability of a new AED with an equivalent dose of an established agent in a randomized, double-blind manner, in people with newly diagnosed epilepsy. Around 50% of this population will respond to their first AED, usually at modest dosing, and so demonstration of superiority would require an unrealistic number of patients. Carbamazepine is the most common choice of comparator, and studies usually involve patients with partial and/or tonic–clonic seizures. Those with other idiopathic generalized epilepsies are specifically excluded. These studies allow a direct comparison without the confounding influence of concomitant agents, thereby negating any positive or negative effects of drug–drug interactions. Data on 6 and 12 month seizure free rates are now requested for monotherapy approval in Europe. Since this patient population typically experiences relatively infrequent seizures, these studies can take upwards of three years to complete.

Withdrawal to Monotherapy

Low dose control monotherapy trials have been designed to meet the demands of the FDA. A therapeutic dose of the study drug is compared with a minimally effective or subeffective dose (pseudoplacebo) of an established AED (often 15 mg kg^{-1} sodium valproate) in a randomized, double-blind design in uncontrolled patients already taking one or two AEDs. Alternatively, low and high doses of the study drug can be compared in the same design. Preexisting AEDs are tapered off, with the aim of maintaining patients on the new monotherapy. Data are typically analyzed on the basis of time to *n*th seizure or percentage of patients exiting the trial. The use of a low dose comparator ameliorates, to some extent, ethical concerns associated with placebo, affords some protection against catastrophic seizures, and provides a paradigm in which superiority can be demonstrated over a relatively short period of time. The use of a pseudoplacebo or a low dose comparator is ethically questionable. Accordingly, the latest version of this design involves comparison of high and low doses of the study drug, with historical controls from previous investigations. Again, one group of patients has to do better (or worse) than the other for the trial to be successful. Many patients withdraw from the study before preexisting AEDs can be successfully tapered off, and as a result, do not actually contribute to appraisal of the test drug as monotherapy. Another concern with this design is that any resulting monotherapy license permits use of the drug in patients with newly diagnosed epilepsy, even though it has not been studied in this population.

Presurgical Studies

Presurgical monotherapy studies enroll patients who have been admitted to specialist centers for assessment of their suitability for epilepsy surgery. In the efforts to localize the seizure focus in these individuals, existing AEDs are withdrawn under careful supervision. During this period, it is possible to undertake a short, randomized, double-blind monotherapy study. Efficacy is typically compared to placebo by measuring the time to *n*th seizure after randomization or the percentage of patients who remain seizure-free throughout the short evaluation period (often no more than 5–10 days). Unsurprisingly, this design has fallen out of favor, predominantly due to concerns about the very short drug exposure and potential interference with the surgical work-up, leading to extended in-patient time and/or failure to localize the seizure focus.

Monotherapy studies designed to satisfy the demands of regulatory authorities have significant limitations. They tend to employ fixed doses, fast titration, and an inflexible design which prevents interindividual adjustment of medication. It is difficult to establish efficacy in an ethical manner and beyond any statistical doubt. It may also be difficult to recruit patients with new onset of seizures, as involvement in research projects is not necessarily their priority when dealing with a potentially life-changing diagnosis of epilepsy. Traditional head-to-head monotherapy studies are unlikely to demonstrate superiority of a novel drug over an established agent at equivalent doses without recruiting an impractical number of patients. Withdrawal to monotherapy studies are ethically dubious; these studies often do not provide efficacy as

monotherapy, and include a patient population different from that in which the new AED will subsequently be licensed.

Novel Designs

The difficulty in proving superiority in monotherapy trials of AEDs has resulted in the search for innovative designs, such as the sequential analysis method. This approach differs from more traditional head-to-head comparisons by incorporating a series of interim analyses of emerging data, which allow for the early termination of the trial at the first sign of a statistically significant difference between treatments. Such designs are likely to require fewer patients than in traditional designs of equal power and can avoid continued recruitment of patients to a trial in which one treatment is evidently inferior. It has been applied in only one AED monotherapy study to date, but that study demonstrated the utility of this approach – to such an extent that it merits further consideration. The sequential analysis method was employed in a randomized, double-blind comparison of remacemide hydrochloride and carbamazepine. The study recruited newly diagnosed epilepsy patients aged 12–75 years, who had experienced two or more partial and/or primary generalized tonic–clonic seizures in the preceding 12 months. Subjects were randomized to either remacemide hydrochloride or carbamazepine in a 1:1 ratio, both being titrated over a 6-week period. Dosage adjustments according to clinical need were allowed, while maintaining the blind. The primary efficacy end-point was time to first seizure after completion of the titration phase. The trial was stopped at the second interim analysis, just 18 months after the first patient had been recruited, when it became apparent that carbamazepine was significantly more effective than remacemide hydrochloride in preventing seizure recurrence. Time to first, second, third, and fourth seizures all significantly favored carbamazepine. This trial, performed over a relatively short duration and including efficacy data on just 449 patients, is the first and only demonstration of superiority of one AED over another at therapeutic doses. Had this trial been performed as a traditional active control monotherapy comparison, it would have required more than 1000 patients studied over a number of years to demonstrate the same effect.

Summary

The clinical development of a new AED follows a well-defined path, through a series of trials that possess a recognized and rigid schedule, which is largely dictated by the demands of regulatory authorities and the desire of the pharmaceutical industry to obtain approval for marketing in as short a time period as possible with the minimum of expense. Phase I studies in healthy volunteers demonstrate tolerability and help determine dose range. Phase II studies in epilepsy patients advance knowledge of tolerability and pharmacokinetics, and offer a preliminary insight into potential efficacy. Phase III studies in large cohorts of people with refractory localization-related epilepsy are fundamental to the demonstration of both efficacy and tolerability; they are the pivotal investigations, two of which provide the minimum requirement for regulatory approval. Phase IV studies and pragmatic trials provide important information about the use of new AEDs in the real world; they serve to address many of the questions that remain unanswered by the development program. Monotherapy studies represent a different problem to add-on trials. Regulatory requirements are geographical, and the inappropriateness of placebo makes demonstration of efficacy as monotherapy more difficult. A number of trial designs have been employed to date but none has, as yet, emerged as the gold standard. Clinical development of novel AEDs is a lengthy and expensive process with no guarantee of success. There is little to suggest that it will become easier in time.

See also: **Antiepileptic Drugs**: Preclinical Drug Development in Epilepsy.

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Gender-Specific Side-Effects of Antiepileptic Drugs

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Introduction

Gender-specific side-effects of antiepileptic drugs (AEDs) affect both men and women at several stages throughout life. These side-effects may have a significant impact on various aspects of life, including pubertal development, fertility, sexuality and self-esteem owing to cosmetic problems, and (at older ages) menopause and bone health. In this article, we discuss the potential problems of chronic AED use at different stages of life, and describe potential side-effect problems of relevance to both men and women. Pregnancy issues and contraception will not be discussed here, as this topic has been reviewed recently in various publications.

Background and Recent Results

Puberty

There is a complex interaction between epilepsy, hormones, and AEDs in puberty, and the isolated effects of AEDs are therefore difficult to ascertain. Concerning the effect of AEDs on pubertal maturation, no effect on different indices of sexual maturation, including linear growth, was found in our study of girls with epilepsy taking valproate (VPA), carbamazepine (CBZ), or oxcarbazepine (OXC). We also studied the menarche age in 265 women with epilepsy debut before the age of 18 and on AED treatment. The study did not confirm clustering of epilepsy debut around menarche; however, the onset of epilepsy was more frequent in adolescents than in children. In contrast, two studies from Egypt have indicated a possible effect of AEDs on pubertal maturation in both sexes, including reduced linear growth. The effects were most pronounced in patients on polytherapy, which generally implies a more severe or active epilepsy than patients on monotherapy. It could therefore not be determined whether this reported effect is due to the drug, the epilepsy, or other factors. However, some animal studies on mice and goats suggest delayed pubertal maturation after chronic VPA treatment, a finding that should encourage further studies directed towards a possible effect of this drug on pubertal maturation.

As for gender-specific, late developing effects of antiepileptic drugs, two of our studies have shown that starting VPA in childhood and adolescence increases the risk of having polycystic ovaries later in life. A subsequent study in the same population of pubertal girls also showed similar findings, with increased frequency of polycystic ovaries and hyperandrogenism at a 5 year follow up. A recent publication verified a higher postmenarche serum testosterone level, but no signs of polycystic ovaries in VPA-treated girls with epilepsy. The possibility of late, gender-specific effects of VPA should therefore be born in mind when treating girls with this drug.

Reproductive Endocrine Dysfunction

Reproductive endocrine dysfunction is common in both women and men with epilepsy. In women, menstrual disorders, polycystic ovaries, and infertility have been described; in men, reduced potency and sperm abnormalities have been found. In both sexes, sexual problems and endocrine changes are frequent. The reasons for these problems are multifactorial, and both epilepsy and AEDs have been causally implicated.

It is well established that epilepsy itself may affect concentrations of reproductive hormones, which can lead to reproductive disturbances. Epileptic seizures are associated with increased secretion of pituitary hormones, including an increase in prolactin and LH pulse frequency (which is important for cycle regularity). Even laterality

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Gender-Specific Side-Effects of Antiepileptic Drugs

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Introduction

Gender-specific side-effects of antiepileptic drugs (AEDs) affect both men and women at several stages throughout life. These side-effects may have a significant impact on various aspects of life, including pubertal development, fertility, sexuality and self-esteem owing to cosmetic problems, and (at older ages) menopause and bone health. In this article, we discuss the potential problems of chronic AED use at different stages of life, and describe potential side-effect problems of relevance to both men and women. Pregnancy issues and contraception will not be discussed here, as this topic has been reviewed recently in various publications.

Background and Recent Results

Puberty

There is a complex interaction between epilepsy, hormones, and AEDs in puberty, and the isolated effects of AEDs are therefore difficult to ascertain. Concerning the effect of AEDs on pubertal maturation, no effect on different indices of sexual maturation, including linear growth, was found in our study of girls with epilepsy taking valproate (VPA), carbamazepine (CBZ), or oxcarbazepine (OXC). We also studied the menarche age in 265 women with epilepsy debut before the age of 18 and on AED treatment. The study did not confirm clustering of epilepsy debut around menarche; however, the onset of epilepsy was more frequent in adolescents than in children. In contrast, two studies from Egypt have indicated a possible effect of AEDs on pubertal maturation in both sexes, including reduced linear growth. The effects were most pronounced in patients on polytherapy, which generally implies a more severe or active epilepsy than patients on monotherapy. It could therefore not be determined whether this reported effect is due to the drug, the epilepsy, or other factors. However, some animal studies on mice and goats suggest delayed pubertal maturation after chronic VPA treatment, a finding that should encourage further studies directed towards a possible effect of this drug on pubertal maturation.

As for gender-specific, late developing effects of antiepileptic drugs, two of our studies have shown that starting VPA in childhood and adolescence increases the risk of having polycystic ovaries later in life. A subsequent study in the same population of pubertal girls also showed similar findings, with increased frequency of polycystic ovaries and hyperandrogenism at a 5 year follow up. A recent publication verified a higher postmenarche serum testosterone level, but no signs of polycystic ovaries in VPA-treated girls with epilepsy. The possibility of late, gender-specific effects of VPA should therefore be born in mind when treating girls with this drug.

Reproductive Endocrine Dysfunction

Reproductive endocrine dysfunction is common in both women and men with epilepsy. In women, menstrual disorders, polycystic ovaries, and infertility have been described; in men, reduced potency and sperm abnormalities have been found. In both sexes, sexual problems and endocrine changes are frequent. The reasons for these problems are multifactorial, and both epilepsy and AEDs have been causally implicated.

It is well established that epilepsy itself may affect concentrations of reproductive hormones, which can lead to reproductive disturbances. Epileptic seizures are associated with increased secretion of pituitary hormones, including an increase in prolactin and LH pulse frequency (which is important for cycle regularity). Even laterality

of epileptic activity may be of importance, as left-sided temporal foci seem to increase the occurrence of polycystic ovaries while right-sided foci increase the frequency of hypogonadotropic hypogonadism in women.

Effect of AEDs on Reproductive Endocrine Dysfunction in Men

Reproductive endocrine dysfunction in men with epilepsy seems to be, at least in part, related to the use of AEDs (Table 1). Enzyme-inducing drugs, in particular phenytoin (PHT), phenobarbital (PB), and carbamazepine (CBZ), reduce the amount of bioactive androgens, including lowering of DHEAS levels. The reason for this effect is primarily an increase in sex hormone-binding globulin (SHBG). In addition, elevated serum estradiol levels have been reported in men taking PHT, and high estradiol levels were found to be associated with sexual dysfunction in some men.

CBZ has been suggested to affect several semen parameters in both animal and human studies. In humans, this effect was first described in an *in vitro* model demonstrating reduced sperm motility. CBZ also inhibited testosterone formation at clinically relevant doses in an *in vitro* Leydig cell model. In two more recent studies, we have shown reduced sperm motility and an increased frequency of morphologically abnormal sperm in men on CBZ. Changes in sperm morphology are considered to have a predictive value for fertilization as well as significant relationships with several sperm function tests.

Valproate (VPA) does not induce liver enzymes. However, VPA does reduce serum gonadotropin levels and increases concentrations of serum androstenedione. This effect may be caused either by a centrally-mediated modification of GABA-ergic neurotransmission in the hypothalamus or by a direct effect of VPA on endocrine tissue, particularly on the testis. A direct effect on the testis is supported by animal studies demonstrating reduction in testicular size and altered testicular morphology after VPA treatment in dogs, rats, and goats. Similarly, small testicular volumes have also been found in humans taking VPA. Further, semen morphology and motility is affected by VPA. In humans, the number of motile sperm is reduced, and several morphological parameters are changed. In contrast to the findings with CBZ, we found that sperm from men treated with VPA have more tail abnormalities. Several studies of infertile men without epilepsy have shown these types of abnormalities; bent or coiled sperm tails were found to be significantly more frequent when a pregnancy did not occur. The sperm tail changes observed with VPA may therefore, at least theoretically, affect fertility. Carnitine is important for sperm motility, and reduced sperm motility has been noted in men on VPA, in whom VPA reduces the free versus total carnitine ratio.

Lamotrigine (LTG) has so far not been associated with reproductive endocrine disturbances in men with epilepsy or in non-epileptic animals. Few studies have been performed on the endocrine effects of newer drugs, and some drugs have not been studied at all in this respect. Some of our studies have suggested that oxcarbazepine (OXC) taken in high doses may affect serum testosterone and SHBG levels, and may also induce changes in sperm morphology. However, this finding must be confirmed in further studies before conclusions on the clinical significance of these possible reproductive effects of OXC can be drawn. No data are at present available for drugs such as felbamate, gabapentin, pregabalin, levetiracetam, tiagabine, vigabatrine, and zonisamide.

Table 1 Endocrine effects of antiepileptic drugs

Drug		*Men*	*Women*
EIAED	Tot. testosterone	↔	↔
	Tot. estradiol	↔(↑?PHT)	↔
	SHBG	↑	↑
	FAI	↓	↓
	DHEAS	↓	↓
	FSH	↔	↔
	LH	↔	↔
VPA	Tot. testosterone	↔	↑
	Tot. estradiol		↓(?)
	SHBG	↔	↑
	Androstenedione	↔/↑	↑
	DHEAS	↑	
	FAI	↔	↑
	Insulin	↑	↑
	FSH	↓	↔
	LH	↓	↔

The table shows the most frequently reported findings. Differences between studies do occur. EIAED: Enzyme-inducing antiepileptic drugs; VPA: Valproate; PHT: Phenytoin; FAI: total testosterone/SHBG × 100.

Effects of AEDs on Reproductive Endocrine Dysfunction in Women

Women with epilepsy experience menstrual cycle irregularities, polycystic ovaries, sexual problems, and reduced fertility. The epilepsy itself definitely contributes to these problems. In women even more than males, AEDs also seem to play an important role (Table 1).

As in male subjects, enzyme-inducing drugs reduce free fractions of steroid hormones, increase SHBG, and may also lower DHEAS. No consistent abnormalities in basal or stimulated serum gonadotropin or prolactin levels have been reported. The clinical consequences of these endocrine changes are unknown. So far, no large study has demonstrated any increased frequency of menstrual disorders or other reproductive endocrine disorder in women treated with these drugs.

The use of VPA has been associated with menstrual disturbances, hyperandrogenism, and polycystic ovaries.

The first, large study to investigate this in some detail was a cross-sectional study from 1993. Altogether, 238 women participated in the study. The major finding was that menstrual disorders were common among women taking VPA monotherapy for epilepsy (45%), and that this effect was frequently associated with polycystic ovaries (PCO) and/or hyperandrogenism. Serum mean androgen levels were significantly increased in women on VPA. We later obtained similar findings in a three-centre study conducted in three European countries (Finland, Norway, Netherlands).

In the original paper from 1993, Isojärvi and coworkers found that PCO and hyperandrogenism were especially common if VPA medication was started before the age of 20. This age dependency was later confirmed by others. Further, increases in serum androgen levels were detected before and during pubertal development in young girls taking VPA for epilepsy. A five year follow-up of these girls showed that 60% of the girls/women who were on VPA (during the follow-up study) had polycystic ovary syndrome (PCOS) as compared to 25% of girls/women taking other AEDs, 5.5% of girls whose medication had been discontinued, and 8.3% of control subjects. These observations indicate that the young ovary is vulnerable to the effects of VPA.

While several human studies have supported an effect of VPA on reproductive endocrine function, others have not shown any differences between CBZ- and VPA-treated women with epilepsy with regard to reproductive endocrine parameters and PCOS. The interpretation of these latter results is difficult because the patients were older, and the duration or doses of medication, or seizure frequency in the different treatment groups were not given.

It has been suggested that obesity and associated hyperinsulinemia could be implicated in the development of PCO and hyperandrogenism in women taking VPA. However, PCO and hyperandrogenism were also found in many lean VPA-treated women without hyperinsulinemia, and hyperandrogenism in girls taking VPA for epilepsy was not associated with hyperinsulinemia. It does appear, however, that obesity and related hyperinsulinemia may exacerbate the VPA-related reproductive endocrine disorders in women with epilepsy.

The original observation that VPA induces reproductive endocrine changes is also supported by other reports. First, the effect seems to be reversible after withdrawal of VPA treatment. Second, similar findings have been observed in women treated for bipolar depression. Third, our studies in non-epileptic animals have demonstrated both ovarian morphological and endocrine changes after VPA treatment.

With regard to our animal studies, long-term VPA treatment of adult rats induced endocrine and ovarian morphological changes. Female Wistar rats were fed perorally with either 200 or 300 $mg\,kg^{-1}$ VPA, 5 $mg\,kg^{-1}$ LTG, or control solution twice daily for 90 days. A significant, dose-dependent increase in the number of follicular cysts in the ovaries after VPA treatment was observed, together with a reduction in the number of corpora lutea. No effects were observed after LTG treatment. A detailed analysis of a series of other tissues, including liver, kidney, pancreas, lymphatic tissue, heart, and brain did not reveal any changes. The study thus showed that the effect of VPA was drug-specific, independent of epileptic activity, and with the ovaries as a possible target organ. This view is further supported by another study showing that VPA (at a total dose of only 300 $mg\,kg^{-1}\,day^{-1}$ for 30 days) treatment in rats leads to an increase in the number of cystic follicles in the ovary.

Long-term VPA treatment in female non-epileptic rats also markedly increased the testosterone-to-estrogen ratio, mainly by decreasing estrogen levels, with no or minor effects on gonadotropins. To investigate in more detail whether this effect is related to a direct action of VPA on follicular steroidogenesis, we studied the secretion of testosterone and estradiol from isolated porcine ovarian follicles. Using VPA concentrations ranging from approximately 600 to 1500 $\mu mol\,l^{-1}$, we showed that VPA increased testosterone and reduced estradiol secretion, and reduced the conversion of testosterone to estradiol at therapeutically relevant concentrations. These observations point to a direct effect of VPA on steroidogenesis in the ovary. A direct effect of VPA is further supported by the findings of Hattori et al. from 2000, demonstrating the presence of the enzyme microsomal epoxide hydrolase (mEH) in human ovaries – an enzyme which is important in detoxification of several substances. Several studies have shown that VPA inhibits mEH activity. Hattori and coworkers showed that human granulosa cells expressed mEH and that the inhibition of mEH suppressed conversion of testosterone to estradiol. Given that VPA is a mEH inhibitor, this may be a mechanism by which VPA reduces serum estrogen levels and thereby increases the testosterone-to-estrogen ratio. This VPA effect would lead to an androgen-dominant microenvironment in the ovary and then possibly to polycystic changes without an increase in LH levels. However, in a study on Rhesus monkeys, no effects of 12–15 months of VPA treatment were found on endocrine function, cycle regularity, or ovarian morphology. However, only seven animals were examined in that study, and it was unclear whether the animals received therapeutic drug concentrations for a sufficient period. Further, the study did find a trend toward increased testosterone-to-estrogen ratio, lower estrogen levels, and increased LH/FSH ratio. In addition, body weight was significantly increased.

LTG has so far not been associated with endocrine side-effects in either humans or in animal studies. Replacement of VPA with LTG resulted in normalization of endocrine function during one-year follow-up in women with a previously identified reproductive

endocrine disorder likely related to VPA medication. This result suggests that LTG therapy is not associated with changes in endocrine and metabolic functions in women with epilepsy.

Effects of Newer AEDs on Reproductive Endocrine Dysfunction

Among the newer drugs, levetiracetam (LEV) is of special interest since it binds to the synaptic vesicle protein SV2A. SV2A is widely distributed in the nervous system and also in endocrine tissue. If LEV binds to SV2A vesicle protein in the ovary and/or in the pituitary gland or hypothalamus, this action may provide a link between the drug and endocrine function at either a central or peripheral level. In a recent paper, we showed for the first time that LEV affected basal, but not gonadotropin-stimulated testosterone and estrogen secretion from porcine ovarian follicular cells. This observation suggests that LEV may affect reproductive endocrine function. The clinical consequences of this preliminary observation are difficult to assess at this time, but it is possible that in humans LEV could only affect reproductive endocrine function in prepubertal female subjects with low serum gonadotropin levels, whereas fertile-aged women might not be at risk because the ovaries are stimulated by higher levels of gonadotropins. It is too early to draw any firm conclusions on the possible clinical relevance of this observation in an in vitro model. Studies in women with epilepsy on LEV are warranted.

Recently, two papers have indicated possible endocrine effects of topiramate. In females, topiramate (100 mg kg^{-1}) for 12 weeks reduced fertility and ovarian weight. The serum concentrations were not measured and the clinical relevance of the observations is uncertain. It does underline, however, the importance of studying all antiepileptic drugs more closely with regard to possible endocrine effects.

Bone Health

People with epilepsy appear to have a 2–4 times increased risk of fractures compared to the general population. Postmenopausal women are the most vulnerable group in relation to untoward effects of drugs on bone health due to their estrogen deficiency. Reduced bone mineral density at the femoral neck has indeed been shown in postmenopausal women on AEDs, and more than 50% of all postmenopausal women taking AEDs have experienced fractures. In a prospective study, AED use increased bone loss at the heel and hip in women older than 65 years. The bone loss was sufficient to increase the risk of hip fracture by 29% over 5 years.

PHT, primidone, and phenorbarbital, the old liver enzyme-inducing AEDs, have most commonly been related to disturbances in bone metabolism, but CBZ and the non-inducer VPA have also been found to affect bone health. For new drugs, there is little information on this topic. Preliminary data indicate that LTG does not cause bone loss, while drugs which inhibit carbonic anhydrase (topiramate, zonisamide, acetazolamide) may theoretically affect bone health by causing a metabolic acidocis.

The mechanisms underlying the bone-depleting effects of AEDs are still only partly known. For many years, the main theory was that enzyme-inducing AEDs (e.g., phenobarbital, phenytoin, carbamazepine) caused an accelerated vitamin D hydroxylation to inactive forms, resulting in hypocalcemia, secondary hyperparathyroidism, increased bone turnover, and higher rates of bone loss. However, later studies have shown that AEDs may cause bone loss in the absence of vitamin D deficiency. Also, resistance to parathyroid hormone, calcitonin deficiency, interference with vitamin K metabolism, decreased intestinal absorption of calcium, and a direct drug effect on osteoblastic function have been suggested. Changes in markers of bone formation and resorption indicate that drug-induced increased bone turnover may play an important role. Enzyme-inducing AEDs may also increase the metabolism of sex steroids, with resulting decreased levels of bioavailable endogenous estradiol and testosterone; an increased rate of bone loss may therefore be a consequence of this increased steroidal metabolism.

Surprisingly few animal studies have been performed to investigate the mechanisms underlying the effect of AEDs on bone. For that reason, we performed a series of experiments in rats. Non-epileptic animals fed with LEV, PHT and VPA for 3 months were studied. PHT and VPA reduced bone mineral density (BMD) and content (BMC) in one or more bone compartments. VPA induced increased bone turnover, whereas modest changes were observed for PHT. LEV did not affect bone mass density or content in these animals. Surprisingly, low dose LEV was associated with reduced biomechanical strength and reduced levels of the bone formation marker, osteocalcin. Histomorphological analyses indicated retention of cartilage. These results demonstrate that AEDs may affect bone health by affecting bone quality, not only bone mass density – which is the parameter studied in the clinic. It is difficult to interpret the clinical relevance of these observed changes, but our findings emphasize the importance of using animal models in trying to reveal the possible mechanisms of action of the different drugs on bone.

Menopause

Little is known about the relationships between menopause and epilepsy. There are indications that epilepsy may affect the time of the menopause, as recent studies have shown that women with epilepsy may reach

menopause at an earlier stage than women in the general population. This effect has been attributed more to the epilepsy itself than the use of AEDs. With regard to seizure frequency, there is generally an increase at perimenopause but a decrease at menopause, especially in those women who had experienced a catamenial seizure pattern during their reproductive years.

Hormone replacement therapy (HRT) to women with epilepsy seems to increase seizure frequency. In a double-blind, randomized, placebo controlled study, a marked dose-related increase in seizure frequency was observed in postmenopausal women with epilepsy when taking HRT in the form of a combination of conjugated estrogen and medroxyprogesterone. This treatment also seemed to decrease serum LTG levels. However, the current understanding of the impact of HRT on epilepsy in post menopausal women is limited. Initiation of hormonal treatment must be thoroughly discussed between the physician and the patient to highlight the advantages and risks of the treatment. If HRT is considered necessary, a combination of a single estrogenic compound (such as 17-β-estradiol) with natural progesterone has been suggested.

Sexuality

The quality of human life is a summation of many factors, and sexuality is – for both men and women – of the greatest importance. Several studies have shown that sexual problems are common in people with epilepsy. It has been estimated that some kind of sexual problem is present in 20–50% of patients with epilepsy in both sexes. A wide range of different problems have been described.

The reasons for the sexual problems are multifactorial, and include psychological, epileptological and medicational issues. The specific role for the different AEDs is therefore difficult to ascertain. In general, enzyme-inducing AEDs will reduce free fractions of peripheral sex steroid hormone concentrations, a change which is well known to affect sexual function (and in men, also potency). Decreased libido and anorgasmia have been reported in women treated with VPA for bipolar disorder. This finding has been attributed to the increased serotonergic transmission associated with VPA treatment. A few cases of anorgasmia have also been described with gabapentin treatment. LTG, however, was shown not to affect sexual function.

AEDs can affect sexuality through different mechanisms. First, certain AEDs induce liver enzymes which lead to reduction of free fractions of peripheral sex steroid hormones. Second, a more general, depressive effect of AEDs on brain excitability may have a negative impact on sexual functions. It is possible that the more sedating AEDs are more harmful in this respect than some of the newer, less sedating AEDs. Further, a negative impact of AEDs on cognitive function may also be important in this respect. More research has to be done in this field to better understand the possible impact of different AEDs on sexuality and to elucidate the underlying mechanisms.

One possible option for treating sexual problems in epilepsy has been offered by Harden and MacLusky. A sixty-one year old man with epilepsy complained of reduced libido. He also had low serum testosterone levels. He was treated with an aromatase inhibitor which blocks the transformation of testosterone to estradiol. As a consequence of this treatment, his testosterone levels increased and his sexual functioning improved. It is possible, therefore, that aromatase inhibitors may offer a treatment option for some epilepsy patients with sexual problems.

Cosmetic Side Effects

Cosmetic side-effects are an issue for both men and women with epilepsy, with potentially significant impacts on gender-related interactions. One obvious such side-effect, obesity, is associated with several drugs including VPA, CBZ, and gabapentin, and is actually more than a cosmetic problem, as it can evolve into a metabolic syndrome with major negative health effects. Conversely, weight loss can be experienced with the use of topiramate and felbamate. Further, hair loss, acne, coarse facial structure and gingival hyperplasia can be seen after treatment with certain AEDs. Cosmetic problems may have a significant impact on medication compliance, especially in women, and should be considered as important problems, not dismissed as trivial difficulties. With the many drugs currently available, such cosmetic side-effects that the patient considers intolerable, can in many instances be avoided by choosing an alternative drug.

A recent publication has shown gender differences in the frequency of rash induced by AEDs. Problems with rash were significantly more frequent in women than in men, and highest during the reproductive years. This observation is important since rash is often more serious than just a cosmetic side-effect.

Conclusion – Current State of the Field

In conclusion, surprisingly many issues in the treatment of epilepsy are gender specific. Most antiepileptic drugs have gender-specific side-effects. Different gender-specific side-effects are of importance at different ages throughout life. For instance, issues such as contraception and fertility are important for fertile-aged subjects, while issues such as HRT and bone health are more relevant in the elderly. Increased awareness of gender- and age-specific side-effects among patients and professionals will make it possible to better tailor the antiepileptic drug treatment to the individual with epilepsy, whatever his/her stage of life.

See also: **Antiepileptic Drugs**: Clinical Drug Development in Epilepsy; **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Seizures.

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CLASSIFICATION

Contents

Classification of Seizures and Syndromes
History of the Classifications of the Epilepsies

Classification of Seizures and Syndromes

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Introduction

Classifications of diseases are not only important for clinical research, but for identifying populations of patients who are likely to share common fundamental structural and functional pathology, for purposes of basic research to reveal fundamental neuronal mechanisms, and for gene discovery. Indeed, the rapid recent advances in identifying the genetic bases of epileptic disorders would not have been possible without well-developed phenotypes. Classifications are also important for creating appropriate experimental animal models, to ensure that basic research is relevant to the human condition. Finally, results obtained from research in the animal laboratory have contributed, and undoubtedly will continue to contribute, to concepts that aid clinicians in devising meaningful classification systems. Consequently, even though classification of epileptic seizures and epilepsy syndromes would appear to be

See also: **Antiepileptic Drugs**: Clinical Drug Development in Epilepsy; **Hormones and Gender**: Hormonal Modulation of Neuronal Excitability; Hormones and Seizures.

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CLASSIFICATION

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Classification of Seizures and Syndromes
History of the Classifications of the Epilepsies

Classification of Seizures and Syndromes

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Introduction

Classifications of diseases are not only important for clinical research, but for identifying populations of patients who are likely to share common fundamental structural and functional pathology, for purposes of basic research to reveal fundamental neuronal mechanisms, and for gene discovery. Indeed, the rapid recent advances in identifying the genetic bases of epileptic disorders would not have been possible without well-developed phenotypes. Classifications are also important for creating appropriate experimental animal models, to ensure that basic research is relevant to the human condition. Finally, results obtained from research in the animal laboratory have contributed, and undoubtedly will continue to contribute, to concepts that aid clinicians in devising meaningful classification systems. Consequently, even though classification of epileptic seizures and epilepsy syndromes would appear to be

an entirely clinical endeavor, it requires continued intensive collaboration with basic scientists.

Epileptic seizures and epilepsy were clearly described in the ancient writings of Mesopotamia and India during the first millennia: BC 2400 years ago, Hippocrates had already recognized that epileptic seizures were due to disturbances in the brain rather than supernatural causes, and 1900 years ago, Galen used the terms 'idiopathic' when referring to seizures generated by the brain, and 'sympathetic' when referring to seizures due to external causes. More modern efforts to characterize and classify epileptic phenomena began in the nineteenth century. Esquirol introduced the concepts of 'grand mal' and 'petit mal' seizures in 1815, and Bravais described focal motor seizures in 1827. John Hughlings Jackson recognized that focal seizures reflected localized epileptic activities within functionally discrete brain areas and, beginning in the 1860s, described a variety of anatomical substrates of ictal signs and symptoms. These clinical observations made it possible in the 1880s to undertake surgical treatment for lesions that were apparent only intraoperatively, and also contributed to our understanding of localization of normal cerebral function.

The history of the recognition of temporal lobe epilepsy illustrates not only how the concept of a specific epilepsy syndrome can evolve, but also the clinical utility of its classification. Although hippocampal sclerosis was described by Bouchet and Cazauvieilh in postmortem investigations of brains of patients with 'alienation' seizures as early as 1826, Hughlings Jackson correlated lesions in the hippocampus with seizures associated with tasting movements and 'dream states' in 1898, and his contemporary pathologists characterized the hippocampal morphology associated with what we now refer to as mesial temporal lobe epilepsy. Surgery was largely limited to neocortical resections implicated by signs and symptoms reflecting primary motor, sensory, and language localizations until the advent of electroencephalography (EEG) in the late 1920s and early 1930s. Jasper and Kershman demonstrated in 1941 that this new electrophysiological technique had the ability to localize epileptiform abnormalities in the brain, with temporal spike foci being most common. They also carried out intraoperative EEG recordings and observed a psychomotor seizure beginning in mesial temporal structures. Bailey and Gibbs were the first to report temporal lobe resections based solely on EEG-localizing evidence in 1951, although Penfield, Jasper, and the Montreal group had already accumulated a large series of patients treated with temporal resections. Following these observations, there was a much greater interest in surgical treatment for epilepsy worldwide, and temporal lobe surgery was the most common procedure performed. As a result, there was considerable interest in temporal lobe epilepsy as perhaps the most common epilepsy syndrome.

To standardize terminology and facilitate discussion among epileptologists, the International League Against Epilepsy (ILAE) created the first classification of epileptic seizures and epilepsies in 1970. Epileptic seizures were distinguished from epileptic disorders, and were divided into 'partial,' beginning in a part of one hemisphere, and 'generalized,' with bilaterally synchronous onset. Generalized seizures included absences, bilateral massive epileptic myoclonus, infantile spasms, clonic, tonic, tonic–clonic, atonic, and akinetic seizures. Partial seizures were divided into those with elementary symptomatology, mediated by primary sensory, motor and language neocortex, and complex symptomatology, mediated by mesial temporal limbic structures. Although the classification was criticized because of insufficient anatomical and pathophysiological evidence to support the localizing assumptions, the term 'complex partial seizure' became synonymous with temporal lobe, psychomotor, or limbic seizure. The 1970 Classification of the Epilepsies merely divided conditions into generalized and partial, depending upon whether the epileptogenic abnormality existed in a part of one hemisphere, or bilaterally. Generalized epilepsies were further divided into 'primary,' referring to conditions characterized by epilepsy alone, and 'secondary' when seizures were due to an identifiable structural or metabolic disturbance. It was believed at that time that all partial seizures were secondary.

Current International Classifications

1981 ILAE Classification of Epileptic Seizures

Epileptic seizures are currently classified according to the system published by the ILAE in 1981 (**Table 1**). This system was intended to be a purely phenomenological classification based on ictal signs and symptoms and EEG correlates, because the authors acknowledged that there was insufficient information concerning pathophysiology and anatomic substrates of most epileptic seizures to do otherwise. This phenomenological classification differed from previous classifications in several ways: it recognized the evolution of simple partial to complex partial, and both to secondarily generalized seizures; divided absences into typical and atypical; deleted infantile spasms (which were considered to be a syndrome and not a seizure type); and also deleted akinetic seizures because their existence was questioned. At the time, there was considerable objection to the redefinition of simple and complex partial seizures determined solely by whether consciousness was impaired, because this classification no longer permitted the term 'complex partial seizure' to be synonymous with temporal lobe, psychomotor, or limbic seizure. Cortical ictal events could now be associated with impaired consciousness without involvement of the limbic system and thus become

Table 1 International Classification of Epileptic Seizures

I. Partial (focal, local) seizures
A. Simple partial seizures
  1. With motor signs
  2. With somatosensory or special sensory symptoms
  3. With autonomic symptoms or signs
  4. With psychic symptoms
B. Complex partial seizures
  1. Simple partial onset followed by impairment of consciousness
  2. With impairment of consciousness at onset
C. Partial seizures evolving to secondarily generalized seizures
  1. Simple partial seizures evolving to generalized seizures
  2. Complex partial seizures evolving to generalized seizures
  3. Simple partial seizures evolving to complex partial seizures evolving to generalized seizures
II. Generalized seizures (convulsive or nonconvulsive)
A. Absence seizures
  1. Typical absences
  2. Atypical absences
B. Myoclonic seizures
C. Clonic seizures
D. Tonic seizures
E. Tonic–clonic seizures
F. Atonic seizures (astatic seizures)
III. Unclassified epileptic seizures

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complex partial seizures, while classical limbic auras (e.g., sensations of epigastric rising and emotions such as fear) occur in clear consciousness and are simple partial seizures. More recent criticisms are based on the fact that this classification is neither completely phenomenological (as it purports to be), nor does it have reliable mechanistic implications. For this reason, Lüders and his colleagues at the Cleveland Clinic have recommended the use of semiological descriptions only for ictal events, in place of the ILAE classification. Their scheme is used by many of their trainees throughout the world; however, it is not a classification in the true sense of the term. Despite these concerns, the 1981 seizure classification has become widely accepted and is universally used.

The ILAE is currently reevaluating the 1981 Classification of Epileptic Seizures for a number of reasons: it is already over a quarter of a century old; a number of additional seizure types have been identified; and advances in understanding pathophysiologic mechanisms and anatomic substrates now make it reasonable to consider using these features for devising a more scientifically-based classification scheme. In addition, a number of conceptual issues need to be addressed – not only the controversial distinction between complex and simple partial seizures, but also the artificial dichotomy between partial and generalized ictal events (explained later).

1989 ILAE Classification of the Epilepsies, Epileptic Syndromes, and Related Disorders

In 1985, the ILAE made its first attempt to create a classification of epileptic disorders that recognized individual syndromes defined as complexes of signs and symptoms that comprise unique epilepsy conditions with variable etiologies. This classification differed from the 1970 concepts in that it recognized that partial epilepsies could also be primary. There were also changes in terminology. Specifically, the terms 'primary' and 'secondary' were replaced with 'idiopathic' and 'symptomatic.' The term 'idiopathic,' unlike in the rest of American medicine, did not indicate cause unknown, but epilepsy itself, *sui generis*, and nothing else, as derived from the Greek '*idios*,' meaning 'self.' The term 'partial' was replaced by the more cumbersome term 'localization-related' because it was felt that the former incorrectly implied a part of a syndrome rather than a syndrome resulting from an abnormality in part of the brain.

This classification was subsequently revised by the ILAE in 1989 (**Table 2**), and the 1989 classification remains in current use. The major change instituted by the newer system was the introduction of the term 'cryptogenic' to define conditions that were presumed to be symptomatic (because they did not fit any of the known idiopathic syndromes), but the cause was hidden. A 1993 report of the ILAE Commission on Epidemiology and Prognosis, however, defined the term 'cryptogenic epilepsies' to mean unknown as to whether the condition is symptomatic or idiopathic. Although this has introduced an unfortunate ambiguity, these distinctions will become moot as research identifies the mechanistic consequences of aberrant gene expression responsible for idiopathic epilepsies and the genetic predisposition for symptomatic epilepsies.

The syndromes included in the 1989 classification were derived predominantly from studies of epilepsies in infants, children, and adolescents, and the classification is clearly useful for these populations. Most adult patients, however, have what in this classification would be referred to as symptomatic or cryptogenic localization-related epilepsies, and there remains no acceptable way to classify or categorize these conditions. Despite the implications of the 1989 classification, frontal lobe epilepsy, parietal lobe epilepsy, occipital lobe epilepsy, and temporal lobe epilepsy are no longer considered discrete syndromes, because a single seizure type or anatomical location is not 'a complex of signs and symptoms.' There are a variety of possible syndromes ascribed to the frontal and temporal lobes, but even the most common and best understood of these – mesial temporal lobe epilepsy with hippocampal sclerosis – may not constitute a discrete syndrome as opposed to a number of different conditions.

Table 2 International Classification of Epilepsies, Epileptic Syndromes, and Related Seizure Disorders

1. Localization-related (focal, local, partial)
1.1 Idiopathic (primary)
- Benign childhood epilepsy with centrotemporal spikes
- Childhood epilepsy with occipital paroxysms
- Primary reading epilepsy

1.2 Symptomatic (secondary)
- Temporal lobe epilepsies
- Frontal lobe epilepsies
- Parietal lobe epilepsies
- Occipital lobe epilepsies
- Chronic progressive epilepsia partialis continua of childhood syndromes characterized by seizures with specific modes of precipitation

1.3 Cryptogenic, defined by
- Seizure type
- Clinical features
- Etiology
- Anatomical localization

2. Generalized
2.1 Idiopathic (primary)
- Benign neonatal familial convulsions
- Benign neonatal convulsions
- Benign myoclonic epilepsy in infancy
- Childhood absence epilepsy (pyknolepsy)
- Juvenile absence epilepsy
- Juvenile myoclonic epilepsy (impulsive petit mal)
- Epilepsies with grand mal seizures (GTCS) on awakening
- Other generalized idiopathic epilepsies
- Epilepsies with seizures precipitated by specific modes of activation

2.2 Cryptogenic or symptomatic
- West syndrome (infantile spasms, Blitz–Nick–Salaam Krämpfe)
- Lennox–Gastaut syndrome
- Epilepsy with myoclonic-astatic seizures
- Epilepsy with myoclonic absences

2.3 Symptomatic (secondary)
2.3.1 Nonspecific etiology
- Early myoclonic encephalopathy
- Early infantile epileptic encephalopathy with suppression bursts
- Other symptomatic generalized epilepsies

2.3.2 Specific syndromes
- Epileptic seizures may complicate many disease states

3. Undetermined epilepsies
3.1 With both generalized and focal seizures
- Neonatal seizures
- Severe myoclonic epilepsy in infancy
- Epilepsy with continuous spike-waves during slow wave sleep
- Acquired epileptic aphasia (Landau–Kleffner syndrome)
- Other undetermined epilepsies

3.2 Without unequivocal generalized or focal features

4. Special syndromes
4.1 Situation-related seizures (*Gelegenheitsanfälle*)
1. Febrile convulsions
2. Isolated seizures or isolated status epilepticus
3. Seizures occurring only when there is an acute or toxic event due to factors such as alcohol, drugs, eclampsia, and nonketotic hyperglycemia

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As with the 1981 Classification of Epileptic Seizures, the 1989 Classification of Epilepsies, Epileptic Syndromes, and Related Seizure Disorders is widely accepted and universally used, despite a number of problems, including the fact that it is almost 20 years old; it does not include a number of recognized syndromes; the artificial dichotomy of localization-related versus generalized conditions exists as it does with seizures – and is joined by an artificial dichotomy of symptomatic versus idiopathic conditions, which is equally unsatisfying; and the term

'cryptogenic' remains ambiguous. By its very structure, this attempt to fit all epilepsy conditions into such a framework implies similarities and differences that are not there, and ignores similarities and differences that are. Consequently, the ILAE has also undertaken a review of this classification with a view toward revising it (explained later).

Recent ILAE Efforts to Evaluate and Revise the International Classifications

Although the ILAE has devoted considerable effort over more than a decade to reviewing the 1981 and 1989 classifications, and numerous reports and discussions have been published, no new classifications have yet been proposed. It has been generally accepted that the 1981 and 1989 classifications remain useful, and that they should not be discarded unless, and until, clearly better classifications have been devised. There has been much debate concerning the difference between a 'gardener's classification,' which is purely pragmatic, and a 'botanist's classification,' which is a taxonomy based on scientific knowledge of natural classes that can be reproducibly distinguished from other natural classes (such as the biological classification of life forms: kingdom, phylum, class, etc.). The work conducted by ILAE so far has resulted in lists of epileptic seizure types and epilepsy syndromes as unique diagnostic entities, consisting, for the present time, as clearly recognizable clinical disorders that could (1) serve as a basis for investigation into underlying causes and mechanisms and (2) be of utility to the clinician in evaluating, treating, managing, and counseling patients. These diagnostic entities should be treated as testable working hypotheses, subject to verification, falsification, and revision. The ultimate intention is to use an evidence-based approach to establish scientifically rigorous criteria for recognizing epileptic seizure types and epilepsy syndromes as unique natural classes. As additional evidence becomes available, individual diagnostic entities will be reevaluated, revised, or discarded as appropriate. These diagnostic entities can then ultimately be categorized into one or more classifications. It is further acknowledged that classifications are used for many purposes, such as clinical diagnosis of individual patients, teaching, epidemiologic studies, clinical trials, selection of surgical candidates, research into basic mechanisms, and gene discovery. Although more than one classification may, therefore, be necessary, an ideal solution would be a modular, flexible, multivariate approach that could be reconfigured for different purposes and revised as necessary when new information becomes available.

In 2001, the ILAE proposed a diagnostic scheme for people with epileptic seizures and with epilepsy (**Table 3**). It must be stressed that this is not a new classification, and does not replace either the 1981 seizure classification or the 1989 epilepsy classification. It is, rather, a recommendation for a standardized approach for describing individual patients based on five features: the ictal phenomenology (Axis 1), the seizure type as a diagnostic entity (which is a new concept) (Axis 2), the syndrome (Axis 3), the etiology (Axis 4), and an optional factor of impairment (Axis 5). In this report, the ILAE made recommendations for refining certain terms (**Table 4**) as part of the ongoing process of ultimately revising the 1981 and 1989 classifications. Among the more important terms is the new concept of epileptic seizure types as unique diagnostic entities, which unlike the electroclinical seizure definitions of the 1981

Table 3 Proposed diagnostic scheme for people with epileptic seizures and with epilepsy

Epileptic seizures and epilepsy syndromes are to be described and categorized according to a system that utilizes standardized terminology, and that is sufficiently flexible to take into account the following practical and dynamic aspects of epilepsy diagnosis:
(1) Some patients cannot be given a recognized syndromic diagnosis.
(2) Seizure types and syndromes change as new information is obtained.
(3) Complete and detailed descriptions of ictal phenomenology are not always necessary.
(4) Multiple classification schemes can, and should, be designed for specific purposes (e.g., communication and teaching, therapeutic trials, epidemiological investigations, selection of surgical candidates, basic research, genetic characterizations).

This diagnostic scheme is divided into 5 parts, or Axes, organized to facilitate a logical clinical approach to the development of hypotheses necessary to determine the diagnostic studies and therapeutic strategies to be undertaken in individual patients:

Axis 1: Ictal phenomenology – from the Glossary of Descriptive Ictal Terminology can be used to describe ictal events with any degree of detail needed.

Axis 2: Seizure type – from the List of Epileptic Seizures. Localization within the brain and precipitating stimuli for reflex seizures should be specified when appropriate.

Axis 3: Syndrome – from the List of Epilepsy Syndromes, with the understanding that a syndromic diagnosis may not always be possible.

Axis 4: Etiology – from a Classification of Diseases Frequently Associated with Epileptic Seizures or Epilepsy Syndromes when possible, genetic defects, or specific pathological substrates for symptomatic focal epilepsies.

Axis 5: Impairment – this optional, but often useful, additional diagnostic parameter can be derived from an impairment classification adapted from the WHO ICIDH.

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Table 4 Definitions of key terms

- *Epileptic seizure type*: An ictal event believed to represent a unique pathophysiological mechanism and anatomical substrate. This is a diagnostic entity with etiological, therapeutic, and prognostic implications. (New concept)
- *Epilepsy syndrome*: A complex of signs and symptoms that define a unique epilepsy condition with different etiologies. This must involve more than just the seizure type; thus, frontal lobe seizures per se, for instance, do not constitute a syndrome. (Changed concept)
- *Epilepsy disease*: A pathological condition with a single specific, well-defined etiology. Thus, progressive myoclonus epilepsy is a syndrome, but Unverricht–Lundborg is a disease. (New concept)
- *Epileptic encephalopathy*: A condition in which the epileptiform abnormalities themselves are believed to contribute to the progressive disturbance in cerebral function. (New concept)
- *Benign epilepsy syndrome*: A syndrome characterized by epileptic seizures that are easily treated, or require no treatment, and remit without sequelae. (clarified concept)
- *Reflex epilepsy syndrome*: A syndrome in which all epileptic seizures are precipitated by sensory stimuli. Reflex seizures that occur in focal and generalized epilepsy syndromes that are also associated with spontaneous seizures are listed as seizure types. Isolated reflex seizures can also occur in situations that do not necessarily require a diagnosis of epilepsy. Seizures precipitated by other special circumstances, such as fever or alcohol withdrawal, are not reflex seizures. (Changed concept)
- *Focal seizures and syndromes*: Replaces the terms partial seizures and localization-related syndromes. (Changed terms)
- *Simple and complex partial epileptic seizures*: These terms are no longer recommended, nor will they be replaced. Ictal impairment of consciousness will be described when appropriate for individual seizures but will not be used to classify specific seizure types. (New concept)
- *Idiopathic epilepsy syndrome*: A syndrome that is only epilepsy, with no underlying structural brain lesion or other neurologic signs or symptoms. These are presumed to be genetic and are usually age-dependent. (Unchanged term)
- *Symptomatic epilepsy syndrome*: A syndrome in which the epileptic seizures are the result of one or more identifiable structural lesions of the brain. (Unchanged term)

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classification, or the purely descriptive signs and symptoms used by the Cleveland Clinic, attempts to begin the use of pathophysiologic mechanisms and anatomic substrates to create a list of natural classes of seizures. Because it often is not possible to make a syndromic diagnosis with certainty, diagnosis of a specific epileptic seizure type would have etiologic, therapeutic, and prognostic implications. It was also stipulated that more than one feature is necessary to define an epilepsy syndrome, and that anatomic localization of a seizure onset is not sufficient to warrant designation of a syndrome. Another important new concept is that of 'epileptic encephalopathy,' which acknowledges that epileptic activity itself can contribute to progressive disturbances in cerebral function, and that certain syndromes are progressive apart from their underlying etiology. It was recommended to return to the term 'focal,' rather than 'partial' or 'localization-related,' when referring to seizures and syndromes, because this term is simpler and still more commonly used. The term 'focal' was rejected in the past because it inappropriately implied a point source of the epileptogenic abnormality. Now that it is generally acknowledged that epilepsy, whether generalized or focal, involves distributed networks (i.e., disturbances over wide areas of brain, which may even be bilateral in the case of so-called partial seizures or localization-related epilepsies), ILAE believes that the danger of misinterpreting the term 'focal' no longer exists. This report also recommends that a future classification no longer use impairment of consciousness as a factor for classifying seizures types.

Phenomenology of Epileptic Seizures

A glossary of standardized terms for describing ictal behavior was also published in 2001 to supplement Axis 1 of the diagnostic scheme. Much of this glossary was derived from the Cleveland Clinic approach. Although impairment of consciousness is an important aspect of seizures with respect to the impairment it creates for individual patients, this feature is an extremely difficult concept to define, and has been replaced in the glossary by the term 'dyscognitive,' which describes more specifically a variety of different disturbances of mental function that are not adequately captured by the more global term 'consciousness'. Unlike the Cleveland Clinic approach, the ILAE diagnostic scheme acknowledges that a detailed description of ictal phenomenology is not necessary for diagnosis or treatment of seizure types in most patients, nor is it usually possible without prolonged video monitoring. Accurate detail regarding phenomenology can provide useful information clinically, however, for localizing the epileptogenic region in patients who are candidates for surgical resection, and it is also important for basic scientists who are interested in identifying the anatomic substrates of ictal behaviors.

Epileptic Seizure Types as Unique Diagnostic Entities

A 2006 ILAE report contained an updated list of recognized seizure types (**Table 5**), that were considered to be unique diagnostic entities based on most recent

Table 5 Seizure types

Self-limited epileptic seizures
I. Generalized onset
  A. Seizures with tonic and/or clonic manifestations
    1. Tonic–clonic seizures
    2. Clonic seizures
    3. Tonic seizures
  B. Absences
    1. Typical absences
    2. Atypical absences
    3. Myoclonic absences
  C. Myoclonic seizure types
    1. Myoclonic seizures
    2. Myoclonic-astatic seizures
    3. Eyelid myoclonia
  D. Epileptic spasms
  E. Atonic seizures
II. Focal onset (partial)
  A. Local
    1. Neocortical
      a. Without local spread
        (1) Focal clonic seizures
        (2) Focal myoclonic seizures
        (3) Inhibitory motor seizures
        (4) Focal sensory seizures with elementary symptoms
        (5) Aphasic seizures
      b. With local spread
        (1) Jacksonian march seizures
        (2) Focal (asymmetrical) tonic seizures
        (3) Focal sensory seizures with experiential symptoms
    2. Hippocampal and parahippocampal
  B. With ipsilateral propagation to
    1. Neocortical areas (includes hemiclonic seizures)
    2. Limbic areas (includes gelastic seizures)
  C. With contralateral spread to
    1. Neocortical areas (hyperkinetic seizures)
    2. Limbic areas (dyscognitive seizures with or without automatisms [psychomotor])
  D. Secondarily generalized
    1. Tonic–clonic seizures
    2. Absence seizures
    3. Epileptic spasms (unverified)
III. Neonatal seizures

Status epilepticus
  I. Epilepsia partialis continua (EPC)
    A. As occurs with Rasmussen syndrome
    B. As occurs with focal lesions
    C. As a component of inborn errors of metabolism
II. Supplementary motor area (SMA) status epilepticus
III. Aura continua
IV. Dyscognitive focal (psychomotor, complex partial) status epilepticus
    A. Mesial temporal
    B. Neocortical
V. Tonic–clonic status epilepticus
VI. Absence status epilepticus
    A. Typical and atypical absence status epilepticus
    B. Myoclonic absence status epilepticus
VII. Myoclonic status epilepticus
VIII. Tonic status epilepticus
IX. Subtle status epilepticus

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pathophysiologic and anatomic evidence. This list, however, remains a work in progress. To create a list of, and ultimately to categorize or classify, epileptic seizures as diagnostic entities based on pathophysiologic mechanisms and anatomic substrates, it is important to recognize that the ictal semiology may be misleading. The obvious example of this problem is brief loss of consciousness, which could represent a typical absence or a focal seizure that manifests as impaired consciousness only. In contrast, a unique seizure type based on a single discrete neocortical mechanism may have entirely different ictal manifestations, depending on whether it occurs, for instance, in motor cortex or calcarine cortex. In this sense, the anatomic substrate used to define unique diagnostic entities refers to large networks that distinguish neocortical from, for instance, thalamocortical mechanisms – but not precentral neocortex from occipital neocortex with the same cytoarchitecture.

There are still a number of problems with the list of seizure types presented in Table 5. Most importantly, it retains the artificial focal versus generalized dichotomy, which remains unsatisfactory. The focal onset types are based largely on the networks involved and most likely need to be revised. The section on status epilepticus also needs further work – for instance, autonomic status epilepticus is not easily incorporated into any of the listed conditions.

Epilepsy Syndromes

The 2006 ILAE report also contained a list of accepted epilepsy syndromes (Table 6), which includes many conditions that were not recognized at the time of the 1989 classification of the epilepsies. As many epilepsy syndromes begin during specific periods of cerebral development, they are listed by age rather than according to the artificial dichotomies of focal versus generalized and idiopathic versus symptomatic. These terms have been retained, however, for certain syndromes, more because of common usage than the accuracy of this description, recognizing that no condition is truly generalized or truly focal. As yet, no better, more accurate descriptive terms have been suggested.

The list of epilepsy syndromes in Table 6 can be used for diagnostic manuals to be applied to individual patients, or to constitute taxonomic classes, but not all can be used for both. Progressive myoclonus epilepsy, for instance, includes a number of distinct conditions and is not appropriate for taxonomic purposes. The next step is to attempt to organize this list into logical categories based on similarities of natural classes that will constitute a scientifically-based taxonomy of the epilepsies. Potential categories that might be considered for future classification systems include autosomal-dominant epilepsies, epileptic encephalopathies, generalized (genetic) epilepsies with febrile seizures plus (GEFS+), idiopathic

Table 6 Epilepsy syndromes by age of onset and related conditions

Neonatal period
Benign familial neonatal seizures (BFNS)
Early myoclonic encephalopathy (EME)
Ohtahara syndrome
Infancy
Migrating partial seizures of infancy
West syndrome
Myoclonic epilepsy in infancy (MEI)
Benign infantile seizures
Dravet syndrome
Myoclonic encephalopathy in nonprogressive disorders
Childhood
Early onset benign childhood occipital epilepsy (Panayiotopoulos type)
Epilepsy with myoclonic-astatic seizures
Benign childhood epilepsy with centrotemporal spikes (BCECTS)
Late onset childhood occipital epilepsy (Gastaut type)
Epilepsy with myoclonic absences
Lennox–Gastaut syndrome
Epileptic encephalopathy with continuous spike-and-wave during sleep (CSWS), including Landau–Kleffner syndrome (LKS)
Childhood absence epilepsy (CAE)
Adolescence
Juvenile absence epilepsy (JAE)
Juvenile myoclonic epilepsy (JME)
Progressive myoclonus epilepsies (PME)
Less-specific age relationship
Autosomal-dominant nocturnal frontal lobe epilepsy (ADNFLE)
Familial temporal lobe epilepsies
Mesial temporal lobe epilepsy with hippocampal sclerosis (MTLE with HS)
Rasmussen syndrome
Gelastic seizures with hypothalamic hamartoma
Special epilepsy conditions
Symptomatic focal epilepsies not otherwise specified
Epilepsy with generalized tonic–clonic seizures only
Reflex epilepsies
Febrile seizures plus (FS+)
Familial focal epilepsy with variable foci
Conditions with epileptic seizures that do not require a diagnosis of epilepsy
Benign neonatal seizures (BNS)
Febrile seizures (FS)

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generalized epilepsies, idiopathic focal epilepsies, and reflex epilepsies. Whatever construct of clustering is devised, it is likely that some syndromes may be represented in more than one grouping, while others may fit into none. Ultimately, a multivariate modular approach may be most appropriate, offering flexibility to use variations of the classification for different purposes.

Etiology

The 2001 ILAE report listed diseases frequently associated with epileptic seizures or syndromes, including those causing progressive myoclonus epilepsies, neurocutaneous disorders, malformations due to abnormal cortical development, other cerebral malformations, tumors, chromosomal abnormalities, monogenic Mendelian diseases with complex pathogenic mechanisms, and inherited metabolic disorders. This list, however, was not intended to be comprehensive. The Centers for Disease Control (CDC) is in the process of proposing a classification of etiologies for epileptic disorders for epidemiological purposes, in an effort to define risk factors (**Table 7**). Although etiology is an essential part of the diagnostic scheme for individual patients, it is unclear to what extent etiologies will become important in devising ultimate classifications for epileptic seizures and epilepsy syndromes. Considerable work remains to be done here, and basic research will be essential to our understanding of how specific etiologies result in epilepsy.

Impairment

The World Health Organization (WHO) has developed a classification of impairment for neurologic disorders, referred to as the International Classification of Impairments, Disabilities, and Handicaps (WHO ICIDH), which has undergone several revisions. This classification, however, is designed for persistent disabilities such as blindness and paralysis, and at the present time is inadequate to define disabilities associated with paroxysmal disorders such as epilepsy. The ILAE is currently adapting this document for use in classifying degrees of impairment among people with epilepsy.

Need for Basic Research

It is clear that concerted efforts to elucidate the underlying pathophysiologic mechanisms responsible for epileptic seizures and epilepsy syndromes, and their anatomic substrates, are essential to the development of a taxonomic classification system based on identification of unique diagnostic entities as natural classes. This work must ultimately be carried out in patients; however, continuing research in the animal laboratory is critical to this effort. Parallel reiterative animal and human experiments include the use of clinical observations to determine questions that need to be investigated through basic research; direct invasive and noninvasive investigations in the clinical setting where possible; extension of this work to appropriate experimental animal models when practical, ethical, and financial considerations prevent experiments in patients; and validation of the relevance of insights gained from the animal laboratory through further studies with patients. In addition, there have been tremendous advances in recent years in characterizing the genetics of epilepsy, not only in identifying genetic disturbances responsible for certain

Table 7 Etiology

A. Predominantly or exclusively of genetic origin
  1. Monogenic epilepsy diseases/syndromes (e.g., ADNFLE, Dravet's, BFNS)
  2. Complex epilepsy diseases/syndromes (IGEs, e.g., CAE, JAE, JME; ILRE, e.g., BCECTS, GEFS+)
  3. Epilepsies associated with other neurological specific syndromes and diseases (most, if not all, monogenic)
    a. Neurocutaneous
    b. In-born metabolic disease
    c. Progressive myoclonus diseases
    d. Other genetic diseases and syndromes (e.g., fragile X, Rett, Angleman, Huntington)
  4. Chromosomal (aneuploidy, e.g., Down syndrome)
B. Predominantly or exclusively of acquired origin
  1. Traumatic
  2. Infectious
  3. Cerebrovascular
  4. Hypoxic–ischemic encephalopathy
  5. Metabolic/toxic because of environmental exposures/insults
    a. Alcohol
    b. Other
C. Specific causes and disorders with either unknown basis (genetic vs. acquired) or mixed genetic–acquired nature
  1. Degenerative diseases of adult onset (e.g., Alzheimer's and Parkinson's disease)
  2. Autoimmune (e.g., multiple sclerosis)
  3. Neoplastic
  4. Malformations
D. Unknown or multiple
  1. With otherwise normal examination, intelligence, imaging (when available), and noncontributory history
  2. With abnormal examination, intelligence, or imaging. If possible, distinguish (a) and (b) below.
    a. As evidenced by developmental static encephalopathy (i.e., CP or MR) documented to be present prior to the onset of epilepsy and consistent with a congenital insult.
    b. As evidenced by other indications of CNS dysfunction acquired postnatally
    c. With imaging demonstrated mesial temporal sclerosis and not known as to when it first occurred
  3. Multiple (>1) potential contributing factors. Any one cause cannot be designated as the primary cause with reasonable confidence.

In addition to assigning etiology to the categories above, the stability of the condition should be specified, i.e., whether the underlying condition is *static* or *progressive*.

ADNFLE, autosomal-dominant nocturnal frontal lobe epilepsy; BCECTS, benign childhood epilepsy with centrotemporal spikes; BFNS, benign familial neonatal seizures; CAE, childhood absence epilepsy; GEFS+, generalized (genetic) epilepsy with febrile seizures plus; IGE, idiopathic generalized epilepsy; ILRE, idiopathic localization-related epilepsy; JAE, juvenile absence epilepsy; JME, juvenile myoclonic epilepsy.

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idiopathic epilepsies (which would not have been possible without a reasonable syndromic classification), but in characterizing groups of susceptibility genes that contribute to classes of epileptic disorders, including conditions currently referred to as symptomatic syndromes. Determining the mechanisms by which these aberrant gene expressions result in epilepsy is also highly dependent upon research with experimental animal models.

Summary

The ILAE created the current International Classification of Epileptic Seizures in 1981, based on electroclinical phenomenology, and the current Classification of the Epilepsies, Epileptic Syndromes, and Related Disorders in 1989. These are generally accepted and widely used. The ILAE, however, has been reviewing these classifications with the intention of revising them according to more rigorous evidence-based criteria, using common underlying pathophysiological mechanisms and anatomic substrates to identify epileptic seizure types, as well as epilepsy syndromes, as unique diagnostic entities, in the same way that other biological classification systems identify and categorize natural classes. The ILAE proposal for a diagnostic scheme for describing individual patients, and lists of recognized epileptic seizure types and epilepsy syndromes, are presented. Classification of pathological phenomena is not only important for clinical purposes, but also for developing appropriate experimental animal models that are relevant to the human condition. Conversely, basic scientists provide the information, from both patient and animal research into fundamental mechanisms and anatomical substrates of pathological phenomena, that make up the basis of biological classification systems. The ongoing effort of classification in epilepsy, therefore, represents a continuing collaborative effort between clinicians and basic scientists.

See also: **Classification**: History of the Classifications of the Epilepsies.

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History of the Classifications of the Epilepsies

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Statement and Significance of the Problem

Classification deals with two major problems. The first problem is one of communication – being sure that in oral or written communication about an 'object' that is not visible, all parties evoke the same picture or concept. To be classified, the entities in question must be labeled as unique identities with unambiguous names. Once a unique name is given, the object is no longer quite so elusive. Name-giving can imply ownership, as can be read even in the Bible. In Isaiah (Chapter 43), the Lord said "I have called thee by thy name; thou art mine." Although this restrictive sense of naming obviously does not apply to diseases, the custom of name-giving using the name of the person who first described a disease entity as eponym (from the Greek *epi* = by, belonging to, and *onima* = name) might suggest some ownership. Further, use of eponyms may stimulate nationalistic tendencies. In neurology, several disorders have multiple and multinational eponyms – e.g., the syndrome of Tooth (Great Britain), Charcot (France), Hoffmann (Germany) – otherwise known as neurogenic muscular atrophy.

The second problem in classification involves the creation of classes and taxonomies through which known or assumed relations between objects or concepts can be traced. It is fair to say that the observation with which Beigel introduced the German translation of J. R. Reynolds' book *Epilepsy* in 1865 still holds:

> No field of medical literature presents . . . such a confused and such reckless application of names and descriptions as in the field of brain and nerve diseases . . . that this culminates in the case of epilepsy concerns the nature of the disease and the ways and modes in which it manifests, and the way the public and doctors relate to it.

Background

Dealing with the problem of classifying epilepsy or epilepsies, distinguishing between epilepsies and seizures, and in turn classifying seizure types are tasks not only typical for our time but – as can be seen from the earliest written accounts – have been problems for all ages. Wherever a sophisticated society existed, similar concerns arose – and were dealt with in various ways. In this article, we focus on developments in what can be briefly described as the 'western world.' Names given to epilepsy in earlier centuries were of several kinds, and involved different frames or reference. For example

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- A surmised cause (morbus lunaticus = moon sickness; epilepsy, derived from the Greek word epilambanein = to seize (by a god))
- The impressiveness of the condition (grand mal = megala nosos)
- The symptomatology (falling sickness)
- Its psycho-social effects (morbus insputatus = the illness at which one spits)
- How the medical understanding of the time was (morbus infantilis = childhood's disease)

Notwithstanding the various names found in ancient literature, these labels did not try to distinguish between seizure types or epilepsies. Most names were for generalized tonic–clonic seizures – called by different names according to the knowledge available, the prevailing worldview, cultural trends, and social order. Early classification systems in other fields reflected, for example, the order that according to Aristotle existed in nature and which he described in the 'Scala Naturae.'

As far as epilepsy was concerned, Hippocrates scornfully mentioned in his work *About the Holy Disease* that certain priests and preachers of penitence described different seizure types, for each of which they held a separate god responsible. According to this system, right-sided hemiconvulsions were caused by the mother of Zeus, Rhea; seizures with shaking of the legs and hypersalivation were caused by the god of war, Ares; when a person had fecal incontinence during a seizure, then it was a seizure caused by Hermes or Enodia; and nightly seizures were caused by Hekate. Hippocrates (460–377 BC) strongly opposed the prevalent concept that seizures were caused by gods. He discussed different symptoms and seizure types, and tried to explain them according to the theory of humoral pathology that he had developed, discerning between blood, phlegm, black bile, and yellow bile. Hippocrates, however, never proposed a classification of seizures.

Almost six centuries later, another highly influential physician appeared on the scene – Galen (Galenos, 131–199 AD) from Pergamon. He distinguished three types of epilepsy, with all three afflicting the brain. The first type of epilepsy, according to Galen, develops in the brain itself, as would be the case with most people who suffer from epilepsy. In the second type, the epilepsy rises from the stomach by sympathy to the brain. And in the third type – also a 'sympathic' form of epilepsy – the onset is from any other part of the body, from which the epilepsy invades the brain. Although these ancient writings are mainly of historic interest, one term introduced by Galen, the 'aura,' is still in use. In a case description, Galen mentions that his patient experienced the invasion of his sympathic epilepsy into the brain as a cold wind (aura) that rises up into his head.

The dominating culture of the Christian Church in Europe in the Middle Ages had no place for the Greco-Roman thinking that was so different from those beliefs based upon the New Testament. Disease was considered a punishment by God for sinful behavior. Similar to the 'mythological classification' so much criticized by Hippocrates, The Church instructed that certain saints were especially to be invoked to ask divine mercy for persons with certain type of seizures. For example, in France those with severe seizures were said to suffer from the 'mal de St. Jean' (Saint John), and those with less-severe seizures suffered from the 'mal de St Gilles' (saint Aegidius, died 720 AD).

Fortunately, Byzantine and Arab physicians had taken over the medical teachings of the Greeks and Romans, and recorded these teachings in numerous books, partly elaborated with their own experiences and knowledge. Through this pathway, Greco-Roman insights returned to Europe in the later part of the Middle Ages and in the Renaissance period. However, as far as classification of the epilepsies was concerned, the know-how had not developed beyond the writings of Galen.

Paracelsus (Theophrastus Philippus Aureolus Bombastus von Hohenheim), the colorful doctor and researcher of the Renaissance, had little esteem for Galen's writings, but nevertheless developed a similar localization-based classification of the epilepsies. He distinguished five types of epilepsy, originating respectively in the head, the heart, the liver, the intestines, or the extremities. Contrary to Galen, he believed that the seizures resulted from what happened in these organs, and that they did not involve the brain (were not sympathic), and accordingly drew up his therapies.

In the eighteenth and nineteenth centuries, a series of publications from France and French-speaking Switzerland made important contributions that can be considered to have formed the foundation from which modern epileptology developed. These works were based upon accurate descriptions of signs and symptoms. Prominent among the authors of this set of publications are: SA-Tissot (1728–1797), JE Esquirol (1772–1840), LF Cameil (1798–1895), Th Herpin (1799–1865), L Bravais (1801–1843), and LJF Delasiauve (1804–1893). Although Tissot championed clinical observation, his classification still used some of Galen's ideas – in particular, that epilepsy could have its onset outside the brain. In his epoch-making *Traité de l'épilepsie* of 1770, he posited that idiopathic epilepsy is the type where the origin of the seizures is in the brain itself, while sympathic epilepsy has its origin outside the brain. For the latter epilepsy type, irritation of the nerves at the locus of origin was transported to the brain, where the irritation elicited a seizure. While epilepsy that had its origin in the brain was labeled idiopathic, Tissot further distinguished what he called 'essential epilepsy' that also had its origin in the brain but did not appear to depend on any pathological change other than a propensity to

seizures (nowadays only these types of epilepsies are labeled idiopathic). About 75 years after Tissot's publication, Delasiauve published a book with the same title as that of Tissot, *Traité de l'épilepsie*. In it, he distinguished idiopathic, symptomatic, and sympathic epilepsies; his descriptions of the first two – idiopathic and symptomatic – covered the same concepts as we presently use for these categories. As regards sympathic epilepsy, however, he began to emphasize an extracerebral trigger – as in those conditions we would now call reflex-epilepsies – or a toxic excitant evoking seizure activity in the brain. However, the definition was not crystal clear. There lingers in this description of sympathic epilepsy the concept of a possible extracerebral seizure generator.

Apart from localization-related distinctions between epilepsy types, Tissot also introduced another discriminator related to the extensiveness of the seizure, thus distinguishing between 'grand accès' and 'petit accès' (later designated as 'grand mal' and 'petit mal'). However, as Herpin posited in a book published in 1867, all seizure types – and he distinguished 39 – were gradations of full-blown epilepsy characterized by the grand mal seizure. This concept is also maintained in Binswanger's chapter in Nothnagels *Specielle Pathologie und Therapie*, published in 1904, wherein epilepsy is discussed under the headings (1) Epilepsia gravior, (2) 'Der unvollständige, rudimentäre Anfall (the incomplete seizure), and (3) Abortive Anfälle (petit mal).

From the eighteenth through the twentieth centuries, important discoveries and research further paved the way for our present-day understanding of the epilepsies and their classification. Pivotal were Galvani's (1737–1798) discovery of animal electricity and Hans Berger's (1873–1941) work, resulting in 1924 with the first electroencephalogram in man.

In the twentieth century, a distinction between lumpers and splitters becomes apparent. The lumpers are those who maintain that epilepsy is a single disorder with graded expression. The splitters are those who prefer to speak about 'the epilepsies' as a related group of disorders that can be so classified. *The Epilepsy Handbook* of Gibbs and Stamps placed the different seizure types in a context expanding from normal brain activity to generalized tonic–clonic seizures (**Figure 1**). Lennox and Lennox's *Epilepsy and Related Disorders* may be taken as exemplary for the lumpers; notwithstanding their long list of adjectives (**Table 1**), they made no attempt at categorization. In contrast, Janz exemplifies the splitters in his book *Die Epilepsien* (1969). Janz made a distinction between three major categories: (1) age-related, and presenting with minor seizures (he retains the epithet 'petit mal'); (2) not age-related, but with minor seizures; and (3) grand mal epilepsies.

In more recent times, the members of the International League Against Epilepsy's Commission on Classification and Terminology can be considered splitters. In 1985, they presented to the General Assembly a 'Classification of Epilepsies and Epileptic Syndromes,' which in a revised form was accepted in 1989. This classification of epilepsies and epileptic seizures had been preceded by a

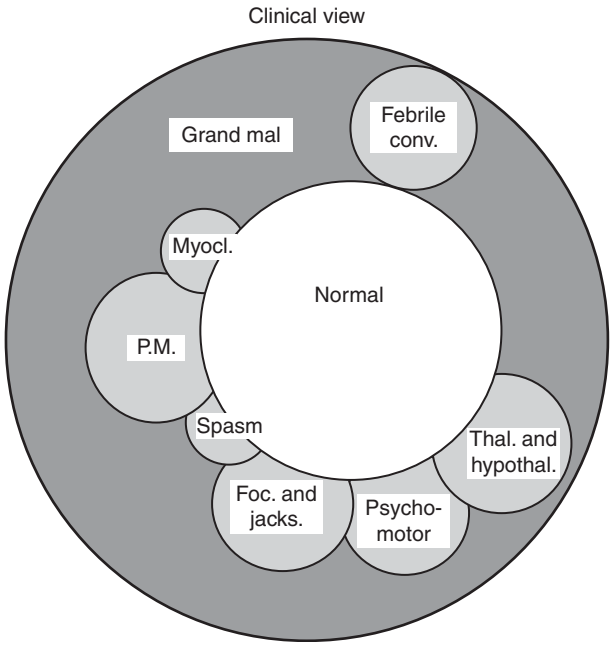


Figure 1 Possible manifestations of epilepsy according to Gibbs and Stamps. Reproduced from Gibbs FA and Stamps FW (1958) *Epilepsy Handbook*. Thomas: Springfield.

Table 1 Adjectives added to epilepsy in index of *Epilepsy and Related Disorders* by William Gordon Lennox and Margaret A. Lennox

Abdominal	Hypothalamic	Psychomotor
Akinetic	Idiopathic	Pyknolepsy
Astatic	Jacksonian motor	Reflex
Atonic	Jacksonian sensory	Respiratory
Automatic	Larval	Rolandic
Autonomic	Laryngeal	Salaam
Centrencephalic	Metabolic	Sonogenic
Childhood	Minor	Spastic
Cryptogenic	Molecular	Static
Diencephalic	Mono-	Striatal
Discontinuous	Musicogenic	Subcortical
Emotional	Myoclonus	Sympathetic
Essential	Optogenic	Symptomatic
Extrapyramidal	Organic	Temporal (lobe)
Fetal	Paranatal	Thalamic
Focal	Partial	Thermal
Genetic	Petit mal	Unilateral
Grand mal	Photogenic	Vasovagal
Hemiplegic	Postencephalitic	Vegetative
Hereditary organic	Postnatal	Vertiginous
Hydraulic	Posttraumatic	Visceral
Hyperthermia-precipitated	Psychic	

'Clinical and Electroencephalographic Classification of Epileptic Seizures' accepted by the General Assembly in 1969 and revised again in 1981.

In 1969, Gastaut introduced a classification scheme crafted by a large international group of epileptologists with the following words:

> The Executive Committee [of the ILAE] is fully aware of the fact that all attempts at classification of epileptic seizures are hampered by our limited knowledge of the underlying pathological processes within the brain, and that any classification must of necessity be a tentative one and will be subject of change with every advance in the scientific understanding of epilepsy.

This expression of the limitations of classification is relevant still, in our-day attempts to improve on prior classifications.

See also: **Models**: Model Characterization in Relationship to Human Disorders.

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EPIDEMIOLOGY

Contents

Epidemiology: Status Epilepticus

Epidemiology: Status Epilepticus

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Introduction and Background

Definitions and Classifications of Status Epilepticus

Clear definition of a condition is a prerequisite for accurate epidemiology. The definition of status epilepticus has proved problematic. Its initial description, by Desiré Bourneville in 1876, confined the term to prolonged tonic–clonic seizures, and this convention was largely retained up until the 1950s. Clark and Prout in 1903/1904, for instance, formulated a definition of status epilepticus – for the 'typical case' – which applied only to convulsive seizures: status epilepticus is 'the maximum development of epilepsy, in which one paroxysm follows

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Introduction and Background

Definitions and Classifications of Status Epilepticus

Clear definition of a condition is a prerequisite for accurate epidemiology. The definition of status epilepticus has proved problematic. Its initial description, by Desiré Bourneville in 1876, confined the term to prolonged tonic–clonic seizures, and this convention was largely retained up until the 1950s. Clark and Prout in 1903/1904, for instance, formulated a definition of status epilepticus – for the 'typical case' – which applied only to convulsive seizures: status epilepticus is 'the maximum development of epilepsy, in which one paroxysm follows

another so closely that the coma and exhaustion are continuous between seizures.' In 1962, the Xth Marseilles Colloquium was the first major meeting devoted to the subject of status epilepticus. It was led by Henri Gastaut who, leaning heavily on EEG data, proposed a comprehensive definition and, for the first time, a systematic classification scheme. A total of 103 participants presented 237 cases, with both clinical and EEG findings "of abnormally prolonged or serially repeated seizures, most of which" (as Gastaut wrote) "did not correspond to the definition of status epilepticus accepted at that time." The conference replaced the 'historical' definition (corresponding to grand mal status) by an 'etymological definition,' which defined status epilepticus as 'a condition characterized by an epileptic seizure, which is so frequently repeated or so prolonged as to create a fixed and lasting epileptic condition.' The seizures (and thus the status epilepticus) could take a variety of forms and, as Gastaut later wrote, implicit in this definition was the view that there are "as many types of status epilepticus as there were types of epileptic seizures."

In parallel to the changing view of status epilepticus were changing views of the classification of epileptic seizures, and the first version of the International Classification of Seizures was in preparation under the direction of Gastaut. In 1964, the proposed classification both of seizures and of status epilepticus was presented to the XIIIth Colloquium in Marseilles, and an approved version of the *International Classification of Seizure Type* was eventually published in 1970. The proceedings of the 1962 colloquium on status epilepticus were published in 1967, and laid out a classification of status epilepticus, which paralleled the *International Classification of Seizure Type*. Status epilepticus was divided into generalized and partial (unilateral) categories: the generalized categories consisted of tonic, clonic, tonic–clonic, absence, and myoclonic status epilepticus; the partial category comprised simple and complex partial status epilepticus. The definition and classification were subsequently included in the World Health Organization (WHO) Dictionary of Epilepsy, where status epilepticus was defined as 'a seizure persisting for a sufficient length of time or is repeated frequently enough to produce a fixed or enduring epileptic condition.' The International League Against Epilepsy (ILAE), in its revision of the seizure type classification in 1981, defined status epilepticus (in an addendum) as follows: 'The term *status epilepticus* is used whenever a seizure persists for a sufficient length of time or is repeated frequently enough that recovery between attacks does not occur. Status epilepticus may be divided into partial (e.g., Jacksonian), or generalized (e.g., absence status or tonic–clonic status).'

The new definitions and classification that were formulated over this period were important steps in the conceptualization of status epilepticus. However, over the next two decades, as the concept of the division of epilepsy into *epileptic syndromes* developed and gained importance, it became also clear that a simple *seizure-type* classification of status epilepticus was becoming obsolete. In the monograph on status epilepticus published in 1994, a more comprehensive composite classification was proposed (albeit 'a provisional classification, necessarily primitive and incomplete, to which future modifications are inevitable, and desirable'). This scheme divided SE into major subdivisions based on age, and within each age group was a mixed classification with categories based on three aspects: clinical and EEG seizure type, pathophysiology and epilepsy syndrome, and clinical phenomenology crossing seizure type and syndromic boundaries. A category of 'boundary conditions' was also included.

The 1973 WHO and the 1981 ILAE definitions of status epilepticus are necessarily rather imprecise, and two questions arise, which are difficult to resolve. The first is the question 'how long should a seizure be considered enduring before it is categorizable as status epilepticus.' The lack of definition of a minimum time period in the early definitions of status epilepticus is problematic from the point of view of epidemiology. Gastaut himself suggested that 60 min was a reasonable minimum period in which to consider a seizure 'enduring,' and this suggestion became the ILAE standard. However, various authors over the next two decades favored a minimum time period of 30 min. These time periods have some physiological justification, as after 30–60 min, major biochemical and physiological cerebral changes begin to occur of ominous import to outcome and therapy. In the 1990s, the suggested minimum time shortened to 20 min (for instance by Bleck in 1991), to 10 min (by Treiman in 1998), and then to 5 min (by Lowenstein in 1999). It was claimed that convulsive seizures lasting longer than 5 min seldom were self-terminating, and this feature was the main reason for proposing a 5-minute minimum time period. This short time interval has some clinical support from a video EEG telemetry study of secondarily generalized tonic–clonic seizures in 47 patients, in which the mean duration of the spontaneously resolving seizures was 62 s, with a range of 16–108 s. However, it is a common clinical experience that many seizures lasting longer than 5 min are self-terminating without therapy. Thus, it seems that the main advantage of defining status epilepticus in this way (i.e., longer than 5 min) is 'operational' rather than physiological. That is, this definition is a response to the perceived need to induce a greater sense of urgency amongst front-line physicians when treating prolonged seizures.

A second issue, which complicates definition of SE, is the definition of 'seizures' in this setting. Prolonged electrographic seizure activity can occur without overt seizures, and the question arises as to the extent to which such states should be included within the rubric of status epilepticus. These are the 'boundary syndromes' in which there is prolonged electrographic seizure

activity, with no signs of a conventional 'epileptic seizure.' There are four main categories in which this issue is relevant: some cases of epileptic encephalopathy; some cases of coma due to acute brain injury, often with myoclonic jerks (*myoclonic status epilepticus in coma*); some cases of epileptic behavioral disturbance or psychosis; and some cases of drug-induced or metabolic confusional states, often with myoclonic jerks (for instance precipitated by toxins or drugs such as tiagabine or metrizamide).

In the 1994 monograph *Status Epilepticus*, a definition of status epilepticus was proposed, which is somewhat more precise and is still widely accepted: "Status epilepticus is a condition in which epileptic activity persists for 30 min or more, causing a wide spectrum of clinical symptoms, and with a highly variable pathophysiological, anatomical, and etiological basis." The question of classification was extensively considered at the recent *London Colloquium of Status Epilepticus* (held in April 2007) and a classification proposed at the congress of convulsive and nonconvulsive forms is shown in **Table 1**.

Results

Incidence and Frequency of Status Epilepticus

The lack of a precise definition of status epilepticus is an obvious bar to accurate estimations of frequency. Most epidemiological studies in recent years have defined status epilepticus as a seizure continuing for 30 min of more, and none (to my knowledge) has adopted the 5-min standard. Five-minute seizures are extremely common, and if status epilepticus was to be defined in this manner, the quoted frequency would be enormously enhanced. Similarly, the epileptic encephalopathies are usually excluded from consideration (although there are sound reasons for including many cases within the rubric of status epilepticus). Another source of major controversy is the inclusion of cases of 'myoclonic status epilepticus in coma' after cerebral anoxia (for instance, after a myocardial infarction) or acute brain injury. These latter cases are common, and have been included in some epidemiological estimates of status epilepticus but not in others.

Table 1 Classification of status epilepticus (SE)

1. SE in the neonatal and infantile epilepsy syndromes
   - West syndrome
   - Ohtahara syndrome
   - Severe myoclonic encephalopathies of infancy (including SMEI)
   - SE in other forms of neonatal or infantile epilepsy
2. SE confined to childhood
   - SE in early-onset benign childhood occipital epilepsy (Panyotopoulos syndrome)
   - SE in other forms of childhood epileptic encephalopathies, syndromes, and etiologies, e.g.: Ring chromosome X, Angelmans syndrome, myoclonic–astatic epilepsy, other childhood myoclonic encephalopathies
   - ESES (Electrical status epilepticus in slow wave sleep)
   - Landau–Kleffner syndrome
3. SE occurring in childhood and adult life

With epileptic encephalopathy
   - Nonconvulsive SE in Lennox Gastaut and similar syndromes [(i) Atypical absence status epilepticus; (ii) tonic status epilepticus]
   - Other forms of nonconvulsive SE in patients with learning disability or disrupted cerebral development (cryptogenic or symptomatic)

Without epileptic encephalopathy
   - Tonic–clonic SE
   - Typical absence status epilepticus
   - Myoclonic status epilepticus
   - Myoclonic SE in coma (NCSE in diffuse cerebral injury)
   - Epilepsia partialis continua
   - Complex partial status epilepticus: (i) Limbic and (ii) nonlimbic
   - Nonconvulsive SE occurring in the postictal phase of tonic–clonic seizures
   - Subtle SE (myoclonic SE occurring at the late stage of tonic–clonic SE
   - Aura continua (simple partial SE)
4. SE confined to late adult life
   - De novo absence status epilepticus of late onset
5. Boundary syndromes[a]
   - Cases of epileptic encephalopathy
   - Cases of acute brain injury, often with irregular myoclonus (myoclonic SE in coma)
   - Drug-induced or metabolic confusional states with epileptiform EEG changes
   - -Cases of epileptic behavioral disturbance or psychosis

[a]Boundary syndromes are defined as cases in which it is not clear to what extent the continuous epileptiform electrographic abnormalities are contributing to the clinical impairment.

Source

Shorvon SD (2007) What is nonconvulsive status epilepticus, and what are its subtypes? *Epilepsia* 48(supplement 8): 35–38.

Epidemiological Studies of Incidence

In the past few years, there have been a series of well-conducted population-based studies in the United States, United Kingdom, Switzerland, Germany, and Italy (Tables 2 and 3). Most have found an incidence of SE of approximately 10–20 per 100 000 per year. Different studies used different inclusion criteria, and the much higher incidence figures (from Richmond, VA) were in part due to the inclusion of cases of myoclonic status epilepticus in coma (see earlier 'boundary syndromes') (Table 2). Extrapolating the figures from this study, the authors estimate that there would be between 126 000 and 195 000 cases of status epilepticus in the United States every year. The study from London was of children (less than 15 years) and of convulsive status epilepticus only. In the studies based in emergency departments, the

Table 2 Six population-based studies of status epilepticus

	Richmond, VA, USA	*Rochester, MN, USA*	*French-speaking Switzerland*	*Hessen, Germany*	*Bologna, Italy*	*London, UK*
Year	1989–1991	1965–1984	1997–1998	1997–1999	1999–2000	2002–2004
Population (denominator)	202 774	1 090 055	1 735 420	743 285	336 876	605 230
Number of cases	166	199	172	150	44	226 total 176 first-ever episode of SE
Incidence of SE (per 100 000 per year)	41 (raw) 61 (adjusted)	18.3 (adjusted)	9.9 (raw) 10.3 (adjusted)	15.0	13.1	17–23 (adjusted) 12.5–14 (adjusted; first-ever episode of SE)
Female:male ratio of cases	1:1.2[a]	1:1.9[b]	1:1.7[b]	1:1.9[c]	1:0.74[b]	1:1.12
History of prior epilepsy	42%	44%	32.8%	33%	39%	7%[d]
Inclusions/ Exclusions	Patients 1 month of age or less were excluded	–	Patients with postanoxic encephalopathy were excluded	Only patients of 18 years of age or over were included	Only patients of 20 years of age or over were included	Only convulsive SE was included; only children (<15 years of age) were included
Case ascertainment	Prospective hospital record review	Retrospective review using record linkage system	Prospective hospital record review	Prospective hospital record review	Prospective active surveillance of hospital admissions	Prosepctive active surveillance through A&E and hospital admissions

[a]Raw data.
[b]Adjusted ratio.
[c]Adjusted figures, from the regions with the best case ascertainment (and least likely to selection bias).
[d]Excluding febrile seizures.

Source

DeLorenzo RJ, Hauser WA, and Towne AR (1996) A prospective, population-based epidemiologic study of status epilepticus in Richmond, Virginia. *Neurology* 46: 1029–1035.

Hesdorffer DC, Logroscino G, Cascino G, Annegers JF, and Hauser WA (1998) Incidence of status epilepticus in Rochester, Minnesota, 1965–1984. *Neurology* 50: 735–741.

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Chin RF, Neville BG, Peckham C, Bedford H, Wade A, Scott RC, NLSTEPSS Collaborative Group (2006) Incidence, cause, and short-term outcome of convulsive status epilepticus in childhood: prospective population-based study. *Lancet* 368: 222–229.

Table 3 Seizure type and epilepsy classification in five population-based studies

	Richmond, VA, USA	*Rochester, MN, USA*	*French-speaking Switzerland*	*Hessen, Germany*	*Bologna, Italy*
Seizure type					
Simple partial	23%	39%	18.1%	13.3%	9%
Complex partial	3%		26.7%	43.3%	16%
Tonic–clonic	70%	48%	33.1%	33.3%	50%
Absence	1%	3.5%	3.5%	6.0%	2%
Myoclonic	1%	9.5%			16%
Other	1%[a]		18.8%[b]		
Not specified				4.0%	7%
Epilepsy type					
Acute symptomatic		50.3%	62.7%		34%
Remote symptomatic		19.6%	28.4%		34%
Idiopathic		13.6%			7%
Other		16.6%[d]			25%[c]
Unknown			8.7%		

[a]Electrographic.
[b]Hemiconvulsive 8.1%, subtle status 1.2%, tonic 2.3%, clonic 0.6%, others 6.4%.
[c]Unprovoked progressive symptomatic 11% and multifactorial 14%.
[d]Progressive symptomatic 8.5%, febrile status epilepticus 8.0%.
Source
DeLorenzo RJ, Hauser WA, and Towne AR (1996) A prospective, population-based epidemiologic study of status epilepticus in Richmond, Virginia. *Neurology* 46: 1029–1035.
Hesdorffer DC, Logroscino G, Cascino G, Annegers JF, and Hauser WA (1998) Incidence of status epilepticus in Rochester, Minnesota, 1965–1984. *Neurology* 50: 735–741.
Coeytaux A, Jallon P, Galobardes B, and Morabia A (2000) Incidence of status epilepticus in French-speaking Switzerland: EPISTAR. *Neurology* 55: 93–697.
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Vignatelli L, Tonon C, and D'Alessandro R (2003) Incidence and short-term prognosis of status epilepticus in adults in Bologna, Italy. *Epilepsia* 44: 964–968.

numbers would not include many cases treated in the community. In all, studies, there was marked underascertainment as nonconvulsive forms.

As suggest earlier, case ascertainment in these studies is likely to have been deficient in the following categories: many of the nonconvulsive forms (and in particular, those that are self-limiting or those occurring in the epileptic encephalopathies), convulsive status epilepticus successfully treated in the community emergency departments, and the boundary syndromes. The true frequency of all forms of status epilepticus is thus unknown, but likely to be much higher than generally reported. The frequency of tonic–clonic status epilepticus of more than 30-min duration, estimated in these studies to be between 10 and 40 cases per 100 000 per year, can be considered a reasonably accurate estimate. This frequency is a key point, given the clinical importance of this form of SE.

Factors influencing frequency were also estimated in these studies and include the following:

Age: Age is a dominant factor. The highest incidence of status epilepticus was in young children and in the elderly. The incidence in the elderly is about 3–10 times that of younger adults.

Socioeconomic status and ethnicity: The importance of these variables were highlighted in the study from Richmond, VA, where the incidence of status epilepticus in the African-American population was found to be three times as high as that in the Caucasian population. In another retrospective study of the incidence of generalized tonic–clonic SE in California, the relative risk of the occurrence of status epilepticus, compared to that in the white population, was 1.92 for African-Americans, 0.5 for Hispanics, and 0.4 for Asians. In North London, Asian children (almost entirely from the Indian subcontinent) had a relative risk of 6.5 (compared with age-matched Caucasian children living in the same area) – due mainly to the higher frequency of prolonged febrile seizures.

Gender: In most studies, the incidence of SE was higher in males, although one well-conducted study from Italy found a higher incidence in females.

Other factors known to influence the frequency of status epilepticus are as follows:

Severity of epilepsy: Status epilepticus is much more common in severe epilepsy than in mild epilepsy, although it can occur in both. In a few cases, recurrent status epilepticus is the only manifestation of epilepsy.

Symptomatic versus idiopathic epilepsy: Status epilepticus is far commoner in symptomatic than in idiopathic epilepsy. Indeed, episodes of severe status epilepticus are extremely unusual in all the subtypes of idiopathic generalized epilepsies.

Learning disability: The frequency of status epilepticus is higher in those with learning disability, reflecting the generally more severe epilepsy.

Location of brain lesion: In lesional status epilepticus, lesions situated in the frontal lobes are more likely to result in status epilepticus than lesions in other cortical regions. Subcortical lesions only rarely result in status epilepticus.

Status Epilepticus as a Proportion of Hospital Admissions and as a Proportion of All Epileptic Cases

Various studies have provided data on the numbers of cases of status epilepticus admitted to hospital, and the proportion of cases in relation to the number of patients with epilepsy. These are important statistics for defining the appropriate allocation of resources. Admission figures, of course, will vary according to medical systems and patterns of practice. However, in general terms, about 1–5% of all neurological admissions, about 0.1–0.2% of all emergency unit consultations, and about 5% of all admissions to neurological intensive care units are due to status epilepticus.

About 5% of all adults with epilepsy and 10–20% of all children with epilepsy will have had an episode of status epilepticus at some point in their lives.

The Timing of Status Epilepticus

Tonic–clonic status epilepticus can occur either as the initial epileptic event or as an intercurrent episode in established epileptic patients; this distinction is important, as the etiology and implications of status epilepticus in the two situations are very different. At least 50% of cases occur due to acute brain diseases in adults without a history of epilepsy – and the figure in children is higher. In his review, Hauser estimated that status epilepticus accounts for 12% of all newly diagnosed cases of epilepsy presenting to hospital with a first epileptic event.

Refractory Status Epilepticus

There has been recent interest in the concept of 'refractory status epilepticus,' defined variously as status epilepticus, which does not respond to initial therapy (usually with benzodiazepines and then phenytoin or phenobarbital), or failure of response to three medications, or has continued for over 2 h. These are the cases that require intensive care therapy. Exact figures for refractory status epilepticus are difficult to obtain, partly because of lack of consensus regarding definition. However, estimates suggest that in 31–43% of patients with tonic–clonic status epilepticus, the SE is refractory, with a mortality rate of 16–60%.

Etiology of Status Epilepticus

The occurrence of convulsive status epilepticus in people without epilepsy is almost always due to a sudden cerebral disturbance. In children, fever is an important cause. In people with existing epilepsy, the range of causes differs, and commonly the status epilepticus is due to injudicious antiepileptic drug reduction or withdrawal.

In the population-based survey of children with first-ever episodes of status epilepticus from London, only 30% of the cohort had a history of prior epilepsy (or neurological abnormality) and the commonest cause of the status epilepticus was febrile illness (febrile status epilepticus). This cause accounted for 32% of all cases. Seventeen percent had acute symptomatic status epilepticus, almost all due to acute metabolic derangement (electrolyte imbalance, hypoglycemia, hypocalcemia, or hypomagnesemia) or acute CNS infection. Of the 95 children presenting with fever and status epilepticus, 12% had bacterial meningitis, emphasizing the importance of this treatable cause in young children.

Amongst adults, almost all studies have shown that the majority of cases of status epilepticus occur in previously nonepileptic patients and are due to an acute brain insult. When convulsive status epilepticus does occur in patients with a previous history of epilepsy, many cases are due to antiepileptic drug reduction/withdrawal. In these patients, an acute brain insult is much less commonly the precipitating factor.

In an analysis of the literature in 1994, of 1679 patients in 13 hospital series, status epilepticus had no obvious cause in only 19% of cases (11% in children and 13% in the elderly). Febrile status epilepticus accounted for 12% of cases, and acute metabolic, tonic, and anoxic causes for 14%. Stroke, tumor, trauma, infection, and perinatal causes accounted for between 7 and 10% each. In 554 patients (in five series of largely adult cases), only 41% had a prior history of epilepsy. An acute cause was found in 89% of patients with status epilepticus in previously nonepileptic patients, and in only 41% of patients with a previous history of epilepsy (**Table 4**).

The more recent population-based studies have reported similar findings to those of the older hospital series. In the studies of adult patients from Hessen in Germany, the following etiologies were recorded: remote stroke 36%, other remote symptomatic 27%, Binswanger disease 17%, acute stroke 14%, tumor 13%, medication induced 11%, metabolic disorders 9%, low antiepileptic drug levels 9%, alcohol 9%, remote trauma 8%, and no cause found 9%. In the Bologna study of adults, of the 41 patients with symptomatic status epilepticus, the following etiologies were recorded: stroke 41%, systemic metabolic disorders 24%, head injury 10%, alcoholism

Table 4 The causes of status epilepticus as the presenting symptom of epilepsy, or as an intercurrent event in established epilepsy[a] (in 554 patients from five case series)

	Status as presenting symptom of epilepsy (%) (n = 327)	*Status as an intercurrent event (%) (n = 227)*
Cerebral trauma	12	17
Cerebral tumor	16	10
Cerebrovascular disease	20	19
Intracranial infection	15	6
Acute metabolic disturbance	12	5
Other acute events	14	3
No cause found	11	41

[a]Excluding precipitating causes.
Source
Shorvon SD (1994) *Status Epilepticus: Its Clinical Features and Treatment in Children and Adults*. Cambridge: Cambridge University Press.

7%, tumors 5%, intracranial surgery 2%, and other factors 27% (undefined acute illness in 20%). These findings probably broadly reflect the situation in most Western European adult populations.

In children, the etiologies identified in the Richmond study were as follows: infection 52%, reduction or withdrawal of antiepileptic drugs 21%, vascular causes 10%, hypoxia 5%, overdose 2%, and remote causes (usually congenital or perinatal) 38%.

The etiology of nonconvulsive forms of status epilepticus clearly depends on the underlying syndrome, age, and type of epilepsy. Amongst the rarer causes of convulsive and nonconvulsive status epilepticus, mitochondrial disease (and especially Alpers disease), inflammation, and rarer infections are all important.

Mortality of Status Epilepticus

Convulsive status epilepticus carries a significant mortality. In 1994, a review of 1686 cases from 12 series, there was an 18% mortality rate. The mortality rate is much higher in patients with acute brain disorders and much lower in patients with existing epilepsy (reflecting the different aetiological profiles). Of the deaths in this series, 89% were thought to be primarily due to the underlying cause (e.g., stroke, CNS infection, etc.) rather than the status epilepticus itself. Death was attributed entirely to the status epilepticus or its treatment in only 2% of cases. Age is also an important determinant of fatality, reflecting largely the different aetiological profile. In the Richmond, VA study, the mortality in pediatric, adult, and elderly populations was 2.5%, 14%, and 38%, respectively. In the London study of 176 children under the age of 15 years with first-ever episodes of status epilepticus, 7 children died. In three of these cases, death was due to acute bacterial meningitis, in one to glutaric aciduria type I, and in three others to progressive neurodegenerative disorders; in none was death due primarily to status epilepticus.

More recent studies have reported generally lower mortality rates associated with status epilepticus, although there have been exceptions. In the population-based epidemiological studies referred to earlier, generally much lower mortality rates were found. The case fatality rate was 9.3% in the study from Hessen, 7.6% in the study from Switzerland, and 3% amongst the children in the study from London. In the study from Bologna, a higher rate (39%) was found (33% if one excluded adults with postanoxic status epilepticus). The poorer outcome in this study was attributed in part to inadequate therapy and selection factors. In the latter study, all deaths occurred in the symptomatic group; there were none in the idiopathic or cryptogenic cases – a constant finding in all studies and consonant with clinical experience. The most common cause of convulsive status epilepticus in children is febrile status epilepticus, which also has low morbidity and mortality rates. In a recent review of all reported outcome studies of febrile seizures, the mortality rate of febrile status epilepticus was found to be 1.6%. This rate can be contrasted to the rates reported in landmark studies of the 1970s, in which the mortality rate was 11%.

The change in rates over time is thought in part to be due to the fact that status epilepticus is now treated earlier and much more vigorously than in the past. The early use of benzodiazepines, the use of prehospital therapy, and the much greater sense of urgency have all contributed to improvements in outcome. This improvement is particularly true of febrile status epilepticus and acute convulsive seizures in the community. However, once a patient has proved refractory to early therapy and requires intensive care and intravenous anesthetic, the mortality rate is between 40 and 50%. Even in this situation, the underlying causal condition remains the primary cause of mortality, and status epilepticus a secondary but important influence.

Nonconvulsive status epilepticus carries much less risk and lower mortality than convulsive SE, but again the outcome depends almost entirely on the underlying condition. Neonatal status epilepticus has a mortality rate of about 15%, infantile spasms of about 5%. Conditions with little or no acute-phase mortality include the syndromes of absence status epilepticus, electrical status epilepticus during slow wave sleep, the Landau–Kleffner syndrome, and the boundary syndromes. Complex partial status epilepticus has a definite but low mortality rate. The 30-day mortality rate of myoclonic status epilepticus in coma is over 60%.

Directions for Future Research

It is clear that the epidemiological data on status epilepticus, and particularly the nonconvulsive forms of SE, are

incomplete, and that the establishment of an accurate database is a challenge for the future. This need is true both for children and adults. A major obstacle is lack of agreement about diagnostic criteria and the inclusion or exclusion of 'boundary syndromes' (for instance, in the epileptic encephalopathies of childhood or the myoclonic status epilepticus in coma in adults). Such variations in inclusion criteria greatly affect the results of investigations and prevent meaningful comparisons between studies. Until accurate figures are available, one of the fundamental goals of epidemiological research, that is, the accrual of data to inform rational service provision, cannot be met. Similarly, there are no data on which one can base identification of environmental factors, knowledge of which could lead to preventative strategies; epidemiology could be usefully employed in this area of investigation. Many aspects of the epidemiological study of status epilepticus would be improved by organization on a multicenter or multinational basis. Such studies carry the potential of providing interesting and valuable epidemiologically based information in areas that cannot be otherwise easily investigated.

See also: **GABA**: Pathophysiology of Status Epilepticus.

Further Reading

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GENETICS

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Clinical Genetics of Epilepsy

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Introduction and Background

The great ancient Greek physician Hippocrates recognized the hereditary component to epilepsy in an era when it was regarded not as a physical disease, but rather as a spiritual visitation. Sadly, the familial nature of epilepsies has contributed to much discrimination over the millennia, and likely also retarded the scientific study of epilepsy genetics. From a contemporary perspective, the vast majority of familial epilepsies are benign and easily

incomplete, and that the establishment of an accurate database is a challenge for the future. This need is true both for children and adults. A major obstacle is lack of agreement about diagnostic criteria and the inclusion or exclusion of 'boundary syndromes' (for instance, in the epileptic encephalopathies of childhood or the myoclonic status epilepticus in coma in adults). Such variations in inclusion criteria greatly affect the results of investigations and prevent meaningful comparisons between studies. Until accurate figures are available, one of the fundamental goals of epidemiological research, that is, the accrual of data to inform rational service provision, cannot be met. Similarly, there are no data on which one can base identification of environmental factors, knowledge of which could lead to preventative strategies; epidemiology could be usefully employed in this area of investigation. Many aspects of the epidemiological study of status epilepticus would be improved by organization on a multicenter or multinational basis. Such studies carry the potential of providing interesting and valuable epidemiologically based information in areas that cannot be otherwise easily investigated.

See also: **GABA**: Pathophysiology of Status Epilepticus.

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GENETICS

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Clinical Genetics of Epilepsy
Large-Scale Mapping and Epilepsy Gene Discovery
Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex

Clinical Genetics of Epilepsy

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treatable, and except in unusual circumstances, the diagnosis of epilepsy should not alter an individual's marital and reproductive choices.

Although the involvement of genetic factors in epilepsy was widely known, systematic study was relatively limited until the latter part of the twentieth century. The major exception to the lack of early data was the contribution of William Lennox, who from the 1930s until 1960 meticulously studied twins and family history in large cohorts of patients with clinical genetic techniques available at that time.

Classification is crucial to genetic analysis, since there is little value in attempts to analyze genetic factors if the clinical classification is imprecisely defined. Indeed, progress in classification and genetics are inherently linked. It took a long time for general recognition of the idea that epilepsy was not one disorder, but rather a group of disorders, more appropriately regarded as 'the epilepsies.' Progress was made with the evolution of the International League Against Epilepsy's (ILAE) classification, which differentiated types of epileptic seizures. The early classification scheme was followed in 1989 by a revised classification incorporating epileptic syndromes. The classification makes the important division of the epilepsies into generalized and partial/focal epilepsies, which are then broken down into idiopathic and symptomatic categories. It provides a framework for research into the genetics of the epilepsies. This classification is currently being updated, incorporating new genetic concepts.

Methods

Problems in Clinical Genetic Assessments

Clinical genetics depends implicitly on quality clinical data, and the quality of clinical data is a major issue in epilepsy research, particularly if the clinical descriptions are based on chart review. Although a family history is usually taken as part of a routine clinical assessment, this history is often only cursory. A detailed family history can be the most important clinical clue to a genetic aetiology.

A formal pedigree can be taken relatively quickly, and a checklist of important information is given in **Table 1**. A history of 'epilepsy' is not sufficient. The clinician should enquire whether family members have had any type of paroxysmal attack, including febrile convulsions, nocturnal attacks, or isolated seizures. The patient often may not know much about his/her family history, but should be encouraged to talk to the mothers in the family and to any elderly matriarchs who may know of early childhood attacks (long since forgotten) in other family members. It is common that the family history is 'fleshed out' by inviting the patient to provide more significant detail at subsequent visits.

Attention to detail is important in constructing a pedigree. It is worth recording the date of birth (if known) of family members, maiden names of partners, and the country of origin of a person's ancestors (**Table 1**). Often first names recur in a family tree, and the date of birth is essential to prevent confusion amongst family members with the same name. The question of possible consanguinity should be raised with each union, as this point may be crucial to understanding the clinical genetics of a seizure disorder. If the parents are not known to be related, but come from the same region or small village, distant consanguinity may be a possibility.

Table 1 Checklist for routine pedigree construction

- Name and date of birth of individuals
- Maiden names of partners
- Countries of origin of ancestors
- Consanguinity
- Obstetric history (miscarriages, stillbirths, neonatal deaths)
- Ask about any seizures (not just epilepsy) including febrile convulsions, nocturnal or paroxysmal attacks
- Ask patient to obtain data from other family members
- Ask older family members, particularly women
- Recheck data on follow-up visits
- Update pedigree when new individuals become affected or new children are born

Source
Scheffer IE and Berkovic SF (2000) Rational diagnosis of genetic epilepsies. In: Schmidt D and Schachter SC (eds.) *Epilepsy: Problem Solving in Clinical Practice*, pp. 111–131. London: Martin Duntz.

It is also important to ask about the patient's mother's obstetric history. If the patient's mother had an undue number of miscarriages or stillbirths, this history may point to a genetic etiology following sex-linked dominant inheritance with lethality in males. Some developmental malformations such double cortex syndrome and periventricular heterotopia have been recognized with this inheritance pattern in certain families – a pattern which is easily missed if the salient questions are not asked.

The familial nature of the patient's epilepsy may only become apparent with time as further extended family history comes to light or new members of the family develop epilepsy. The older generations may initially be reticent to discuss a family history of seizures, as they feel guilty about 'passing on' a disorder. With gentle education regarding genetic issues, as well as time, they may volunteer carefully guarded family secrets of major relevance.

Pedigrees with males solely affected raise the question of X-linked inheritance that may be seen in Fragile X syndrome, ARX mutations, and many other rare disorders. Where only females are affected, X-linked dominant inheritance must be considered; a clue may be an excess of male fetal losses (discussed earlier). Another disorder with only females affected – where paradoxically males are spared – is epilepsy and mental retardation limited to females (see Further Reading).

Methodological Approaches

The clinical genetic assessment described above is a starting point for analyzing epilepsy genetics as subjects present to clinicians or clinical researchers. In approaching the issue of clinical epilepsy genetic studies more broadly, a number of research strategies are used. The choice of strategy depends on the question being addressed and the clinical sample under evaluation.

Genetic Epidemiology

These studies provide a population-based view and seek to measure genetic factors operative across an unselected population. Evidence of a genetic contribution to disease is obtained by examining aggregation in families. Familial aggregation can be assessed by comparing the prevalence of epilepsy in a specific group of relatives (e.g., siblings, second-degree relatives) to the prevalence in the general population. The ratio of these is the recurrence risk ratio. For example, a sibling recurrence risk ratio of 2 means that siblings of patients are at twice the risk compared with the general population.

A common problem with such studies is that it can be difficult to obtain detailed and uniform information about phenotypes across a population. Nonetheless, they provide an important 'big picture' view of the problem.

Multiplex Family Studies

Here, large families with multiple affected individuals (typically 10 or more individuals) are specifically targeted. To date, these studies have provided the most robust advances in clinical epilepsy genetics, with identification of many new syndromes and subsequent molecular genetic discoveries.

Recognition of a new familial syndrome, which is likely to be under the control of a single genetic mutation rather than a random cluster of cases with multifactorial causes, is clinically challenging. Some phenotypes, such as febrile seizures, are relatively common in the general population, so that not all patients may have febrile seizures due to the same underlying mutation in a multiplex family, despite an apparently dominant inheritance pattern. Researchers must hypothesize which individuals are likely to have the familial syndrome in order to enable successful linkage and subsequent gene discovery.

Assessment of smaller multiplex families (e.g., 3–6 affected individuals) can be valuable in understanding the clinical relationship between epilepsy phenotypes. Some families might have a major dominant gene, but most are likely to have a complex genetic structure where multiple genes are involved.

Although multiplex families are not typical of the general population, discoveries about their clinical and molecular genetics have provided insights into the genetics of the common epilepsies.

Endophenotyping

In attempts to simplify molecular analyses in disorders with complex inheritance, endophenotyping may have a role. Here, rather than using the epilepsy syndrome as indicating affected status, a heritable subclinical marker is chosen and attempts are made to map this trait through families. Ideally, such markers should be easily and objectively measured and be present outside the age range of the syndrome under scrutiny. Endophenotypes could include EEG features such as photosensitivity, neuroimaging changes, or subtle differences in seizure phenomena.

Twin Studies

Twin studies, which were first extensively utilized in epilepsy by Lennox, remain a powerful method for demonstrating a genetic contribution in diseases with complex genetics. A comparison is made between concordances in identical (monozygotic) and nonidentical (dizygotic) twin pairs. Concordance refers to the presence of a disease in both twins. The degree of genetic contribution in a particular syndrome is judged by the completeness of the concordance in monozygous twins and by the extent to which it exceeds that in dizygotic twins.

Twin studies allow the distinction of the effects of shared genetic factors from those of shared environmental factors. Moreover, significant results can be achieved in relatively small samples in which detailed and consistent phenotyping is feasible. Study of twins can also aid in classification and nosology, addressing the issue of 'lumping' versus 'splitting.' If two apparently different syndromes (splitter's view) occur in monozygotic twins, then the syndromes are quite unlikely to be genetically different, providing evidence that they would be better regarded as a single entity (lumper's view). Finally, twins can also be used in matched pair designs, where one twin has had an exposure and the other not. Examples include effects of anticonvulsant exposure or postnatal insults.

In assessing twin studies, care must be taken to account for specific ascertainment biases. These biases include an excess of female monozygotic twins in volunteer twin registries, and the tendency to over-report cases of concordant monozygotic twins.

Single Cases and Case Series

Although it may seem counterintuitive to regard isolated cases as useful in genetic studies, it is becoming increasingly apparent that new (de novo) mutations, where there is no family history, form an important part of the genetic architecture of epilepsies.

This has long been known in classical chromosomal syndromes associated with epilepsy, such as trisomy 21, ring 20 syndrome, Angelman syndrome, etc. More recently, it has become clear that most cases of Dravet syndrome have de novo mutations in the sodium channel alpha 1 subunit gene *SCN1A*. De novo mutations are found in a variety of severe epilepsies, but are also know to occur in mild syndromes such as benign neonatal seizures.

Finally, it should be noted that strategies for clinical genetic research are changing with the power of new technology in molecular genetics. On the one hand, discoveries may now be made on only a few patients' samples. For example, the gene for the rare recessive disorder of action myoclonus-renal failure syndrome was identified using only three affected individuals. On the other hand, the current wave of genome-wide association studies, which may detect common variants as susceptibility genes for epilepsy, will need thousands of DNA samples from unrelated individuals with well-phenotyped genetic epilepsies.

Recent Results

Recent reviews highlight the major advances in this rapidly moving field (see Further Reading). Genetic epilepsies continue to be newly described or refined. In 2008, familial occipitotemporal lobe epilepsy and migraine with visual aura was described, POLG1 mutations were identified as an important cause of occipital lobe epilepsy, and the phenotype of epilepsy and mental retardation limited to females was extended.

Pharamcogenetic studies have largely been disappointing or controversial to date, but one finding has emerged of practical clinical importance. The risk of the severe skin reactions, Stevens Johnson syndrome or toxic epidermal necrolysis, in individuals of Asian descent appears to be markedly increased if they carry the HLA-B*1502 allele.

Table 2 shows some gene discoveries made in idiopathic epilepsies to date. The vast majority of these have been in monogenic epilepsies, but the difficult problem of identifying susceptibility genes is beginning to be tractable.

Future Goals

There is an exciting bright future for clinical genetic research in epilepsy. New syndromes continue to be discovered. By collaborating with molecular geneticists, the

Table 2 Some genes implicated in idiopathic epilepsies

	Monogenic Disorders	*Susceptibility Genes*	*Gene product*
Autosomal Dominant seizure syndromes of the first year of life			
Benign familial neonatal seizures (BFNS)	*KCNQ2* *KCNQ3*		Neuronal voltage-gated potassium channel subunits, responsible for M-current
Benign familial neonatal-infantile seizures (BFNIS)	*SCN2A*		Neuronal voltage-gated sodium channel subunit
Febrile Seizures, Generalized Epilepsy with Febrile Seizures Plus (GEFS+) and Dravet Syndrome			
GEFS+, FS, Dravet syndrome	*SCN1A*		Neuronal voltage-gated sodium channel, α_1- and β_1-subunit
GEFS+	*SCN1B*		
GEFS+, FS, Dravet syndrome	*GABRG2*		γ_2-Subunit of the neuronal $GABA_A$-receptor
GEFS+		*GABRD*	δ-Subunit of the $GABA_A$-receptor
Idiopathic Generalized Epilepsies (IGE)			
Childhood absence epilepsy	*GABRG2*[#1]		γ_2-Subunit of the neuronal $GABA_A$-receptor
Juvenile myoclonic epilepsy	*GABRA1*[2]		α_1-Subunit of the neuronal $GABA_A$-receptor
Various IGE phenotypes	*CLCN2*[#]		Voltage-gated chloride channel
Childhood absence epilepsy		*CACNA1H*[3]	Neuronal voltage-gated T-type calcium channel subunit
Juvenile myoclonic epilepsy		*EFHC1*[4]	Protein of unknown function with Ca^{2+}-binding EF-hand motifs
Partial Epilepsies			
Autosomal dominant nocturnal frontal lobe epilepsy	*CHRNA4* *CHRNB2* *CHRNA2*		Neuronal nicotine receptor subunits
Autosomal dominant partial epilepsy with auditory features	*LGI1*		Implicated in the assembly of presynaptic potassium channels

Source

Helbig I, Scheffer IE, Mulley JC, and Berkovic SF (2008) Navigating the channels and beyond: Unravelling the genetics of the epilepsies. *Lancet Neurology* 7: 231–245.

clinical researcher can be a key player in a vital team that unlocks fundamental neurobiological discoveries that come from isolating genetic defects.

The relationship of phenotype to genotype is becoming more challenging. Far from the initial vision of particular genes being associated with particular clinical syndromes, it is now clear that many clinically homogeneous genetic epilepsies can be caused by more than one gene, and that defects in one gene may cause different phenotypes. A new level of analysis and interaction between clinical researchers and their molecular genetic colleagues is required to attack this puzzling problem.

Large multicentre studies, with carefully phenotyped cases, are required for genome-wide association studies. It is clear from studies in other disorders that subtleties in phenotyping may be crucial to the success or failure of such endeavors.

Pharmacogenetics of epilepsy is a growing field and will need careful clinical research to use the benefits potentially afforded by the emerging technologies. In the future, it may be possible to determine the likelihood of treatment success and the probability of specific side-effects with particular drugs in individual subjects.

See also: **Genetics**: Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Genetic Contributions to Brain Structure and Epileptogenesis; Human Epilepsy as a Complex Genetic Trait: Lessons from Animal Models and Prospects for the Future; Large-Scale Mapping and Epilepsy Gene Discovery; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; Single Gene Mutations in Inherited and Sporadic Epilepsy.

Further Reading

Berkovic SF, Dibbens LM, Oshlack A, *et al.* (2008) Array based gene discovery with three unrelated subjects shows SCARB2/LIMP-2 deficiency causes myoclonus epilepsy and glomerulosclerosis. *American Journal of Human Genetics* 82: 673–684.

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Large-Scale Mapping and Epilepsy Gene Discovery

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Introduction

How does one identify Mendelian genes (traits whose genetic basis is a single gene), and complex non-Mendelian genes (traits whose genetic bases are multiple genes) in epistases that are responsible for at least half of all epilepsies? These genetic epilepsies represent 25–50 million people worldwide, ranging from the full epilepsy phenotypes (grand mal, absence, and myoclonic seizures), to endophenotypes (lateral temporal lobe auditory hallucinations, nocturnal rolandic bucco-oral, and appendicular motor seizures), including a variety of EEG abnormalities (diffuse polyspike waves, focal temporal spikes, centrotemporal rolandic spikes), speech impairment, oromotor apraxia, and cognitive deficits. These full seizure phenotypes and some endophenotypes endanger life, increase morbidity and mortality, produce social disadvantages, compromise quality of life, and can cost a single nation $11.1–12.5 billion a year. Most researchers now agree that the clear and surest way to develop cures

clinical researcher can be a key player in a vital team that unlocks fundamental neurobiological discoveries that come from isolating genetic defects.

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and repairs for these epilepsies is to define their epilepsy-causing mutations, the biochemical pathways in which these epilepsy genes act, and their consequent pathogenetic mechanisms. Mapping (finding) the chromosomal locations of these epilepsy genes is the first step toward their molecular definition.

Background

Most idiopathic generalized epilepsies and some focal epilepsies show a strong genetic component. In juvenile myoclonic epilepsy (JME), the 56–65% concordance rate for monozygotic twins (who share 100% of their DNA) and 21–12% for dizygotic twins (who share on average 50% of their DNA) suggest a strong genetic influence in its etiology. Janz and colleagues reported about one-third of JME patients had a positive family history for epilepsy; 3.4% of parents were affected with epilepsy, 7.0% of sibling, and 6.6% of children. In a recent review of our sample of 186 JME cases, 49% of our JME probands had an immediate family member with epilepsy or the EEG polyspike-wave trait. About 3.3% of parents, 6.7% of siblings, 3.4% of offspring, and 1.6% of second degree relatives of classic JME, have JME. In contrast, only 0.7–3.4% of the same relatives of classic JME have childhood absence. In terms of relative risks, or risk ratio to a relative, as explained by Risch in 1990 (risk ratio represents the recurrent risk or percent of a specific epilepsy (in this case JME) in a family member divided by prevalence of a specific epilepsy (in this case, JME) in the normal population), JME is 100 times more prevalent in parents and offspring and 200 times more prevalent in siblings, while 50 times more prevalent in second-degree relatives than in the normal population. In contrast, childhood absence is only 0.7–3 times more prevalent in relatives of classic JME patients. The low drop-off rate of recurrence risk from first degree relatives to second degree relatives is highly suggestive of a monogenic disorder. Almost all genetic modes of inheritance have been proposed for JME, including autosomal dominant (AD) transmission, a two-locus dominant–recessive model, and an autosomal recessive (AR) mode of inheritance. We used a dominant model with reduced penetrance (70%) to successfully map a JME gene to 6p12 (Myoclonin1/EFHC1); JME appears to segregate as an AD trait in these samples.

Historically, a strong genetic contribution to the spike-and-wave traits and absence epilepsies have also been supported by 84% concordance for EEG traits and 75% concordance for absence seizures in monozygotic twins. These twin studies support the concept of a major gene, working with additional genetic and environmental factors, in the phenotypic expression of absence epilepsy. In Rolandic epilepsy with centrotemporal spikes, complex genetic inheritance as well as AD inheritance has been observed. The syndrome has been mapped to chromosomes 16p12–11.2, 15q14, and 11p, while SRPX2 mutations have been linked with disorders of language cortex and cognition.

Despite family studies showing heritability and a clear genetic etiology, only 40 Mendelian epilepsy-causing mutated genes (mutations sufficient and necessary by themselves to cause epilepsy) have been defined. These genes account for 1%–10% of all Mendelian epilepsy genes. Moreover, the multiple susceptibility variants or alleles that contribute to the common complex or sporadic epilepsies remain undefined. To address these shortcomings, large scale mapping is now being done by various genetic consortia, through whole genome scans using 440 short tandem repeat polymorphisms (STRPs) (also called microsatellites in family-based cohorts) or 500 000 single nucleotide polymorphisms (SNPs) (in case association studies and linkage disequilibrium studies in trios). Most recently, 500 000 SNPs have also been used in linkage analyses of large families.

Methodological Approaches and Results to Date

Mendelian epilepsy genes, and their mutations that cause monogenic idiopathic epilepsies, are revealed by linkage analyses and positional cloning, using STRPs (microsatellites). Genetic linkage analysis (also called recombination mapping or crossing-over mapping) orders genetic loci (e.g., a disease allele and a DNA marker) on a chromosome and estimates genetic distances among them using the phenomenon of crossing over or recombinations. During meiosis (a stage of cell division), the two homologous chromosomes separate at most places but also maintain one or more zones of contact where crossing over of DNA strands occur. With increasing distance between two loci, an increasing number of crossovers occur. The number of crossovers between two loci serves as a measure of distance (in units of Morgans) between the loci. In a disease state, one can visualize crossovers between markers on a chromosome and place a disease locus into an interval between markers that crossed over together. We observe such phenomena when a disease allele and a marker is passed from a grandparent to a parent and from the parent to an offspring; the disease allele and the DNA marker are genetically coupled and linked. We call this phenomenon genetic linkage. Many new statistical algorithms determine the crossover frequencies occurring between two points on a gamete and the presence of linkage between two loci using the original Elston–Stewart algorithm.

Likelihood for linkage has loosely been called odds for linkage, and a given value indicates a ratio of how much higher the likelihood of the data is under linkage than under

absence of linkage. It is usually convenient to work with the logarithm of this likelihood ratio, which is the so-called Logarithm of Odds (LOD) or LOD score. The maximum of the LOD score denoted Z max serves as a measure for the weight of the data in favor of the hypothesis of linkage. When that maximum exceeds a certain critical value, the data are said to convey significant evidence for linkage. According to Morton, the critical value is three for autosomal loci and two for X-linked loci. As mentioned in the later text, with dense maps and 200–500 microsatellite DNA markers, the pointwise critical LOD scores required for an overall genome wide significance level of 0.05 varies according to the number of tests done with the markers and the diagnostic models used. With five diagnostic models used, the critical LOD score should be raised to 4.0.

There are two ways in which families can be used in linkage analysis: (1) In advanced socioeconomic countries where large multiplex and multigeneration families are rare, small, and medium sized families (with 3–5 affected members) are usually studied in 30–60 families for linkage analyses. LOD scores from each of the families are then pooled together. (2) Dominant Mendelian traits typically occur clustered in large pedigrees, with affected individuals being found in many generations. In developing socioeconomic countries, such large multiplex and multigeneration families are common. In dominantly inherited epilepsies, the ideal situation is to have one large family or a set of large families, each with at least nine affected members over at least three generations. For rare dominant traits, only one parent is heterozygous for the disease allele and potentially informative for linkage. Such a family can independently prove linkage to a given chromosome site (LOD score >3.35 at theta $= 0$ during genome wide scan using microsatellites spaced 1 cM, or LOD score >3.63 at $\theta = 0$ during genome wide scan using microsatellites spaced 0.1 cM) (see **Table 1**).

In recessive epilepsy traits, six affected offsprings belonging to consanguineous matings in one large kindred will usually reveal homozygosities that point to the chromosome site (supported by significant LOD scores). Lander and Botstein called this homozygosity mapping. An affected offspring provides approximately five times more linkage information than an unaffected offspring. With tight linkage of a recessive trait and a marker, each offspring independently contributes 0.602 and each unaffected offspring contributes 0.125 toward the expected LOD score. Using both approaches, we have identified two AD mutation harboring epilepsy genes (myoclonin1/EFHC1 for JME, and GABRB3 for remitting childhood absence) and two AR epilepsy genes (laforin/dual specificity phosphatase and malin/E3 ubiquitin ligase for Lafora progressive myoclonus epilepsy).

In genetically complex epilepsies that are clearly heritable yet do not follow any of the known modes of inheritance and are thought to be due to interaction (epistases) of multiple genes with small genetic effects, at least

Table 1 Large scale mapping of epilepsy chromosome loci and epilepsy gene discovery

	Mendelian epilepsy genes	*Complex non-Mendelian epilepsy genes*
Method for locating chromosome site	Genetic linkage analyses	Case association studies or transmission disequilibrium test (TDT)
Epilepsy subjects	For dominantly inherited epilepsies: • Large families (>9 affected in at least three generations) and • Medium sized families (>2 affected in at least three generations) For recessive epilepsies: • Six offsprings of consanguineous matings in a large kindred	For case association studies: • Two cohorts of 1 200–2000 unrelated index cases and matched controls of equal number For transmission linkage disequilibrium: • Two cohorts of >300 trios (parents and index cases)
Markers used	>400 microsatellites	Universal tag set of SNPs (>500 000 SNPs)
Refinement/reduction of size of chromosome site by recombination and haplotype analyses and linkage disequilibrium	Necessary	SNP haplotype may directly lead to the putative epilepsy variants
Mutation analyses	Mutations segregate with affected members of many generations in many unrelated families from separate cohorts, ideally of different geographic and ancestral origin	For case association studies: • SNPs associate with index cases more than by chance For transmission linkage dysequilibrium: • SNPs are transmitted to index case more than by chance
Replication	Usual	Difficult but possible using two-stage approach
Proof of causality	Knockout or knockin mice reproduce one or all phenotypes of epilepsy syndrome	Difficult and presently not achieved

Modified from Delgado-Escueta AV (2007) Advances in genetics of juvenile myoclonic epilepsies. *Epilepsy Currents* 7(3): 61–67.

1200–2000 unrelated index cases (preferably 10 000–20 000 cases) and an equal number of age, sex, and racial/geographically matched controls would be ideal for a case control association study during a genome – wide scan using a single universal tagging set of SNPs (300 000–500 000 SNPs) aided by the 2005 international HapMap. This situation would allow construction of tag SNP haplotypes that often travel together through generations of recombinations and could directly identify the many common DNA variants that underlie susceptibility to epilepsy in many samples around the world. For this approach, a two-stage analysis is commonly used. In stage one, a test cohort of 1200 individuals is genotyped, and then (stage two) a replication cohort of another 1200 persons is genotyped. Stringent criteria to satisfy the presence of significant association should be followed. An association exists between a disease and a DNA tag SNP when they occur together in the same individuals with the disease more often than expected by chance. To investigate whether the association is present, two groups are collected, the disease group and a control sample. In each group, the proportion of individuals with the disease SNP allele is determined and the usual chi-square test for 2×2 tables is applied to determine significance. The strength of an association is then measured by the relative incidence or relative risk.

For case–control association studies, allele and genotype frequencies are calculated using the gene counting method. Hardy–Weinberg equilibrium calculations are carried out for all SNPs in each test and control population. A population is said to be in Hardy–Weinberg equilibrium when the genotype frequencies in the population depend only on the gene frequencies, under conditions of random mating and in the absence of disturbing forces such as mutations, migration, and selection at the gene in question. Allele and genotype association between epilepsy probands and controls are tested using the Transmission Disequilibrium Test (TDT), whose statistic as discussed earlier is tested using chi square (χ^2). Correction for multiple statistical testing is commonly done using the Bonferroni correction. If different populations from different geographic areas are used, combined cross-population p-values are calculated using Stouffer's weighted-Z-method.

Family-based association study (one offspring and two parents or trios) can also be done for epilepsy families with two or more generation members, both under the null hypothesis of no association and no linkage, and under no association in the presence of linkage with an extension of the TDT.

Mendelian Epilepsy Genes

Other investigators have suggested that since genetic markers no longer constitute a constraint, the greatest limitation to the study of human heredity disease is finding enough families with multiple affected members. In our experience, this limitation can be overcome in cultures such as in Hispanics from Los Angeles (CA, USA), Mexico, Honduras, El Salvador, Brazil, and Peru, since large families are common in these societies and cultures. Among those populations, we can attain the ideal situation for independently proving linkage – a large family with multiple members affected with epilepsy across at least three generations. With marker spacing <0.1 cM, the appropriate critical LOD score limits of 3.3 will give an overall genome-wide significance level of 0.05.

Large families can have rare mutations that are unique to that particular family, and thus may not represent the common forms of idiopathic epilepsies found in the more common smaller families. Hence, steps 1 and 2 of our strategy (see **Figure 1**) require that linkage found in one large family be replicated in another data set of one or two other large families of the same racial ethnic group. Because replication does not carry the burden of multiple testing as a genome screen, the significance level, when replicating a previously significant finding, may be set anywhere from 0.01 to 0.05.

On a practical level, a second large family should replicate a first family's linkage. However, we usually have to screen 5–10 large families before we are able to replicate a linkage claim. Thus, step 1 in our strategy requires that we enlarge the existing original family significantly linked to a chromosome locus by enrolling fourth generation or fifth generation offspring of previously genotyped members. With each new generation of affected members, LOD scores should increase. Otherwise, the significant linkage findings may be spurious.

Once we have two families that each independently prove significant linkage, pooled LOD score will be >5 ($\theta = 0$) and recombinations/crossovers can be confidently used to localize and reduce the size of the candidate chromosome regions. Haplotypes from these two families can then be used with 20–40 surrounding microsatellites and tSNPs as screening sets to narrow the critical gene region, to look for more recombinations in large and medium size families and determine if a common affected haplotype exists segregates with affected members in a set of 30–50 medium size multigeneration families. This approach gives us even more convincing evidence that an epilepsy gene contained within the haplotypes is segregate with epilepsy affected members and also gives us a glimpse of how common the putative candidate region and contained epilepsy gene is amongst familial epilepsy.

With more recombinations, the critical gene interval can be reduced to <1 cM, setting the stage for heteroduplex analyses and sequencing of candidate JME genes. Usually the critical gene interval will contain transcripts, known genes, full-length cDNAs, ESTs, and predicted

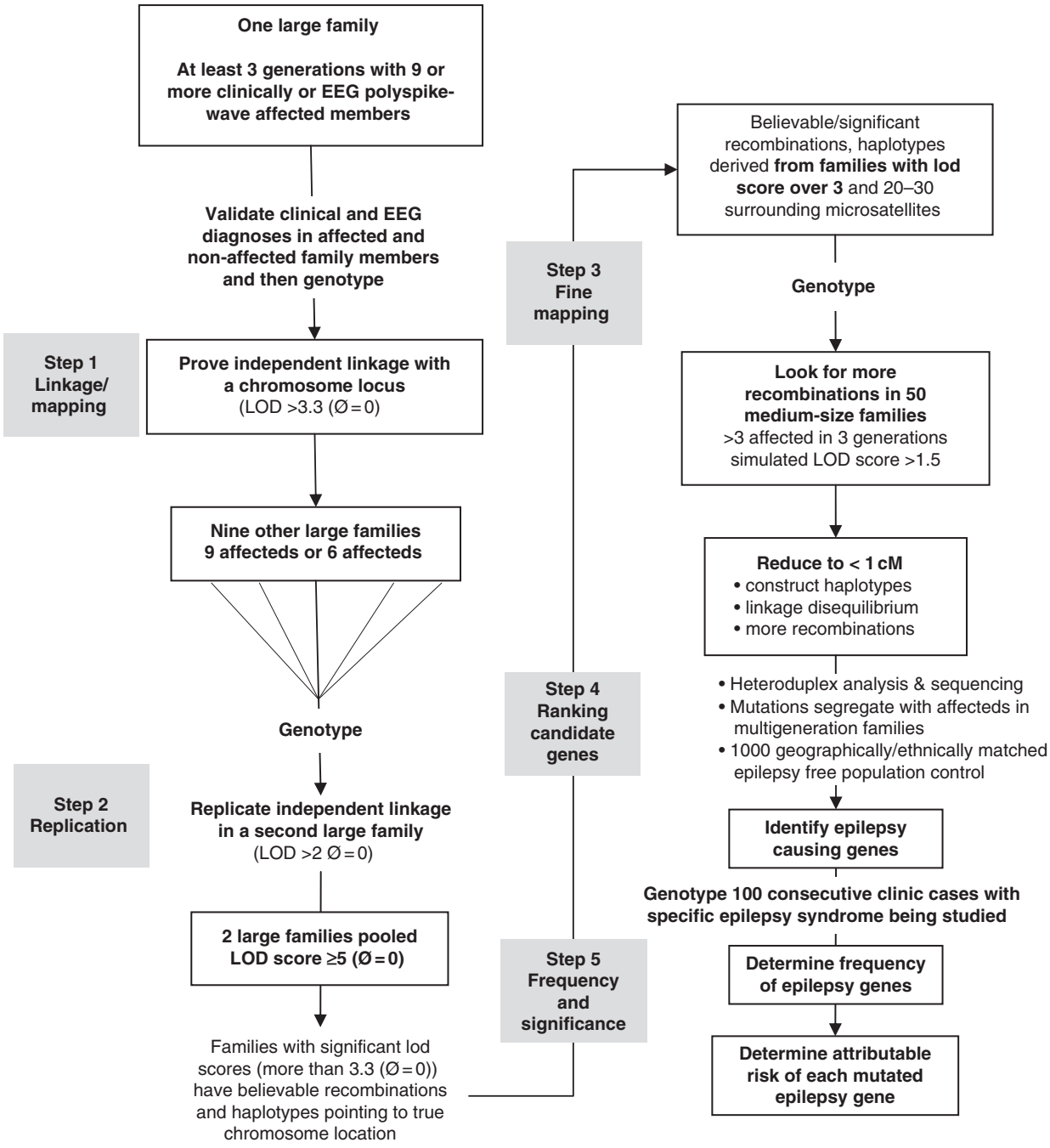


Figure 1 Strategy for search of mutation harboring Mendelian epilepsy genes.

proteins. To reduce the gene list, predicted proteins without a start codon and stop codon should not be studied. In addition, homology searches for translated sequences (by use of BLAST, a program that reveals genes with sequences that are similar to a gene in question, sequences conserved in evolution within various animal species) should be performed to eliminate additional putative genes. Remaining candidate genes must be expressed in brain to receive our attention for direct sequencing or heteroduplex analyses. This outlined strategy has served us well and helped us identify Laforin/DSP (the disease causing gene of Lafora epilepsy), myoclonin/EFHC1 (the epilepsy/polyspike wave EEG causing gene of JME), and GABRb3 (the seizure causing gene of remitting childhood absence epilepsy).

Tables 2–5 list the chromosome loci and mutation containing Mendelian genes found so far in idiopathic epilepsies.

Table 2 Chromosome loci and genes identified in JME as of 2008

Locus	*Gene*	*Country/ethnic group*	*No. of families*	*Mode of inheritance*	*Phenotype*
6p12–11	Myoclonin 1/EFHC1[a]	Native Americans mixed with Europeans (Hispanic) [UCLA]	22	AD	Classic JME and pCAE evolving to JME
		Native Americans mixed with Europeans (Hispanics) [Belize]	1	AD	Classic JME
		Native Americans mixed with Europeans (Hispanics) [Mexico[b]]	31	AD	Classic JME
		Native Americans mixed with Europeans (Hispanics) [Honduras]	2	AD	Classic JME and pCAE evolving to JME
		Japanese [Japan]	2	AD	Classic JME
6p12; 16p13; and 7q32	–	Netherlands Netherlands	18	AD	JME Photosensitive JME
6p21.3	BRD2[b]	Americans of European descent [Los Angeles, California]	24	AD	Classic JME
		Americans of European descent [New York[c]]	85	AD	Classic JME mixed with pCAE evolving to JME
6p21.3	–	Germany	29	AD	JME
15q14	CX36[b]	United Kingdom, Denmark, France, Greece, Portugal, Sweden	25	AR	JME
6q24	–	Saudi Arabia	34	AR	JME, some nonproband members with mild gait ataxia and tremors
2q22–2q23	CACNB4[a]	Germany	1	?	Classic JME with absences
5q34 GABR1	GABRA1[a]	French Canadian (Quebec, Canada)	1	AD	4/8 affected family members had pCAE. one with epilepsy onset at 5 year
3q26	CLCN2[a]	Germany	1	AD	Classic JME
18	ME2[b]	American of European descent (NY, USA)	–	?	Classic JME
10q25–q26	–	India (New Delhi)	11	AD	JME
5q12–q14	–	India	1	AD	JME

[a]Mutations.
[b]SNP associated variants; AD, autosomal dominant; AR, autosomal recessive; JME, juvenile myoclonic epilepsy; pCAE, pyknoleptic childhood absence epilepsy.
Modified from Delgado-Escueta AV (2007) Advances in genetics of juvenile myoclonic epilepsies. *Epilepsy Currents* 7(3): 61–67. (Article includes references for the studies).

Table 3 Chromosome loci of idiopathic epilepsies of infancy, childhood, and adulthood

Phenotype	*Genotype*[a]	*Country of origin & No. of families*
Familial febrile seizures (FEB3), (possibly GEFS+)	2q23–24	One family from Utah, USA
Generalized epilepsies with febrile seizures plus (GEFS + type 2)	2q23–q31	One family from Northern Victoria, Australia; SCN1A mutation in two families from France
Generalized epilepsies with febrile seizures plus (GEFS + type 1)	19q13	SCN1B mutation in one Tasmanian family from Australia
Generalized epilepsies with febrile seizures plus (GEFS + type 3)	5q	GABRG2 mutation in one family from France
Febrile seizures and childhood absence	5q	GABRG2 mutation in one family from Australia
AR idiopathic myoclonic epilepsy of infancy	16p13	One family from Sardinia, Italy
Remmitting childhood absence epilepsy	15q11–12; GABARB3 Exon 1a and Exon 2 mutations	Four families from Mexico and Honduras
Childhood absence epilepsy persisting with grand mal	8q24	One large family from India and medium size families from Argentina, Spain, and Los Angeles
Photogenic childhood absence with eyelid myoclonia evolving to JME	Unknown	Five families Mexico and California (USA)
Benign Adult Familial Myoclonic Epilepsy	8q23.3–q24.1	Five families from Japan

[a]Unless indicated all evidence acquired by model dependent linkage analyses.
Modified from Delgado-Escueta *et al.* (2002) *Advances in Neurology* 89: 161–184. (Article includes references for the studies).

Table 4 Chromosome loci of other generalized epilepsies of infancy and childhood

Phenotype	*Geno-type*[a]	*Country of origin & no. of families*
Infantile spasms syndrome (MIM308350)	Xp11.4–Xpter	Two families from Leuven, Belgium
Benign familial neonatal convulsions (EBN1)	20q	KCNQ2 mutation initially detected in one family from Sweden and then in six families from Newfoundland, Sweden, Quebec (Canada), USA, six families from France and one family from Japan
Epilepsy and mental retardation limited to females (EFMR)	Xq22	PCDH19 mutations in seven families from Australia
Ohtahara syndrome	9q33.3–q34.11	STXBP1 mutation in four patients from Japan
Benign familial neonatal convulsions (EBN2)	8q24	KCNQ3 mutation in one family USA (Texas) and one family from Japan
Benign familial infantile convulsions	19q13	Families from Italy
Familial infantile convulsions and Paroxysmal choreoathetosis	16p12–q12	Four families from France and one family from China
Familial febrile seizures(FEB1)	8q13–21	One family from Australia
Familial febrile seizures (FEB2)	19p	One family from the Midwest, USA
Familial febrile seizures (FEB4) by nonparametric GENEHUNTER and transmission linkage disequilibrium	5q14–q15	One large and 39 nuclear families from Japan
Childhood absence epilepsy with or without grand mal	8q24 (ECA1)	Seven families from Bombay, India, Argentina, LA, CA (USA), Spain, Saudi Arabia
Idiopathic generalized epilepsy with generalized spike waves	3p14.2–p12.1	One family from Italy
Adolescent-onset idiopathic generalized epilepsies with generalized spike-waves (NonJME random grand mal and juvenile absence seizures)	8p11–12	23 families (15 grand mal and eight juvenile absence) from New York

[a]Chromosome loci.

Modified from Delgado-Escueta AV (2002) Genetics of idiopathic myoclonic epilepsies: An overview. *Advances in Neurology* 89: 161–184. (Article includes references for the studies).

Table 5 Chromosome loci of partial epilepsies

Phenotype	*Geno-type*[a]	*Country of origin & no. of families*
Autosomal dominant nocturnal frontal lobe epilepsy	20q	NACHR-α4 subunit mutation in two families from Australia, one family from Norway and one family from Japan
Autosomal dominant nocturnal frontal lobe epilepsy	15q24; 1cen	Three families from Quebec, Canada, Italy, and England; Calabria,Southern Italy
Autosomal dominant nocturnal frontal lobe epilepsy		
TLE with auditory symptoms	10q	One family from New York; one family from Basque region, Spain
Autosomal dominant partial epilepsy with variable foci	2q (preliminary)	One family from Australia
Familial partial epilepsy with variable foci	22q11–q12	Two families from Quebec; one family from Spain
AR Rolandic epilepsy with paroxysmal exercise induced dystonia and writer's cramp	16p12–q12	One family from Italy
Centrotemporal spikes in families with Rolandic epilepsy	15q14	22 families from Germany
Rolandic epilepsy and speech dyspraxia	?	Australia
Familial TLE	?	Australia
Familial TLE with hippocampal scleroses and interleukin (IL)-1β, IL-1α, and IL-1 receptor antagonist gene	2 (association studies)	50 patients with TLE + HS from Japan
Familial Spastic paraparesis and epilepsy	14q; 15q	One family from Italy
Episodic ataxia type I (EA-1) and epilepsy	Mutation in potassium channel gene KCNA1	One family from Glasgow, Scotland
Frontotemporal dementia Parkinosonism-17 (FTDP-17)	17q21–22 (missense mutation at nucleotide 1137 C–T in tau gene)	One family from Germany

[a]Chromosome loci.

Modified from Delgado-Escueta AV (2002) Genetics of idiopathic myoclonic epilepsies: An overview. *Advances in Neurology* 89: 161–184. (Article includes references for the studies).

Genetically Complex Epilepsies

Genes for complex epilepsies can be found through case-control association studies. However, with the exception of one recent report, most association studies on genetically complex epilepsies have been underpowered in relation to likely effect sizes for risk alleles. Previous claims of associations have either not survived replication or the Bonferroni correction. Three exceptions appear to be: (1) *BRD2* in chromosome 6p21.3 as a risk factor for JME; (2) *Cx-36* in chromosome 15q14 as a risk factor for JME; and (3) *ME2* in chromosome18 as a risk factor for idiopathic generalized epilepsies.

SNPs within the *BRD2* (RING3) gene in chromosome 6p21.3 have been suggested to be susceptibility alleles (odds ratio 6.5) for AR JME families from New York. Linkage disequilibrium found two strongly JME-associated SNP variants in the promoter region of *BRD2*, and a common variant haplotype in over 50% of 20 probands from families that had produced positive LOD scores for 6p21 during linkage analyses. A recent study has confirmed the association between rs3918149 promoter variant of BRD2 with JME samples from Britain, but not in German, Australian, or Indian populations, suggesting the BRD2 risk factor is population-specific.

Both case–control and family-based association studies were used by Greenberg's group in New York in identifying malic enzyme 2 (*ME2*)-centered nine-SNP haplotype in chromosome 18q. When present in the homozygous state, the *ME2*-centered nine-SNP haplotype increased the risk for idiopathic generalized epilepsy (odds ratio 6.1). Studies have suggested that the *BRD2* gene in 6p21.3 epistatically interacts with the *ME2* gene in 18q to produce JME. Another study showed that alleles of *BRD2* also conferred susceptibility to the EEG photoparoxysmal response in JME/idiopathic generalized epilepsy families from Germany; allelic and haplotypes associations were found between the photoparoxysmal response and six BRD2 polymorphisms, but the polymorphisms appeared to be protective against, instead of increasing risk for photoparoxysmal response.

As in the New York population mentioned earlier, susceptibility SNPs in JME families have been found in families from the United Kingdom and Sweden. Investigators sequenced the gene, *Cx-36*, in 29 JME probands previously linked to the 15q14 locus and did not find any epilepsy-causing mutations that segregated with affected members. Instead, a case–control study found significant association between JME and the c.588T polymorphisms within exon 2 of *Cx-36*. With significant differences in both alleles ($p = 0.03$) and genotype ($p = 0.017$) frequencies, subjects with the T/T genotype at position 588 had a significantly increased risk for JME (odds ratio 4.3) compared to those with a C/C genotype. HAP2, a haplotype containing c.588T, was significantly associated with JME ($p = 0.03$). This association of c.588T (dbSNP:rs3743123, S196S) within *Cx-36* was replicated in 247 German JME patients (vs. 621 controls). Investigators observed an increase in homozygotes (13.4%) in JME compared with controls (8.7%), suggesting the allele increased the risk for JME in the homozygous state.

A recent study attempted to remedy the lack of power that plagued previous association studies in epilepsies. They enrolled 2717 cases and 1118 controls collected from four separate research centers from the United Kingdom, Ireland, Australia, and Finland. No clear indisputable common genetic risk factors were found that contributed to selected epilepsy subphenotypes across multiple populations. However, numerous SNPs in four genes contributed to predisposition in an apparent population-specific manner. These genes were (1) KCNAB1 (voltage gated potassium channel subunit beta-1) predisposing to 'secondarily generalized tonic–clonic seizures,' 'all epilepsy phenotype,' and 'partial seizures' in the Finnish cohort. (2) GABRR2 (γ-aminobutyric-acid C receptor subunit rho-2 precursor) predisposing to 'JME,' 'absence,' 'myoclonic seizure,' and 'nonJME grand mal seizures' in British and Irish populations. (3) KCNMB4 (calcium activated potassium channel, subfamily M subunit beta 4) predisposing to the syndrome of medial temporal lobe epilepsy (TLE) with hippocampal sclerosis in the Irish cohort. (4) SYN2 (Synapsin (2) predisposes to 'febrile seizures' in the UK, Irish, and Finnish cohorts). The authors recommend an even larger and more syndromically focused patient cohort be used for association studies exploring intermediate phenotypes and endophenotypes.

Challenges for the Future

Mendelian Epilepsy Genes

Depending on how one measures the epilepsy subsyndrome phenotype, only 1–10% of Mendelian epilepsy genes, have been identified. Large-scale genome mapping of large multigeneration, multiplex families from developing countries, as well as medium sized multigeneration, multiplex families from developed countries, should continue to define more and new chromosome loci for idiopathic epilepsies. The epilepsy genes of more than 10 known chromosome loci in JME, 5 chromosome loci for febrile seizures and infantile convulsions, 6 chromosome loci for partial epilepsies, and 3 chromosome loci for absence epilepsies still remain to be defined. These Mendelian genes will undoubtedly be discovered using the power of linkage analyses and positional cloning.

Genetically Complex Epilepsies

Linkage disequilibrium-based case control association studies, powered to a sufficiently large cohort (perhaps 5000–10 000 syndromically defined epilepsy cases and an equal number of controls), and performed in two stages

for replication or transmission linkage disequilibrium analyses of 300 trios or family based association studies, have not been successfully executed in genetically complex epilepsies. Instead, SNP susceptibility alleles in previously linked chromosome sites have been associated with small cohorts of epilepsies, as discussed earlier.

These proposed susceptibility polymorphisms (i.e., *BRD2* in chromosome 6p21.3; *Cx-36* in chromosome 15q14; and *ME2* in chromosome18) are hypothesized to act in epistases as minor epilepsy genes in the homozygous state, necessary but not sufficient to produce a JME phenotype. The challenge with these SNPs is to show what changes in cell function are altered so as to produce the JME phenotypes. No such tests have yet been performed. A second, more formidable challenge is proving causality (see **Table 1**). It is a difficult challenge to insert SNPs of putative JME genes in a bacterial artificial chromosome (BAC) vector – together with other SNPs of putative JME genes with which they are hypothesized to interact in epistases – and then transgenically reproduce one or all of the JME phenotypes. The *BAC* gene targeted approach rarely has been used to prove that interacting polymorphisms can cause a given disease phenotype.

See also: **Genetics**: *GABRB3* and Childhood Absence Epilepsy; Genetic Association Studies in Human Epilepsy and Pharmacogenetics of Antiepilepsy Drugs; Genetics of Lafora and Juvenile Myoclonic Epilepsies.

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Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex

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Introduction

The development of the human cerebral cortex is a dynamic process that can be divided into partially overlapping stages that occur during several gestational weeks. During the first stage, stem cells proliferate and differentiate into young neurons or glial cells deep in the forebrain, in the ventricular and subventricular zones lining the cerebral cavity. During the second stage, after their final mitotic division, cortical neurons migrate away from their place of origin. Most cells migrate in a radial fashion, along the radial glial fibres from the

for replication or transmission linkage disequilibrium analyses of 300 trios or family based association studies, have not been successfully executed in genetically complex epilepsies. Instead, SNP susceptibility alleles in previously linked chromosome sites have been associated with small cohorts of epilepsies, as discussed earlier.

These proposed susceptibility polymorphisms (i.e., *BRD2* in chromosome 6p21.3; *Cx-36* in chromosome 15q14; and *ME2* in chromosome18) are hypothesized to act in epistases as minor epilepsy genes in the homozygous state, necessary but not sufficient to produce a JME phenotype. The challenge with these SNPs is to show what changes in cell function are altered so as to produce the JME phenotypes. No such tests have yet been performed. A second, more formidable challenge is proving causality (see **Table 1**). It is a difficult challenge to insert SNPs of putative JME genes in a bacterial artificial chromosome (BAC) vector – together with other SNPs of putative JME genes with which they are hypothesized to interact in epistases – and then transgenically reproduce one or all of the JME phenotypes. The *BAC* gene targeted approach rarely has been used to prove that interacting polymorphisms can cause a given disease phenotype.

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Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex

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Introduction

The development of the human cerebral cortex is a dynamic process that can be divided into partially overlapping stages that occur during several gestational weeks. During the first stage, stem cells proliferate and differentiate into young neurons or glial cells deep in the forebrain, in the ventricular and subventricular zones lining the cerebral cavity. During the second stage, after their final mitotic division, cortical neurons migrate away from their place of origin. Most cells migrate in a radial fashion, along the radial glial fibres from the

periventricular region towards the pial surface, where each successive generation passes one another and settles in an inside–out pattern within the cortical plate. A subset of cells (interneurons) migrate tangentially, primarily from the ganglionic eminences. When neurons reach their destination, they stop migrating and order themselves into specific 'architectonic' patterns, according to a complex signalling pathway guiding cells to the correct location in the cerebral cortex. This third phase involves final organization within the typical six layers of cortex, associated with synaptogenesis and apoptosis. When cortical development is complete, the cortex is a six-layered structure, with each layer comprising different types of neurons that form discrete connections within the CNS and perform distinct functions (**Figure 1**). In humans, the proliferation stage ranges from weeks 5–6 to weeks 16–20, migration from weeks 6–7 to weeks 20–24, and organization from week 16 until well into postnatal life. Only a few of the gene products that influence such complex mechanism have been identified in humans and specific disorders arising from mutations in some of these genes have been described.

Abnormal cortical development is increasingly recognized as a cause of developmental disabilities and epilepsy. This recognition is due, in part, to the improved use of magnetic resonance imaging (MRI), which makes it possible to assess the distribution and depth of cortical sulci, cortical thickness, the boundaries between gray and white matter, and variations in signal intensity. Abnormalities of any or all of these features may be observed in different cortical malformations, which may be restricted to discreet cortical areas or may, alternatively, be diffuse. Epilepsy is often present in patients with cortical malformations and tends to be severe, although its incidence and type vary in association with different malformations. The physiopathological mechanisms relating cortical malformations to epilepsy remain elusive.

In the following sections we will discuss the most frequent cortical malformations causing epilepsy and those for which the causative gene has been cloned (see **Table 1**).

Background

Lissencephaly

Definition and histopathology

Lissencephaly (LIS) (derived from the Greek words "lissos" meaning smooth and "enkephalos" meaning brain) is a neuronal migration disorder characterized by absent (agyria) or decreased (pachygyria) convolutions, producing a smooth cerebral surface. Several different types of LIS have been recognized. The most common type, known as classical LIS or type 1 LIS, features a very thick cortex (10–20 mm vs. the normal 4 mm) and no other major brain malformations. The cytoarchitecture consists of four primitive layers, including an outer marginal layer that contains Cajal-Retzius neurons (layer 1), a superficial cellular layer that contains numerous large and disorganized pyramidal neurons (layer 2) corresponding

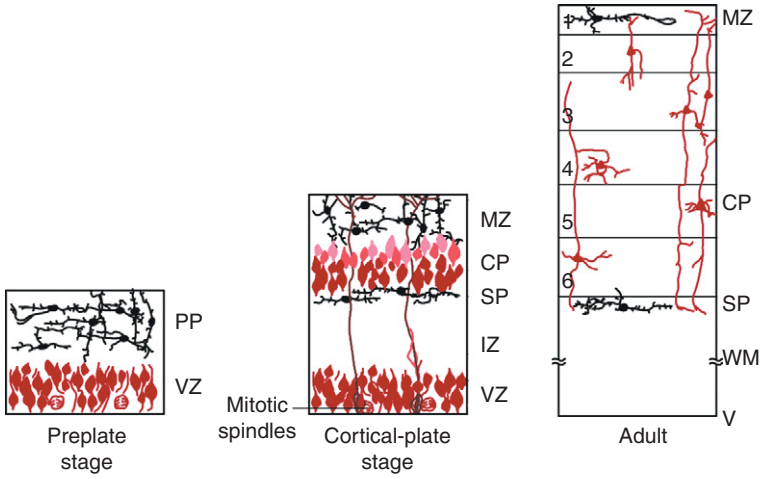


Figure 1 Normal cortical development. Cortical development can be divided into three stages. The preplate stage is marked by the formation of the preplate (PP), a plexiform layer that forms around embryonic-day 10–12 in mice and weeks 8–9 in humans. The cortical-plate (CP) stage is marked by proliferation of neuroblasts in the ventricular zone (VZ) (indicated by mitotic spindles) and migration of these cells along radial glial fibers (brown processes). Migrating neurons penetrate the preplate, split the preplate into the subplate (SP) and the marginal zone (MZ), and deposit in the cortical plate (CP). Neurons generated later must pass through the subplate and past previously deposited neurons to reach the outer limits of the developing CP (indicated by the progressive lighter shades of red in more-superficial CP neurons). The cortical-plate stage lasts from E10–E17 in mouse and weeks 10–18 in humans. The adult stage is marked by the lamination of the CP into a six-layered structure. Abbreviations: IZ, intermediate zone; V, ventricle; WM, white matter. Modified from Gleeson and Walsh, 2000.

Table 1 Most frequent cortical malformations causing epilepsy and their, currently known, causative genes

Cortical malformation	*Inheritance*	*Locus*	*Gene*	*Gene product/function*
Bilateral periventricular nodular heterotopia	X-linked	Xq28	*FLNA*	F-actin-binding protein; coagulation and vascular-related functions
	AR	20q13.13	*ARFGEF2*	Proliferation and migration
Isolated lissencephaly	AD	17p13.3	*LIS1* or *PAFAH1B1*	LIS1: Platelet-activation factor acetylhydrolas E, isoform 1B, α subunit; regulates the level of platelet-activating factor in the brain; interaction with dynein, dynactin, NUDE, and NUDEL proteins
	X-linked	Xq22.3	*DCX*	
	AD	12q12-q14	*TUBA1A*, *TUBA3*	Involved in microtubule formation
Double cortex (subcortical band heterotopia)	X-linked	Xq22.3	*DCX*	Interact with LIS1; effect on microtubules and cytoskeleton
	AD	17p13.3	*LIS1* or *PAFAH1B1*	
Miller–Dieker syndrome	Microdeletion disorder	17p13.3	*LIS1*, *14–3-3*ε and contiguous genes	
Lissencephaly with cerebellar hypoplasia	AR	7q22	*RELN*	Control cell-cell interactions critical for cell positioning in the brain.
			VLDLR	Very low density lipoprotein receptor; involved in reelin signalling pathway
Lissencephaly with abnormal genitalia	X-linked	Xp22.13	*ARX*	Aristaless-related homeobox gene; tangential migration & differentiation of GABAergic interneurons in the ganglionic eminence and neocortex
Fukuyama congenital muscular dystrophy (FCMD)	AR	9q31	*Fukutin (FCMD)*	Maintenance of muscle integrity, cortical histiogenesis, and normal ocular development
Walker-Warburg syndrome (HARD +/-E syndrome)	AR	9q34.1	*POMT1*	Similar to O-mannosyltransferases
		14q24.3	*POMT2*	Similar to O-mannosyltransferases
Muscle-eye-brain disease (MEB)	AR	19q13.3	*FKRP*	Modification of dystroglycan
		1p34-p33	*POMGNT1*	Involved in O-mannosyl glycan synthesis
Polymicrogyria				
Bilateral perisylvian	AD	11p13	*PAX6*	Transcription factor.
	X-linked	Xq21.22-q23	*SRPX2*	Expressed in fetal and adult brain, involved in rolandic seizures (?)
Bilateral frontoparietal	AR	16q13	*GPR56*	G Protein coupled receptor; role in regional patterning of the cerebral cortex
Holoprosencephaly	AD	7q36	*SHH* (Sonic Hedgehog)	Involved in differentiation of ventralized progenitors into motor neurons or interneurons, defining the pattern of neuronal cell types generated in the neural tube

to the true cortex, a variable cell sparse layer (layer 3), and a deep cellular layer (composed of medium and small neurons) that extends more than half the width of the mantle (layer 4) (Figure 2).

Subcortical band heterotopia (SBH) is a related disorder in which there are bilateral bands of grey matter found interposed in the white matter between the cortex and the lateral ventricles. SBH may appear as a solid band of heterotopic tissue or as numerous islands of radially oriented gray matter separated by white matter. Pyramidal cells in the heterotopic band may be smaller than normal compared with those in the overlying cortex, which is usually structurally normal with the exception of shallow sulci.

Genetics

Two major genes have been associated with classical LIS and SBH. The *LIS1* gene (OMIM: *601545) on chromosome 17p13.3 is responsible for the autosomal form of LIS, while the doublecortin gene (*DCX* or *XLIS*) (OMIM: *300121) is X-linked. Although mutations in either gene can result in either LIS or SBH, most cases of classical LIS are due to deletions or mutations of *LIS1*, whereas most cases of SBH are due to mutations of *DCX*.

LIS1 mutations result in more severe LIS in the posterior brain regions (posterior > anterior gradient), whereas *DCX* mutations result in more severe LIS in the anterior brain regions (anterior > posterior gradient) (**Figure 3**). Truncating *LIS1* mutations cause severe LIS, while missense mutations, cause less severe malformations such as pachygyria and SBH usually accompanied by milder motor and cognitive impairment. Mosaic mutations of *LIS1* also cause SBH in the posterior brain.

DCX mutations classically cause the SBH phenotype. Women with *DCX* mutations have anteriorly predominant band/pachygyria. However, rare carrier women harboring missense mutations with normal brain MRI, due to either favourable X-inactivation skewing or to mutations with mild functional consequences, have been described. Mutations of *DCX* have been found in all reported pedigrees, including families in which females have SBH and males have LIS, and in ~80% of sporadic females and 25% of sporadic males of SBH. Genomic deletions of the DCX gene have been identified in females with sporadic SBH and in males with X-linked LIS. Maternal germline or mosaic DCX mutations may occur in about 10% of

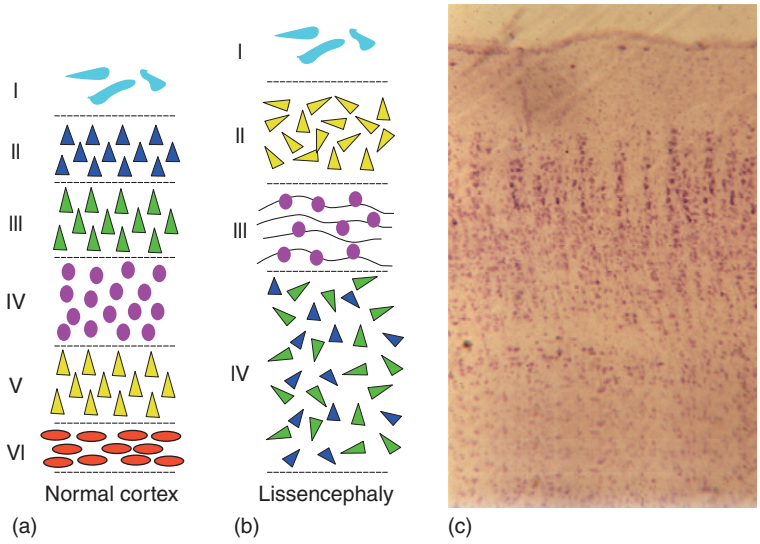


Figure 2 Scheme of normal (a) cortical laminar organization (6 layers) and cortical layering in classical LIS (4 non-organized layers) (b). (c) Histopathological section from a patient with LIS. Modified from Golden JA and Harding BA (2004) Pathology & Genetics. Developmental Neuropathology. Basel: ISN Neuropath Press, with permission from Wiley-Blackwell.

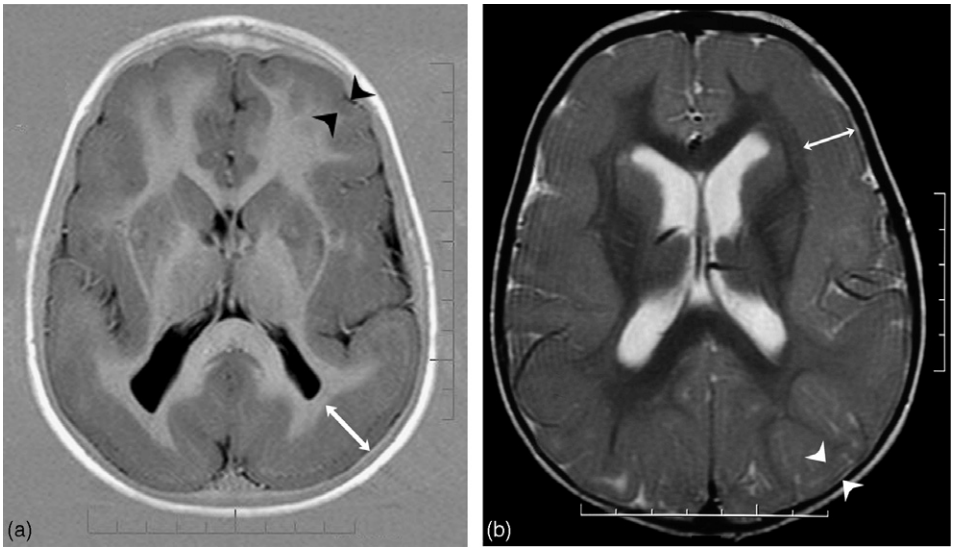


Figure 3 (a) Brain MRI scan; axial section: classical LIS in a boy with *LIS1* gene mutation. There is a typical posterior > anterior malformative gradient with relative preservation of the gyral pattern and cortical thickness in the anterior brain; cortical thickness is around 6 mm in the frontal lobes (black arrows; normal cortical thickness = 4 mm) and around 3 cm in the posterior brain (white arrow). (b) Brain MRI scan; axial section: LIS in a girl with *DCX* mutation. There is a typical anterior > posterior malformative pattern, cortical thickness is around 2 cm in the frontal lobes (single white arrow) and around 4 mm in the posterior brain (double white arrows).

cases of either SBH or XLIS. Hemizygous males with *DCX* mutations have classical LIS, but rare boys with missense *DCX* mutations with an anteriorly predominant SBH have also been described.

Recently, a mutant mouse was generated with abnormalities in the laminar architecture of the hippocampus and cortex, accompanied by impaired neuronal migration. This animal had a mutation in the guanosine triphosphate (GTP) binding pocket of alpha-1 tubulin (Tuba1). *TUBA1A* is one of three α-tubulin genes in a cluster on human chromosome 12q, and is highly homologous to the mouse and rat *Tuba1* gene. Mutations in this human homolog of *Tuba1* (*TUBA1A* or *TUBA3*) were subsequently identified in patients with LIS. The clinical and anatomic spectrum of severity TUBA1A-related LIS appears to be wide.

Experimental models and pathogenesis

LIS1 encodes a 45 kDa protein (PAFAH1B1), which is similar to the α-subunit of heterotrimeric G proteins and functions as a regulatory subunit of platelet-activating factor acetylhydrolase (PAF-AH). PAFAH1B1 homozygous mutant mice die at the blastula stage of embryonic development, whereas heterozygous mice are grossly normal but show a dose-dependent histopathological disorganization of cortical lamination as well as hippocampal and cerebellar cortical defects. The LIS1 protein has been shown to co-localize with microtubules and to promote their stabilization; thus, the LIS1 protein probably exerts its effects on migration through microtubules.

DCX encodes a 40 kDa microtubule-associated protein (DCX) that is expressed in migrating neuroblasts of the fetal brain. The DCX protein contains two tandem evolutionarily conserved repeats (doublecortin domain). Each of the repeats binds to tubulin but not to microtubules, so that both repeats are necessary for microtubule polymerization and stabilization. Mice in which DCX was disrupted did not have any cortical defects. However, in a recent study, DCX function was reduced by RNAi in a rat model, resulting in SBH resembling that seen in humans. Lis1 and Dcx display overlapping localization and may interact.

TUBA1A belongs to the α-tubulin protein family. α- and β-Tubulin heterodimers assemble to form microtubules that are essential for neuronal migration, neurite extension, and nucleokinesis. The pattern of expression of TUBA1A is limited to early-born postmitotic migrating neurons throughout development, and to specific neurogenic regions in the adult brain.

Epidemiology and clinical features

Classical LIS is rare, with a prevalence of about 12 per million births. Patients with severe LIS have early developmental delay, early diffuse hypotonia, later spastic quadriplegia, and eventual severe or profound mental retardation. Some children with LIS have lived more than 20 years, although anecdotal experience suggests that the lifespan is less than 10 years in most patients. Patients with less severe cortical malformations have moderate mental and motor impairment and may have a normal life-span expectancy. Seizures occur in over 90% of LIS children, with onset before 6 months in about 75% of cases. About 80% of children have infantile spasms, although EEG does not show typical hypsarrhythmia. Most LIS children subsequently have multiple seizure types. The EEG shows diffuse, high-amplitude, fast rhythms, which are considered to be highly specific for this malformation.

The main clinical manifestations of SBH are mental retardation and epilepsy. Epilepsy is present in almost all patients and is intractable in about 65% of cases. About 50% of these epilepsy patients have focal seizures, and the remaining 50% have generalized epilepsy, often within the spectrum of Lennox–Gastaut syndrome. Those with more severe MRI abnormalities have significantly earlier seizure onset and are more likely to develop Lennox–Gastaut syndrome. Depth electrode studies have demonstrated that epileptiform activity can originate directly from the heterotopic neurons, with nearly constant simultaneous involvement of the 'true' cortex. Callosotomy has been associated with worthwhile improvement in drop attacks in a few patients. Epilepsy surgery for focal seizures yields poor results.

Miller–Dieker syndrome (MDS) is caused by a contiguous deletion in the *LIS1* gene. Classical LIS is accompanied by distinct dysmorphic facial features, including prominent forehead, bitemporal hollowing, flattened ear helices, mild hypertelorism, epicanthic folds, short nose and anteverted nares, prominent lateral nasal folds, and round philtrum. Additional malformations can be observed. Affected children have severe delay, epilepsy, and feeding problems. Deletions of 17p13.3, including the *LIS1* gene, are found in almost 100% of patients. Deletion of two additional genes, *CRK* and *14-3-3ε*, telomeric to *LIS1*, may contribute to the most severe LIS grade and dysmorphic features observed in MDS.

X-linked LIS with absent corpus callosum and ambiguous genitalia (XLAG) is a severe malformation syndrome that is only observed in boys (**Figure 4**). The anatomoclinical spectrum includes LIS with posterior-to-anterior gradient and only moderate increase of the cortical thickness (only 6–7 mm), absent corpus callosum, poorly delineated and cavitated basal ganglia, postnatal microcephaly, neonatal-onset epilepsy, hypothalamic dysfunction, including deficient temperature regulation, chronic diarrhoea, and ambiguous genitalia with micropenis and cryptorchidism. Early death is not uncommon. Brain neuropathology reveals an abnormally laminated cortex exclusively containing pyramidal neurons, with a pattern suggesting disruption of both tangential and radial migration, dysplastic basal ganglia, hypoplastic olfactory bulbs and optic nerves, abnormal gliotic white matter containing

numerous heterotopic neurons, and complete agenesis of the corpus callosum without Probst bundles.

Mutations of the X-linked *aristaless*-related homeobox gene (*ARX*) (OMIM: *300382) were identified in individuals with XLAG, and in some female relatives. Females carrying *ARX* mutations usually have normal cognition and may either have normal brain MRI scan or show partial or complete agenesis of the corpus callosum. However, mild mental retardation and epilepsy have been reported in rare carriers. Mouse *Arx* and human *ARX* are expressed at high levels in both dorsal and ventral telencephalon. The murine *Arx* gene is specifically expressed in the GABAergic neural lineages, both during development and in adulthood. *Arx*-deficient mice show deficient tangential migration and abnormal differentiation of interneurons containing γ-aminobutyric acid (GABAergic interneurons) in the ganglionic eminence and neocortex. These findings suggest that the different *ARX* mutations may affect the overall GABAergic system functionality, leading to the clinical features of XLAG in humans; this deficit might account for the severe neonatal epileptic encephalopathy with suppression burst EEG that is often observed in affected boys. The mutations of the *ARX* gene in XLAG patients tend to be premature termination mutations.

Autosomal recessive LIS with cerebellar hypoplasia associated with severe abnormalities of the cerebellum, hippocampus, and brainstem was mapped to 7q22, and mutations were identified in the *RELN* gene (OMIM: *600514). Patients presented dysmorphic facial features and generalized seizures. *RELN* encodes a large (388 kDa) extracellular matrix protein that acts on migrating cortical neurons by binding to the very low-density lipoprotein receptor (VLDLR), the apolipoprotein E receptor 2, and α3-β1 integrin and cadherins-related receptors (CNRs). In mice, *Reln* mutations cause cerebellar hypoplasia, abnormal cerebral cortical neuronal migration and abnormal axonal connectivity. Neurons in affected mice fail to reach their correct location in the developing brain, disrupting the organization of the cerebellar and cerebral cortices and other laminated regions. In this animal model, the cortical layering appears inverted. Thus, Reln is thought to control cell–cell interactions critical for cell positioning in the brain.

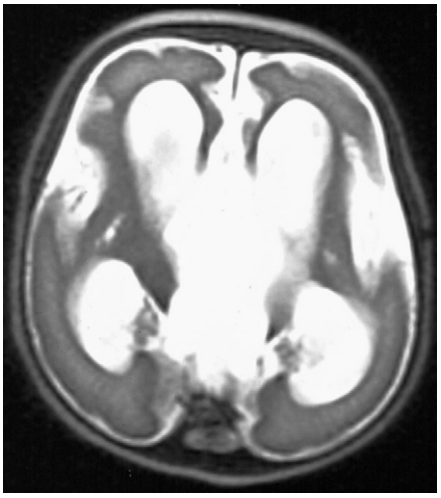


Figure 4 Brain MRI scan; axial section. 1 year-old boy with X-linked LIS, with corpus callosum agenesis and ambiguous genitalia due to mutation of the ARX gene. Note absence of the corpus callosum with ventriculomegaly and LIS.

Periventricular Nodular Heterotopia

Definition and histopathology

Periventricular nodular heterotopia (PNH), most often bilateral, consists of nodules of grey matter located along the lateral ventricles; these neurons have failed to migrate into the cortex (**Figure 5**). PNH can be highly variable in extent, ranging from isolated single nodules to confluent bilateral lesions. PNH is most common near the angle of the lateral ventricles and around the temporal horns. Nodules of PNH may sometimes show some rudimentary lamination, and neurons within the nodules show change in size consisting with developmental age. Although most patients with PNH come to medical attention because they have focal epilepsy of variable severity, there is a wide

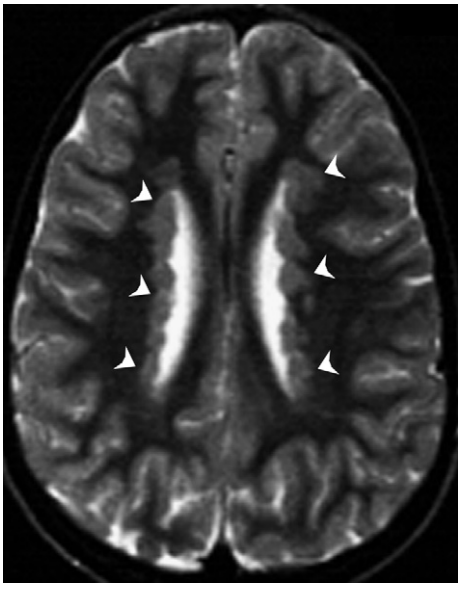


Figure 5 Brain MRI scan; axial section. Typical, classic bilateral periventricular nodular heterotopia in a woman with a missense mutation of the *FLNA* gene. Bilateral nodules of subependymal heterotopia are contiguous and rather symmetric, extensively lining the ventricular walls (white arrows). Patients with periventricular heterotopia different from that reproduced in this picture have very low chances of carrying a mutation on the *FLNA* gene.

spectrum of clinical presentations, with some correlation between the size of PNH and the likelihood of concomitant structural abnormality of the cortex and clinical severity. Approximately 90% of patients with periventricular nodular heterotopia have epilepsy, which can begin at any age. Functional MRI studies suggest that heterotopic nodules can assemble in functionally active aggregates and can participate in integrated networks. Indeed, depth electrode studies have demonstrated that either the heterotopic cortex or the overlying cerebral cortex, or both, can be epileptogenic. In one series, temporal lobectomy, which did not include the area of heterotopia, did not result in any significant improvement.

Genetics

PNH occurs much more frequently in women as an X-linked trait. It is associated with high rates of prenatal lethality in male fetuses, and in a 50% recurrence risk in the female offspring of affected women. Almost 100% of families with X-linked bilateral PNH, and 26% of sporadic patients, harbor mutations of the *filamin A* gene (*FLNA*) (OMIM: +300017). The low percentage of *FLNA* mutations in sporadic cases might be explained by somatic mosaicism, although this mechanism remains hypothetical. Heterozygous females have normal to borderline intelligence and epilepsy. Coagulopathy and cardiovascular abnormalities have been observed in some patients. Only a few living male patients with bilateral PNH owing to *FLNA* mutations are on record.

Other genes can cause PNH. A rare recessive form owing to mutations of the adenosine diphosphate-ribosylation factor guanine nucleotide exchange factor 2 (*ARFGEF2*) (OMIM: *605371) has been reported in two consanguineous pedigrees. *ARFGEF2* encodes for the protein brefeldin A-inhibited GEF2 (BIG2), which is involved in proliferation and migration during cortical development. Affected children have microcephaly, severe delay, and early-onset seizures, including infantile spasms. PNH may also be observed in patients with chromosomal rearrangements, such as duplication of 5p15.1 or 5p15.33.

Experimental models and pathogenesis

FLNA maps to Xq28 and codes for the F-actin-binding cytoplasmic cross-linking phosphoprotein Filamin A (FLNA). Filamin homologues are involved in regulation of cell stability, and filopodial protrusion and motility, across various biological systems. The mouse *Filamin A* ortholog is abundantly expressed in the cell soma and leading processes of migratory neurons, and is widely distributed across the cortical mantle, reaching the highest levels in the ventricular zone within the cortex during neurogenesis. FLNA likely influences neuroblast migration during cortical development in vertebrates, and PH in humans likely results from disruption of this process. FLNA also promotes orthogonal branching of actin filaments and is important for coagulation and vascular development. These coagulation and vascular-related functions of FLNA might account for the prenatal male lethality observed in most pedigrees. Cardiovascular or gut malformations may also account for prenatal male lethality. Complete loss of Flna in mice results in embryonic lethality with severe cardiac structural defects involving ventricles, atria, and outflow tracts, as well as widespread aberrant vascular patterning.

Polymicrogyria Phenotypes

The term 'polymicrogyria' refers to an excessive number of small and prominent convolutions separated by shallow and enlarged sulci, giving the cortical surface a lumpy aspect. Cortical infolding, as well as secondary, irregular, thickening due to packing of microgyri, is visible on MRI – although mild forms are difficult to recognize on neuroimaging. Two histological types are recognized. In 'unlayered polymicrogyria,' the molecular layer is continuous and does not follow the profile of the convolutions; the underlying neurons have radial distribution but no laminar organization. In 'four-layered polymicrogyria,' there is a layer of intracortical laminar necrosis with consequent impairment of late migration and post-migratory disruption of cortical organization. The two subtypes do not necessarily have a distinct origin as both may coexist in contiguous cortical areas.

Several syndromes featuring bilateral polymicrogyria have been described, including bilateral perisylvian polymicrogyria, bilateral parasagittal parietooccipital polymicrogyria, bilateral frontal and frontoparietal polymicrogyria, and unilateral perisylvian or multilobar polymicrogyria. These different forms might represent distinct entities that reflect the influence of regionally expressed developmental genes.

In *bilateral perisylvian polymicrogyria* (BPP; *OMIM 300388), the polymicrogyric malformation is limited to the cortical area surrounding the sylvian fissure (**Figure 6(a)**). Typically, this malformation will be associated with mild to moderate mental retardation, epilepsy (often of the symtomatic generalized type), and difficulties with speech. Most cases are sporadic; however, numerous families have been described, exhibiting heterogeneous inheritance patterns including X-linked, autosomal recessive, and autosomal dominant. Recently, a missense mutation in the Xq22 gene *SRPX2* was found in a 15-year-old Australian male with rolandic seizures and BPP. The same mutation was also found in his unaffected mother and aunt, as well as in two maternal aunts with mild mental retardation but normal brain MRI. A second mutation in the *SRPX2* gene was also identified in a 3-generation French family presenting with rolandic seizures, oral and speech dyspraxia,

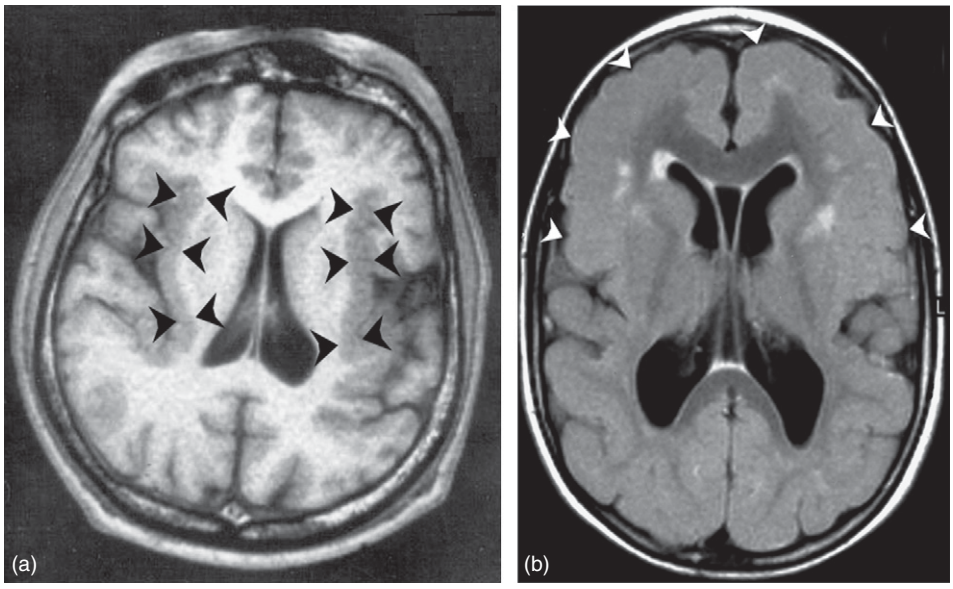


Figure 6 (a) Brain MRI; T1weighted axial section. *Bilateral perisylvian polymicrogyria*. Sylvian fissures are open and the perisylvian cortex is thickened and irregular (black arrows). Young man with Lennox–Gastaut syndrome. (b) Brain MRI; T1 weighted axial section, *bilateral frontoparietal polymicrogyria* (black arrows) in a girl with the *GPR56* gene mutation and Lennox–Gastaut Syndrome.

and a variable degree of mental retardation. In cultured cells, both mutations were associated with altered patterns of intracellular processing that suggested protein misfolding. SRPX2 is a secreted sushi-repeat containing protein, which carries a signal peptide and three consensus sushi-repeat motifs. Expression of SRPX2 has been detected in most human tissues, including both the fetal and adult brain, particularly in neuronal perikarya of the rolandic area. In the murine brain, Srpx2 protein expression appeared in neurons at birth, suggesting that SRPX2 may play an important role in the development and/or function of the perisylvian region critical for language and cognitive development.

Bilateral frontal polymicrogyria was described in children with developmental delay, mild spastic quadriparesis, and epilepsy. Indeed, *frontoparietal polymicrogyria*, a malformation extending a few centimeters back into the parietal lobes (**Figure 6(b)**), has been reported in several consanguineous and non-consanguineous families with recessive pedigrees, and was initially mapped to chromosome 16q12.2-21 and subsequently associated with mutations of the G protein-coupled receptor gene 6 (GPR56). GPR56 belongs to the G-protein-coupled receptor family – the largest gene family in the human genome, representing about 1% of all genes. The pattern of expression of mouse Gpr56, as well as the topography of the cortical abnormality in patients harboring homozygous mutations, strongly suggests that Gpr56 regulates cortical patterning. Epilepsy, seen in the majority of patients, is mainly associated with partial seizures and atypical absences, and can be of variable severity.

Unilateral multilobar polymicrogyria is often observed as a sporadic malformation. However, familial cases with unilateral polymicrogyria, or both unilateral and bilateral polymicrogyria, are on record. In some children with unilateral or bilateral polymicrogyria, epilepsy with electrical status epilepticus during sleep can develop, exhibiting the typical age-related course.

Other Gene-Related Malformation Syndromes

Several other genes, including fukutin (FCMD) and Sonic Hedgehog (SHH), have been associated with complex malformation syndromes that involve abnormal cortical development and epilepsy. The malformation spectrum of the most well-known syndromes, causative genes, and gene product function are reported in **Table 1**.

Methods

Since 1992, we have been collecting clinical, neurophysiological, and neuroimaging information on a cohort of 900 patients with epilepsy and malformations of the cerebral cortex. Since 1999, we have performed mutation analysis of the main genes causing cortical malformations in order to define the spectrum of genotype–phenotype correlations. In particular, we have studied *LIS1*, *DCX*, *FLNA*, *ARX*, and

GPR56 genes, using denaturing high-performance liquid chromatography (dHPLC) and direct sequencing.

X-inactivation studies were performed using the androgen receptor (AR) assay with a fluorescent-labeled primer to test.

The rate of mutant allele in patients with somatic mosaicism was quantified by carrying out single nucleotide primer extension (SnuPE) studies associated with dHPLC.

Multiplex ligation-dependent probe amplification (MLPA) was performed to search for whole-exon deletions or duplications of the DCX gene, using the 'Lissencephaly probe kit,' which includes seven paired probes specific for DCX.

Recent Results

Our laboratory has contributed to better define the clinical, anatomic, and molecular features of some genetically determined epileptogenic malformation of the cerebral cortex.

To offer new insights into the nosology of PH, we examined the clinical and brain MRI characteristics of a large cohort of individuals with PH, including bilateral PNH and other types of PH. These studies led to the definition of 15 distinct malformation patterns. Classical bilateral PNH represented the largest group. The 14 additional phenotypes included: PNH with Ehlers–Danlos syndrome (EDS), temporo-occipital PNH with hippocampal malformation and cerebellar hypoplasia, PNH with fronto-perisylvian or temporo-occipital polymicrogyria, posterior PNH with hydrocephalus, PNH with microcephaly, PNH with frontonasal dysplasia, PNH with limb abnormalities, PNH with fragile-X syndrome, PNH with ambiguous genitalia, micronodular PH, unilateral PNH, laminar ribbon-like and linear PH. We found *FLNA* mutations in 100% of familial cases with X-linked PNH and in 26% of sporadic patients with classical bilateral PNH. Furthermore, we did not observe significant differences between the type and location of mutations, and the severity of the associated phenotype. The overall information that could be drawn from that study is that PH is a heterogeneous disorder in which clinical and brain imaging presentation and genetic causes are variable.

We have observed that missense germline mutations and distal truncating mutations of the *FLNA* gene are compatible with survival of affected males; however, insertions, deletions, or truncating mutations are lethal in males. Partial or residual function of the protein presumably accounts for male viability. We have also demonstrated that somatic mosaicism in patients with truncating mutations may attenuate the severity of the phenotype. We have described a mosaic mutation in a man with a moderate PNH phenotype and an healthy daughter, demonstrating that, although maternal transmission is much more likely, father-to-daughter transmission is also possible (i.e., either parent can transmit the mutation to a female proband). Lack of transmission may mask the X-linked nature of the disorder.

We have characterized a family in which a deletion in the *FLNA* gene led to early death of boys. Postmortem neuropathologic study in an affected member of this family revealed bilateral PNH similar to that observed in female patients. The boy had multiple congenital abnormalities, including short and malrotated intestine and coagulopathy. Therefore, it seems likely that lethality in men is caused by the phenotypic effects of *FLNA* mutations on the cardiovascular system, homeostasis, or internal organs rather than on severity of the brain malformation.

We also investigated how specific genetic mechanisms may explain the mild phenotypes observed in some patients with *DCX* or *LIS1* mutations. We described four families segregating missense mutations of *DCX*. In each family, one or more carrier women had normal brain MRI, mild mental retardation, or borderline IQ, with or without epilepsy. These women were ascertained through their affected sons or brothers, all of whom had pachygyria–SBH. Molecular studies indicated that the mechanisms leading to seemingly normal neuronal migration in carrier women may result from either mild loss of gene function or skewed X-inactivation.

We also reported patients with SBH and epilepsy with somatic mosaicism for mutations of the *LIS1* gene. Coexistence of two genetically distinct neuronal populations, resulting from somatic mosaicism, may explain the unusually mild phenotype observed in these patients.

We applied the MLPA technique to evaluate the frequency of deletions or duplications encompassing one or few exons of the *DCX* gene in patients with SBH in whom gene sequencing had failed to reveal any mutation. The MLPA assay uncovered exon deletions in 3 out of 11 women (27%) with SBH, and raised the percentage of *DCX* mutations from 52% to 65% in our series. Our data indicate that deletions of *DCX* appear to be an underascertained cause of SBH, making MLPA a useful complement to DNA sequencing for mutation detection. These results offer additional tools for genetic counseling to be offered to the families of patients with SBH.

Future Directions

Genetic studies of cerebral cortex development and neuronal migration have been remarkably successful over the past few years, uncovering several genes that, when mutated, cause disorders of neuronal migration and

cerebral cortical development. However, the genetic etiology for patients with cortical malformations, but not harboring mutations in the known genes, remains unexplained. New genes or cryptic chromosomal abnormalities might be involved in these phenotypes. Array CGH can now be used as a molecular genetic tool to search for new genes associated with cortical malformations. Array CGH is a method based on the hybridization of the genomic DNA with clones or oligomers (targets) covering the entire genome; this method is able to reveal cryptic chromosome abnormalities. Screening of candidate genes that map to specific target regions, and are deleted or duplicated, can then be performed in order to identify their causative role in individuals with similar phenotypes but no visible rearrangements. Expression analysis and functional studies in experimental animal models can be then employed to further investigate the candidate gene association to biological pathways linked to the malformation conditions. In particular, advanced molecular biology techniques such as RNA interference and/or targeted deletion allow investigators to 'knock down' the expression of the targeted gene and investigate the functional consequences in experimental models, both in vitro and in vivo. Furthermore, expression studies in human tissue, both from malformed and normal brains, could provide insight into the pathophysiology of the malformation conditions.

See also: **Development/Malformations**: Epilepsy-Associated Reelin Dysfunction Induces Granule Cell Dispersion in the Dentate Gyrus; **Genetics**: Clinical Genetics of Epilepsy; Genetic Contributions to Brain Structure and Epileptogenesis; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy.

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Relevant Website

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IMAGING

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Imaging Approaches to Seizure Analysis: PET Analysis

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Introduction

Of the 0.5–1% of the population suffering from epilepsy, half of the cases have no identified etiology. Moreover, in almost 25% of patients with epilepsy, the seizures are medically-refractory; that is, they do not respond to multiple anti-epileptic medications. In these patients, surgical resection of the epileptogenic brain tissue may alleviate the seizures provided that the preoperative evaluation identifies the brain region to be resected so as to achieve seizure control. Electrographic determination of epileptogenic brain regions, combined with various types of neuroimaging, often provide reasonable localization of epileptic cortex. In this article, we discuss the role of functional neuroimaging with positron emission tomography (PET) in the evaluation and management of epilepsy and epileptic syndromes.

PET is a medical imaging technique, which has an unparalleled capability to generate images of body function and metabolism. Quite often, internal biochemical or physiological changes predate or exceed changes in the structural appearance of tissue or organ; in such cases, PET can detect alterations earlier than changes in anatomy. In PET, positron-emitting radionuclides, such as ^{18}F, ^{11}C, ^{15}O, and ^{13}N, are used to label various natural biological substrates and drugs or pharmaceuticals. These substrates all contain hydrogen, carbon, oxygen, or nitrogen, which can be replaced with their radioactive positron-emitting counterpart. The resulting radioactive substance, also known as a radiotracer or PET tracer, will show similar behavior and will follow the same physiological pathways as the biological substrate do. At the same time, they will be emitting paired high-energy (511 Kev) photons traveling in opposite direction as a result of positron decay of the radioactive atom; these photons can be detected by external detectors and the whole process can be traced or 'imaged.' Further, as the process is captured temporally in a dynamic fashion, kinetics can also be calculated. Therefore, virtually any physiological or metabolic process, such as glucose metabolism, protein synthesis, enzymatic processes, or receptor–ligand interactions, can be studied using an appropriate radiotracer. This capability is particularly useful in studying epileptic brain, in which basic anatomical features or physiological processes undoubtedly contribute to, or reflect, the epileptogenic nature of the tissue.

Background

The most commonly used PET tracer in epilepsy is 2–^{18}F-fluoro-2-deoxy-D-glucose (FDG), which is an analogue of glucose. Since glucose is widely used as a substrate for obtaining energy, metabolism of various organs can be assessed by imaging FDG uptake of that organ. Other PET tracers with the potential for detecting epileptic brain regions include ^{11}C-alphamethyl-L-tryptophan (AMT), which measures tryptophan metabolism; ^{11}C-flumazenil (FMZ), which binds to α subunits of the $GABA_A$-benzodiazepine receptor; and other radioligands, which bind to opioid receptors, histamine receptors, *N*-methyl-D-aspartate (NMDA) receptors, peripheral benzodiazepine receptors, serotonin receptors, or

Table 1 PET tracers, their targets and uptake pattern in epileptogenic lesions

PET tracer	*Target*	*Uptake pattern in epileptogenic region*
2–^{18}F-fluoro-2-deoxy-D-glucose (FDG)	Glucose Metabolism	Interictal decrease Ictal increase
^{15}O-H_2O (Water)	Blood flow	Interictal decrease Ictal increase
^{11}C-Alpha Methyl-L-Tryptophan (AMT)	Tryptophan metabolism to serotonin or quinolinic acid	Interictal increase
^{11}C-Leucine	Protein synthesis	Variable
^{11}C-Flumazenil (FMZ)	$GABA_A$/Benzodiazepine receptor (antagonist)	Interictal decrease
^{11}C-PK-11195 (PK)	Peripheral benzodiazepine receptor (antagonist)	Increased
^{11}C-(S)-[*N*-methyl]-Ketamine	NMDA receptor (antagonist)	Decreased
^{11}C-L-Deprenyl	MAO-B (inhibitor)	Increased
^{11}C-Doxepin	H_1 receptor (antagonist)	Increased
^{11}C/^{18}F-FCWAY	$5HT_{1A}$ receptor (antagonist)	Decreased
^{18}F-MPPF	$5HT_{1A}$ receptor (antagonist)	Decreased
^{18}F-Altanserin	$5HT_{2A}$ receptor (antagonist)	Decreased
^{11}C-Carfentanyl	μ-Opioid receptor (*agonist*)	Increased
^{11}C-Methylnaltrindole	δ-Opioid receptor (antagonist)	Increased
^{18}F-Cyclofoxy	μ,κ-Opioid receptor (antagonist)	Increased
^{11}C-Diprenorphine	μ,δ,κ-Opioid receptor (antagonist)	Decreased
^{11}C-NMBP	$_m$Ach receptor (antagonist)	Decreased
^{123}I-Iododexetimide	$_m$Ach receptor (antagonist)	Decreased

GABA: Gamma aminobutyric acid; PK11195: 1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methylpropyl)-3-isoquinoline carboxamide; NMDA: *N*-methyl D-aspartic acid; MAO-B: Monoamine oxidase-B; H: Histamine; FCWAY: trans-4-fluoro-*N*-2-[4-(2-methoxyphenyl) piperazin-1-yl] ethyl-*N*-(2-pyridyl) cyclohexanecarboxamide; HT: Hydroxytryptamine; MPPF: 4-(2-methoxyphenyl)-1-[2-(*N*-2''-pirydynyl)-*p*-fluorobenzamido] ethylpiperazine; NMBP: 4-*N*-methylpiperidinyl benzilate; Ach-Acetylcholine.

acetylcholine receptors (Table 1). Normal age-related uptake patterns of the three most important PET tracers used in epilepsy are given below, since knowledge of the normal pattern is necessary to appreciate any change from the norm.

Normal FDG uptake pattern

The metabolic rate of glucose utilization in the newborn brain is markedly different from that in adults. During birth, the child's local cerebral metabolic rate of glucose (LCMRglc) in cortex is usually 30% less than that in adult. LCMRglc then increases and reaches adult values by the second year of life. However, LCMRglc exceeds adult values by the third year, reaches the peak value by 3–4 years of age and then plateaus. This plateau persists until about 10 years of age, and then gradually declines to adult values by the age of about 16–18 years. The spatial glucose metabolism pattern across the brain, and thus the visual appearance in PET scans, also changes with age. In the newborn, glucose metabolic activity is most prominent in primary sensory- and motor cortex, thalamus, brain stem, and cerebellar vermis. More recent studies with high-resolution PET scanners have suggested that the cingulate cortex, amygdala, hippocampus, and striatum may also show relatively prominent activity in the newborn period (Figure 1).

Between age of 2 and 4 months, glucose utilization increases in parietal, temporal, and primary visual cortex (medial occipital or calcarine cortex), as well as in the basal ganglia and cerebellar hemispheres. Between 6 and 8 months of age, glucose utilization increases in lateral and inferior prefrontal regions, with medial and dorsal frontal cortex becoming active between 8 and 12 months of age. The adult pattern of glucose utilization is seen by 1 year of age (Figure 1), although the absolute metabolic rates for glucose utilization undergo a more protracted nonlinear course, as described already.

Normal FMZ binding pattern

FMZ binding, or FMZ VD (volume of distribution), is high at 2 years of age and then decreases exponentially with age until adult values are reached at around age 20. Since no data are available for children less than 2 years of age, it is difficult to say when FMZ binding is highest. It is possible that binding is highest at the time of, or even before birth, and then decreases to reach adult values. In adults, FMZ VD is 10% higher in females than in males. The order of brain region (from highest to lowest FMZ binding) at 2 years of age is as follows: primary visual cortex, superior frontal cortex, medial temporal cortex, temporal lobe, pre-frontal cortex, cerebellum, basal ganglia, and thalamus. Medial temporal lobe structures,

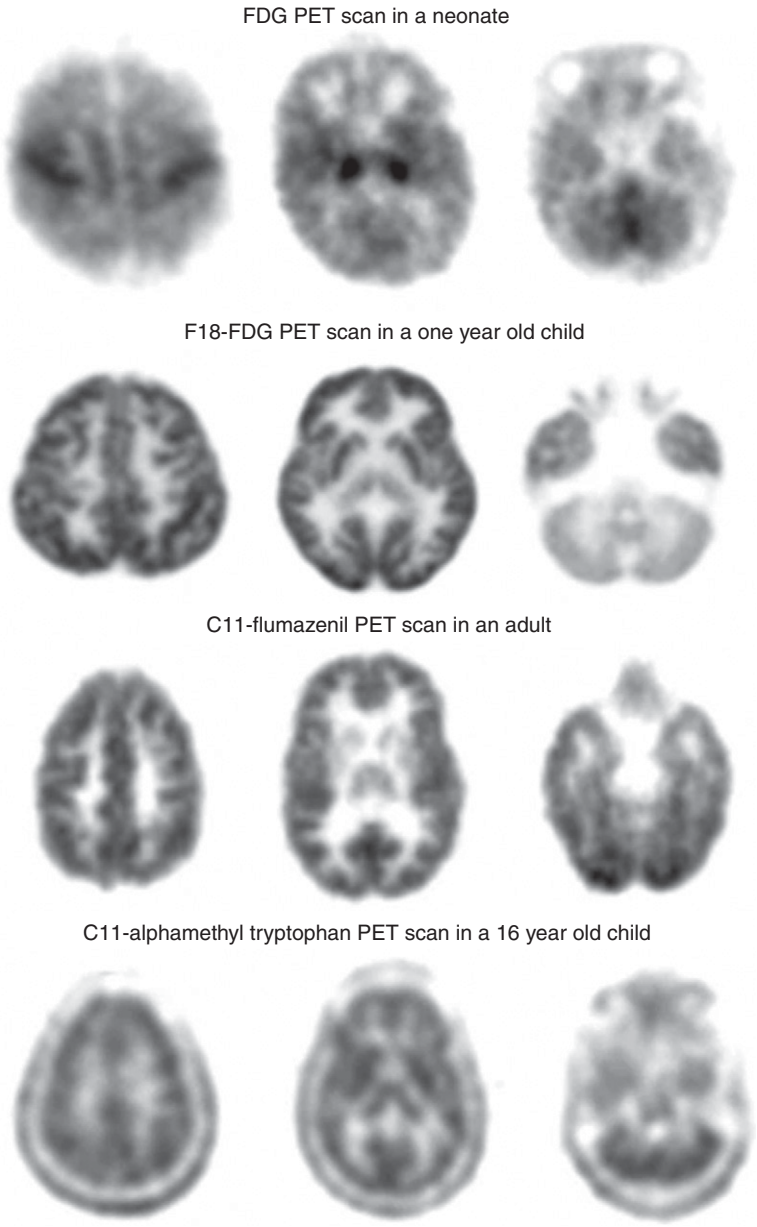


Figure 1 Normal FDG PET scan in a 7 day old newborn (first row) and a1 year old child (second row), normal FMZ PET scan in an adult (third row), and a normal AMT PET scan in a 16 years old child (lowermost row).

primary visual cortex, and thalamus show the largest temporal decrease in FMZ VD (approximately 50% decreases) compared with basal ganglia, cerebellum, and other cortical regions (25–40% decreases). Subcortical brain regions reach adult values earlier (14–17.5 years) than the cortical regions do (18–22 years) (Figure 1). The medial temporal region shows the fastest decline and has the lowest adult FMZ VD value among cortical areas. Nevertheless, FMZ binding is robust enough for its good visualization (compared with FDG PET, where the medial temporal region, particularly hippocampus, is often not well visualized). Thus, FMZ PET can play an important role in evaluating epileptic patients in whom medial temporal lobe abnormalities are suspected.

Normal AMT uptake pattern

AMT is an analog of tryptophan, the precursor for serotonin synthesis. However, unlike tryptophan, AMT is not incorporated into protein in significant amounts. AMT is converted to alpha-methyl-serotonin (AM-5HT) by tryptophan hydroxylase, but because AM-5HT (unlike serotonin) is also not a substrate for the degradative enzyme monamine oxidase (MAO), it accumulates in neurons and

nerve terminals along with the releasable pool of serotonin. After intravenous injection, AMT accumulates in the brain for the first 20 min (less than 2% of the injected dose is present in the brain at peak values), after which a plateau is reached and maintained for up to 60 min, with no right-left asymmetry in normal brain. Since AMT PET does not allow the measurement of absolute rates of serotonin synthesis, the term 'serotonin synthesis capacity' (an index of serotonin synthesis) has been applied. This measurement increases after birth, reaches twice the adult value by 3 years of age, remains at a plateau until 5 years of age, and then declines, reaching adult values by 14 years of age. In adults, the highest serotonin synthesis capacity values are seen in putamen, followed by caudate nucleus, transverse temporal gyrus, thalamus, anterior cingulate cortex, cerebellar hemispheres, cerebellar vermis, posterior cingulate cortex, hippocampus, rectal gyrus, middle temporal gyrus, brain stem, inferior and superior temporal gyrus, frontal cortex, primary visual cortex, parietal cortex, and lateral occipital cortex (**Figure 1**). Values in women are 10–20% higher throughout the brain, and particularly higher in the transverse temporal gyrus, anterior cingulate cortex, and cerebellar vermis.

Methods

Standard Protocol for Doing FDG PET Scan

The patient should be fasted for 4 h before the FDG PET scan, and blood glucose level should be below 100 mg dl^{-1}. In a diabetic patient with a high blood glucose level and having an urgent need for a FDG PET scan, insulin can be used – under the supervision of a trained physician or pediatrician – to bring down the blood glucose to an acceptable level. EEG electrodes are placed on the head (if EEG monitoring is required, such as in epilepsy where seizure during the FDG uptake period – usually the first 30 min after the injection – may change its uptake value) and FDG is injected IV in a quiet, dimly lit room. The patient and any family members in attendance are asked to remain quiet. Thirty minutes after the injection, electrodes are removed and the patient is brought to the scanner room and placed supine, with the head positioned (in a head holder) in the field of view of the scanner. If required, the patient is kept under sedation. Subsequently, a 30 min emission scan of the brain is acquired. A calculated attenuation correction is performed. In PET-CT, a CT scan of the head is acquired for attenuation correction, followed by a 20 min emission scan.

Standard Protocol for Doing FMZ PET Scan

No fasting is required for an FMZ scan unless the patient is to be sedated. EEG electrodes are placed on the head and the patient is brought into the scanner room. If needed, the patient is sedated. A transmission scan of the head is acquired to be used for the attenuation correction. Subsequently, radiotracer is injected and a 60 min emission scan is acquired.

Standard Protocol for Doing AMT PET Scan

Children are advised to fast for 6 h, and adults for 8 h prior to the AMT PET scan. EEG electrodes are placed on the head and the patient is brought into the scanner room. Two IV lines are started; one IV line is used to take blood samples to be used in tracer kinetic analysis and the other for the injection of the radiotracer. If needed, the patient is sedated. A transmission scan of the heart (15 min) is then acquired, to be used for the attenuation correction. Subsequently, with patient's heart in the field of view of the scanner, the AMT radiotracer is injected and an emission scan of heart is acquired for 20 min. The transmission and emission scan of the heart is necessary to calculate the initial input function for tracer kinetics. After the completion of the transmission and emission scans of the heart, the patient's head is positioned under the scanner and emission scan for the head is started (typically 25 min after the injection of the radiotracer and continuing for another 40 min). In the end, a 15 min transmission scan of the head is acquired for the attenuation correction of the head. Fiducial markers are also placed on the patient's forehead and behind each ear for motion correction. Blood sampling is performed at 20, 30, 40, 50, and 60 min post-injection of AMT. If tracer kinetic analysis is not planned, and one is interested in only seeing the uptake pattern, IV blood sampling and heart transmission and emission scans are not required. After the radiotracer is injected, an emission scan of the head for 40 min is acquired followed by a transmission scan for 15 min.

The patient/parent/guardian must fill out the medication and information list and sign the informed consent form for all research procedures.

Results – Applications of PET

The various applications of PET in the evaluation and management of epilepsy are summarized in **Table 2**.

Evaluation of Seizure Etiology

An underlying cause for epilepsy is apparent in only 50% of cases, where it is linked to infection, head trauma, stroke, brain tumors, brain malformations, and other causes. In most of these conditions, PET does not contribute further to the etiology. However, in mild cortical dysplasia not evident on structural imaging (including MRI), FDG PET shows hypometabolism in the area of cortical dysplasia and can be a useful guide in the

Table 2 Role of PET scan in epilepsy

1. To evaluate the underlying cause, such as subtle brain malformations
2. To search for cellular (including synaptic) and molecular (including receptors and transmitters) changes associated with epilepsy
3. To assess seizure effects on the brain
4. To help in treatment selection (by demonstrating altered cellular or molecular processes for specific targeted medical therapy or focal lesions in cases of cryptogenic epilepsy for possible surgery)
5. Evaluation of treatment response
6. To predict seizure intractability
7. In epilepsy surgery
   a. To delineate (lateralize and/or localize) epileptogenic regions along with secondary epileptic foci, particularly in cases of normal structural imaging or discordance between structural and electrophysiological data or multiple lesions shown by one or other investigative modalities.
   b. To search for dual pathology
   c. To evaluate for the presence of potential secondary epileptic foci
   d. To evaluate the functional integrity of the rest of the brain
   e. To evaluate eloquent cortex
   f. Postsurgical evaluation
8. To evaluate neonatal seizures



Figure 2 FDG PET scan showing an area of hypometabolism in the Lt parietal lobe (arrow) in a 10 year old child with intractable seizures and normal MRI. Post-surgical histopathology revealed mild cortical dysplasia.

placement of intracranial EEG electrodes, if scalp EEG also shows ictal epileptic discharges arising from that general area (Figure 2). If intracranial EEG confirms and defines the extent of the epileptogenic cortex, this area can then be removed, even if MRI appears normal. Many times these resected cortical regions show evidence of microdysgenesis (heterotopic neurons in white matter can contribute to epileptogenesis by providing an aberrant circuitry). Similarly, FMZ PET can help in identifying microdysgenesis, which can show either decreased or increased FMZ binding, depending upon the number and subunit composition of $GABA_A$-benzodiazepine receptors in that tissue.

Evaluation of Seizure Pathology

An injury to neurons may cause a wide variety of changes, from subtle membrane change to neuronal loss, followed by gliosis. Injury may lead to structural and functional changes in various receptors and ion channels. There may also be a loss of inhibitory interneurons, synaptic changes with increased or decreased density, or abnormal axonal arborization with associated changes in synaptic strength. All these changes may alter neuronal firing thresholds, and lead to loss of hyperpolarization and progressive depolarization, which may be further augmented by extracellular ionic changes, ultimately leading to the formation of an epileptic focus. By applying appropriate ligand radiotracers, PET can be used to assess the receptors and ion channels. To study the change in number or distribution of various receptors, some of the PET tracers listed in table-1 can be used. However, if there is a structural and configurational change in the receptor, as in the case of suspected channelopathies, new PET ligands must be developed. Neuronal and glial proliferation can be imaged by applying specific cell markers tagged with positron-emitting isotopes. As synaptic reorganization is usually associated with new protein synthesis, so ^{11}C-leucine PET scaning can be used – at least theoretically – to measure the altered protein synthesis rate.

Neuroinflammation may be the underlying cause for intractable epilepsy in some cases, such as in Rassmussen's disease. Neuroinflammation is mediated by activated microglia, which secrete a number of proinflammatory molecules such as cytokines (IL-1, IL-6, TNF-α), chemokines (MIP-1α,β, MCP-1) and neurotoxins, free radicals, nitric oxide, proteinases, eicosanoids, and excitotoxins – many of which may play an important role in epileptogenesis. Although the exact mechanisms are still unclear, it appears that the inflammatory mediators may act by increasing glutamatergic neurotransmission, decreasing GABA-mediated currents, inducing neovascularization, and damaging the blood-brain barrier. Detection of microglia is not possible with current radiological methods or biochemical techniques, but requires histopathological examination of CNS tissues, which is quite invasive or possible only post-mortem. Since activated microglia express peripheral-type benzodiazepine receptors, they can be imaged with PET using ^{11}C-PK11195, a radioligand which binds specifically to the peripheral-type benzodiazepine receptors, thus making the in vivo detection of neuroinflammation possible (Figure 3).



Figure 3 PK11195 PET scan showing increased radiotracer binding in the right fronto-parietal area, indicating microglial mediated neuroinflammation.

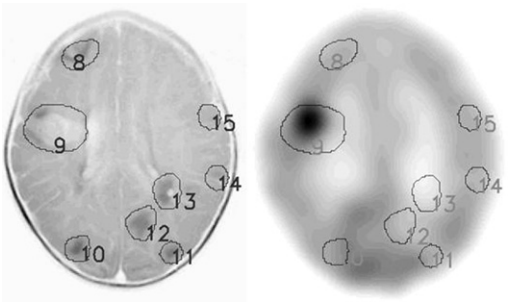


Figure 4 MRI scan showing multiple tubers (left image) in a child with tuberous sclerosis and intractable seizures with the majority of seizures coming from right parietal lobe. AMT PET scan showed intense uptake in a right parietal tuber (right image), corresponding to the EEG focus.



Figure 5 FDG PET showing significantly reduced glucose metabolism in the thalamus and lenticular nuclei with some sparing of the caudate nucleus in a neonate with hypoxic ischemic injury. Compare pattern of glucose metabolism with the normal pattern shown in **Figure 1**.

Patients with tuberous sclerosis may have multiple brain tubers. However, only one or two of them may be epileptogenic. It has been found that these tubers and/or the tissue around them contain a high amount of quinolinic acid, a product of tryptophan metabolism by indolamine 2,3-dioxygenase through increased activity of kynurenine pathway (induced under certain pathological conditions). Quinolinic acid is a strong convulsant through its agonist action at excitatory NMDA receptors, thus probably confering epileptogenicity to these tubers. AMT PET shows increased AMT uptake in these tubers and can help identify epileptogenic tubers (**Figure 4**).

PET can also be useful in investigating the structural basis for seizures associated with specific neurological conditions. For example, in neonates with some types of hypoxic ischemic brain injury in the perinatal period, FDG PET shows either absent or significantly reduced glucose metabolism in the thalamus and lenticular nuclei, with some sparing of the caudate nucleus (**Figure 5**). The cerebral cortex is also relatively spared compared to the subcortical structures, but may show focal areas of hypometabolism corresponding to the seizure foci. These findings are typical of children with the choreoathetoid or dystonic form of cerebral palsy and are consistent with the clinical observation that most of these children have relative preservation of cognitive function compared with their severe motor impairment (with chorea, athetosis, or dystonia). MRI may not be able to show all these details. Therefore, FDG PET can be used to provide a better understanding of anatomical-functional correlations in these children.

In patients with infantile spasms, FDG PET may reveal a single or multiple areas of cortical abnormality and can provide evidence of complex cortico-subcortical interactions believed to be important in the secondary generalization of focal cortical discharges, resulting in spasms. PET studies have shown that, regardless of the type or extent of cortical abnormality, increased glucose metabolism in lenticular nuclei and brain stem is a common feature in infants with spasms (**Figure 6**), consistent with the hypothesis that the spasms result primarily from the interaction of cortical abnormalities with brain

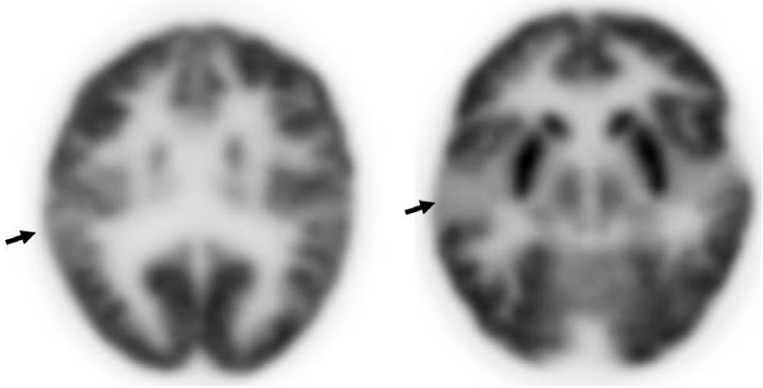


Figure 6 FDG PET scan showing an area of hypometabolism in right superior temporal region (arrows) and increased metabolism in bilateral basal ganglia in a child with infantile spasms.

stem and lenticular nuclei – and that this pathway of generalization accounts for the bilateral motor involvement and relative symmetry of the majority of spasms (even in the presence of a discrete focal lesion).

Assessment of Seizure Effects on Brain

FDG PET can be used to assess the effect of seizures on function in the rest of the brain. The spread of hypometabolism to neighboring areas from the presumed epileptogenic area is usually associated with some degree of functional suppression, and its extent has been shown to increase with time and depends upon overall seizure control. Hypometabolism of the ipsilateral thalamus and basal ganglia can be seen in cases of focal epilepsy (mostly from frontal and temporal cortex) (**Figure 7**). Contralateral cerebellar hypometabolism (diaschisis) is usually seen in cases of frontal or parietal lobe seizures. Occasionally, hypometabolism is seen in a contralateral 'mirror' region and represents the functional suppression of these regions through callosal fibers (transcallosal diaschisis) or through the fornix in the case of contralateral hippocampus. After long-standing seizures or in cases of severe status epilepticus, FDG PET scan shows diffuse hypometabolism of the entire cerebral cortex, particularly of the primary sensory areas, and is probably related to a temporary functional suppression (**Figure 8**). Therefore, whenever possible, it is important not to perform an FDG PET scan immediately after a seizure, but to wait at least 24 h. This delay is often not possible in young children with intractable epilepsy who have many seizures per day.

Treatment Selection

By achieving a better understanding of the altered cellular or molecular processes in human epilepsy *in vivo* using PET, new specific targeted therapies can be developed. Furthermore, response to treatment can be monitored

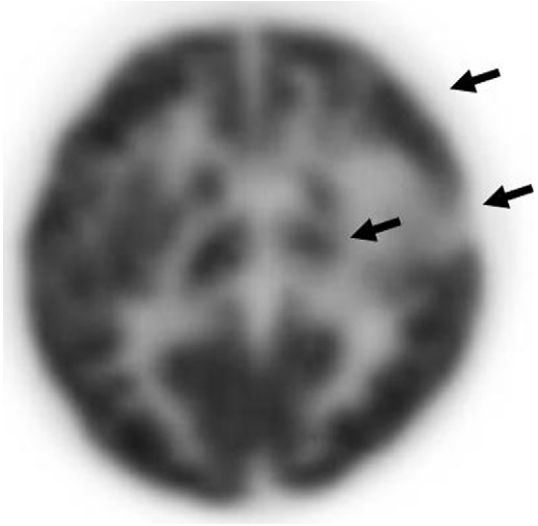


Figure 7 FDG PET scan in a child with a left fronto-temporal epileptic focus showing hypometabolism in the seizure onset zone (arrow). Note also the left thalamic hypometabolism (arrow).

using PET. Similarly, application of FDG PET has improved our understanding of the possible pathological mechanisms of epilepsy in various pediatric epilepsy syndromes, and has had an impact on their clinical management. For example, on PET scanning, most infants diagnosed with 'cryptogenic' *infantile spasms* will have focal or multifocal cortical hypometabolism. In cases of intractable spasms and a single focal PET abnormality that corresponds to the EEG focus, resective surgery can be planned with good results. Even in some cases showing multifocal areas of hypometabolism, but with all seizures arising from one area, resective surgery can sometimes be performed as a palliative procedure with improved quality of life; however, the developmental outcome may be less favorable than if a single focus were present. When the pattern of glucose hypometabolism is generalized and symmetric, a lesional etiology is not likely and



Figure 8 FDG PET scan showing diffuse hypometabolism of the entire cerebral cortex compared to basal ganglia in a child with status epilepticus.

neurometabolic or neurogenetic disorders should be considered in further evaluation and management.

Children with *Lennox-Gastaut syndrome* (triad of multiple seizure types including tonic seizures, developmental delay, and 1–2.5 Hz generalized 'slow' spike and wave EEG pattern), may have four metabolic patterns on FDG PET scan: unilateral focal, unilateral diffuse, or bilateral diffuse hypometabolism, as well as normal. Patients with unilateral focal and unilateral diffuse patterns may be occasionally considered for cortical resection, provided there is concordance between PET and ictal EEG findings.

In cases of *Sturge-Weber syndrome* (SWS), FDG PET usually identifies additional areas of abnormal cortex extending beyond the lesion visible on MRI. Serial FDG PET can be used to assess the dynamic nature of the disease process and can help in treatment selection. In some patients, serial FDG PET scans may show rapidly progressing and severe hypometabolism in the affected area, probably because of rapid demise of the brain tissue associated with the angioma: these patients will have improvement in seizure status and cognitive function and therefore may not require surgical intervention. Early and rapid progression in unilateral cases of SWS forces early and more efficient reorganization in the brain due to plasticity mechanisms. In contrast, persistent mild hypometabolism of the cortex underneath the lesion may indicate ongoing functional disturbance, and these patients may show persistent seizures and developmental arrest. These are the patients who require surgical intervention for seizure control and possible cognitive improvement, by promoting effective reorganization in the contralateral hemisphere while brain plasticity is still at a maximum during development.

PET scanning using FDG as well as ^{11}C-PK-11195 can help in the early diagnosis and management of Rasmussen's syndrome or other inflammatory conditions with intractable seizures, where CT and MRI are often normal for several months after the clinical manifestation of the disease. Treatment measures may include anti-inflammatory drugs, steroids, immunoglobulins, plasmapharesis, or surgical removal of the affected region. Localization of the most affected brain regions may also provide a guide in deciding the site of brain biopsy in order to avoid sampling errors.

Treatment Response Evaluation

Although treatment response is best evaluated clinically, cellular and biochemical substrates can be monitored and followed, either non-specifically with FDG PET (e.g., improvement versus worsening of hypometabolism, or appearance of any new area of hypometabolism) or more specifically using relevant PET tracers (such as receptor-ligands, radio-labeled neurotransmitters, and ligands for ion channels). In cases of suspected neuroinflammation as an underlying cause of seizure, ^{11}C-PK11195 is currently being tested to monitor its evolution (by imaging activated microglia). Similarly, dynamics of neurons, astrocytes and other glial cells can potentially be imaged using specific radio-markers as they become available.

Almost one third of epileptic patients will develop refractory epilepsy. P-glycoprotein (P-gp) and members of the multidrug resistance-associated proteins have been found to be increased in the seizure focus of patients with intractable epilepsy. These proteins actively extrude various lipophilic drugs, including anticonvulsants, out of the cell, and they may be responsible for a diminished or failed response to treatment. There is no way to detect in vivo the presence of increased expression of these drug transporters, except by PET. By using appropriate and specific radioligands, such as ^{11}C-verapamil for P-gp, one can measure the expression of these proteins in vivo and predict treatment response or help in selecting some treatment for P-gp modulation.

Prediction of Seizure Intractability

As in the case of PET scanning for P-gp, if any receptor or ion-channel change can be shown to be associated with seizure refractoriness, specific PET tracers could be developed and used to image their expression in vivo. Likewise, if some genetic changes are the cause of intractable seizures, PET potentially can be used to assess gene expression either

at the mRNA or at the protein expression level, using specific molecular imaging probes to study the dynamic processes in vivo in real-time and in a quantitative and noninvasive manner. For example, reporter gene imaging can be used to measure gene expression levels in tissues outside of the central nervous system. When cloned into promoter/enhancer sequences or engineered into fusion proteins, imaging reporters can be used to study the expression levels of any gene product. However, at present, engineering these molecules so that they cross the blood-brain barrier remains problematic.

Role in Epilepsy Surgery

In cases of intractable seizures, surgery may be considered. The most important aspect of presurgical evaluation is to identify a discrete epileptogenic region, which can be resected without causing an unacceptable loss of neurological function, and the removal of which will lead to complete seizure control. PET has little localization role in cases in which MRI-visible structural lesions are concordant with electrophysiological and clinical data. However, in cases of discordant results, or normal MRI, or multiple lesions with inconclusive EEG, PET can play a very important role. In cases of multifocal lesions associated with partial seizures showing a consistent semiology, the goal of PET (using selected radiotracers) is to identify the epileptogenic lesions. Sometimes, the epileptogenic lesion may be medially-located and be associated with generalized epileptiform activity due to secondary bilateral synchrony. In such cases, PET may allow a surgical option when otherwise the patient may not be considered for surgery. Further, FDG PET can provide some useful prognostic information; for example, when contralateral thalamic hypometabolism or bilateral temporal metabolism is present, the surgical outcome is usually poor.

Epileptogenic Region

2–^{18}F-fluoro-2-deoxy-D-glucose PET scan (FDG PET)

Interictal FDG PET typically shows reduced radiotracer uptake (hypometabolism) in the epileptogenic region. Although the exact mechanism of hypometabolism is unknown, it may result from a variety of mechanisms, including neuronal loss, diaschisis, suppressed function or reduction in synaptic density. It appears that cortical hypometabolism may be associated with duration, frequency and severity of the seizures, as hypometabolism is usually found in only one fourth of children with new onset temporal lobe epilepsy compared to 80–85% of adults with intractable seizures. Persistent or increased seizure frequency may lead to enlargement of the hypometabolic area, whereas seizure control may be associated with decrease in the size of hypometabolic cortex or even its resolution. The time interval between PET acquisition and the most recent seizure also may affect the extent and severity of the cortical abnormality; FDG PET done a short interval after a seizure may show more extensive and severe hypometabolism.

The sensitivity of FDG PET to identify the epileptogenic temporal lobe is estimated to be around 85–90%, and can show temporal lobe hypometabolism in more than 50% of patients with non-lateralized scalp ictal EEG findings. FDG PET can provide additional information about the epileptogenic zone in 77% of cases, and can impact surgical decision making in almost 50% cases. Although it rarely provides additional clinical information when hippocampal atrophy is obvious on MRI, FDG PET can lateralize the epileptic focus in temporal lobe epilepsy patients with subtle or absent quantitative MRI abnormalities. It has been found that interictal hypometabolic regions are not strictly confined to the presumed temporal epileptogenic zone or to the brain tissue showing pathologic changes. They commonly extend beyond temporal structures to ipsilateral parietal and frontal cortex as well as thalamus and also occasionally to the contralateral temporal lobe. Since this extension may reflect the epileptogenic network involved in the seizure propagation, these extratemporal regions should be investigated with intracranial EEG if scalp EEG over these areas are not completely normal.

In case of extratemporal lobe epilepsy, the sensitivity of FDG-PET in localizing the epileptogenic zones has been reported in the range of 45–73%. However, using a high-resolution PET scanner, we found a sensitivity of 92% and a specificity of 62.5% for FDG PET in the detection of the epileptic foci in children with frontal lobe epilepsy. In cases of occipital lobe epilepsy, a lower localization value (60%) for FDG PET has been reported.

As FDG PET usually shows an area of hypometabolism extending beyond the epileptogenic region, it can not be reliably used to precisely determine the surgical margin. However, it can be used for lateralization and general localization of the seizure focus. Further, this information can help in making a priori plans for subsequent subdural electrode placement, which may be very useful, particularly in cases of normal MRI. However, use of more specific tracers can help in providing a more precise delineation of the epileptogenic tissue; this enhanced localization becomes particularly important when the epileptic focus potentially involves eloquent brain regions (primary motor, speech or visual areas).

^{11}C-Flumazenil PET Scan (FMZ PET)

Among various neuro-receptor PET tracers used in epilepsy, FMZ has been the most widely applied clinically and appears to be quite promising. FMZ PET shows

decreased FMZ binding in the presumed seizure focus of both temporal and extratemporal lobe epilepsy; the extent of decreased binding is usually less than the area of hypometabolism shown by FDG PET. Rarely, FMZ PET may show increased uptake in some cases of cortical malformation and dysplasia, depending upon the number and/or affinity of the $GABA_A$/benzodiazepine receptors expressed in the abnormal tissue. Using ^{3}H-FMZ autoradiography on surgically resected human epileptic brain cortex, we found increased receptor density in cortical layers V–VI of spiking cortex; however, there was also decreased receptor affinity, and this change outweighed the increased density such that the net effect was a decrease in volume of distribution. At the PET level, this net decrease appeared as an area of decreased FMZ uptake on images obtained prior to surgery. FMZ PET is highly sensitive in temporal lobe epilepsy and shows decreased FMZ binding in the sclerotic hippocampus in patients with medial temporal lobe epilepsy that is largely confined to the affected temporal lobe. After correcting for partial volume effects, the sensitivity of FMZ in detection of unilateral hippocampal sclerosis has been reported to be 100%, with contralateral abnormalities in one-third of patients. In neocortical epilepsy, FMZ PET has been reported to have 60–100% sensitivity when compared to intracranial ictal EEG. In MRI negative patients, FMZ PET has been found to be abnormal in 84% and 70% of patients with temporal lobe and extratemporal lobe epilepsy, respectively. FMZ PET appears to be more sensitive than FDG PET in identifying an epileptogenic region and is associated with excellent surgical outcome both in temporal and extratemporal lobe epilepsy, even when the MRI is normal.

^{11}C-Alpha-Methyl Tryptophan PET Scan (AMT PET)

Another PET tracer, AMT, appears to have strong clinical applications in selected cases of epilepsy. In fact, AMT PET was the first and is still the only PET tracer capable of differentiating between epileptogenic and nonepileptogenic lesions in the interictal state in children with tuberous sclerosis. Subsequently, it was found to be useful also in patients with other types of neocortical epilepsy, showing increased uptake of AMT in the epileptogenic cortex. While the specificity of AMT PET is very high, its sensitivity is suboptimal, and appears to be related to the underlying pathology as well as the method of image analysis. For example, in patients with tuberous sclerosis and intractable epilepsy, we found that MRI-based quantitative assessment increased the sensitivity of AMT PET to 79% from 44.4% with visual assessment. This increased sensitivity is due to the fact that nonepileptogenic tubers typically show decreased AMT uptake, and that some epileptogenic tubers showing relatively increased AMT uptake cannot be easily differentiated from adjacent normal cortex without quantitative analysis.

Although clinical experience with AMT PET is limited in patients with medial temporal lobe epilepsy, this method does not appear to be particularly useful in cases of hippocampal sclerosis. However, we found that histologically-verified (macroscopic or microscopic) cortical dysplasia is associated with a higher occurrence of increased AMT uptake as compared to cases with nonspecific gliosis. Also, the area of increased AMT uptake is significantly more restricted than the extent of corresponding glucose hypometabolism. In some instances, AMT PET can identify epileptogenic cortex even if FDG and/or FMZ PET scan is normal.

Other PET Tracers

Clinical experience with opiate, histamine, NMDA, acetylcholine, dopamine and other neuro-receptor PET tracers is limited. Their uptake patterns, based on the studies available so far, are given in table-1. However, because of the paucity of data, their current role in presurgical evaluation is not yet established.

Dual pathology

Undiagnosed dual pathology (co-existence of neocortical lesion and hippocampal sclerosis) can be a source of surgical failure, since resection of both the cortical lesion and the affected hippocampus is necessary to optimize the surgical results. Although FDG PET can be used to evaluate for an abnormally functioning hippocampus, the higher sensitivity of FMZ PET makes it more useful in such cases, particularly when the MRI does not reveal an abnormal signal or atrophy in the hippocampus.

Secondary epileptic foci

A 'secondary' epileptic focus is an additional focus that is located at a different site from the primary focus, along the path of seizure propagation. Secondary epileptic foci evolve over a period of time. In the beginning they may be dependent upon the primary focus, in which case they tend to disappear after removal of the primary focus. With passage of time, they may become independent and are one of the major contributors to surgical failure in patients with longstanding intractable epilepsy. It is not known, however, at which stage these foci become independent and whether these areas of so called secondary epileptogenic foci require a critical period of seizure or some other aggravating factors for their activation. Histopathological examination of these secondary foci usually shows gliosis. FMZ PET can play a very important role in the detection of secondary epileptic foci. It appears that complete removal of the primary *and* secondary foci improves the surgical outcome.

Functional status of the rest of the brain

FDG PET can be very valuable in assessing the integrity of brain regions outside of the epileptogenic zone during the surgical planning process, and allows certain prognostic implications. These non-epileptogenic but dysfunctional areas are often associated with specific clinical manifestations.

For example, in patients with unilateral temporal lobe epilepsy based on EEG, the presence of bitemporal glucose hypometabolism is associated with poor memory performance and prefrontal hypometabolism is associated with impaired executive function. Similarly, there may be associated hypometabolism and/or reduced FMZ binding in the ipsilateral insular cortex (in 60% of the patients), usually correlating with emotional or somesthetic symptoms (depending upon the involvement of the anterior versus the posterior part of the insula). Bilateral lateral temporal (neocortical) and bilateral medial prefrontal hypometabolism (**Figure 9**) may be associated with interictal aggressive behavior, probably because of the removal of the inhibitory effects of temporal neocortex (and perhaps medial prefrontal cortex) on medial temporal lobe. In infants with epileptic spasms, bilateral temporal hypometabolism (affecting both hippocampus and superior temporal gyri) is strongly associated with an autistic or pervasive phenotype.

In children with hemimegalencephaly, FDG PET often shows additional less pronounced abnormalities in the opposite hemisphere, which probably accounts for the suboptimal cognitive outcome even with complete seizure control after surgical removal of the profoundly abnormal hemisphere. Thus, FDG PET can be useful in such cases to assess the functional integrity of the contralateral hemisphere prior to hemispherectomy and help predict cognitive outcome.

Eloquent cortex

Although ^{15}O-water PET has been used in the past to assess eloquent cortex (e.g., motor and language cortex), this type of evaluation has been, for the most part, replaced by functional MRI. The latter can be repeated and offers the advantage of no radiation exposure. As a result, PET has a very limited role in activation studies for presurgical evaluation in epilepsy.

Postsurgical evaluation

In surgical failures where a second surgery is being considered, there are very few neuroimaging options to pinpoint remaining epileptic tissue. Occasionally, ictal SPECT may be helpful in this regard, but in most cases the epileptologist is left with the scalp EEG and seizure semiology to guide placement of intracranial electrodes. We have found AMT PET to be particularly useful in this setting. Unlike MRI, interictal FDG or FMZ PET, AMT PET can differentiate epileptogenic cortex from non-epileptic tissue damage caused by the initial surgery in about half of cases (**Figure 10**); clearly, more work is required in this difficult group of patients undergoing reoperation.

FDG PET can be also useful in the post-surgical evaluation and monitoring of previously hypometabolic non-epileptogenic brain regions (i.e., regions that had no EEG correlate) in the pre-surgical FDG scan. Resolution of this hypometabolism, which occurs in some of these cases, will suggest the functional nature of the suppression. Further, persistence of a remote but connected region, or appearance of a new area, of hypometabolism may suggest a secondary potentially epileptogenic focus.

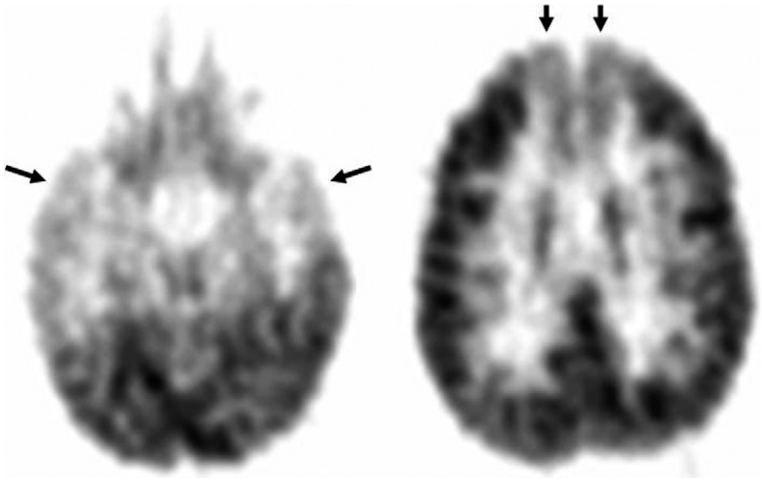


Figure 9 Bilateral temporal neocortical and bilateral medial prefrontal hypometabolism (see arrows) on FDG PET scan in a child with temporal lobe epilepsy and interictal aggressive behavior.

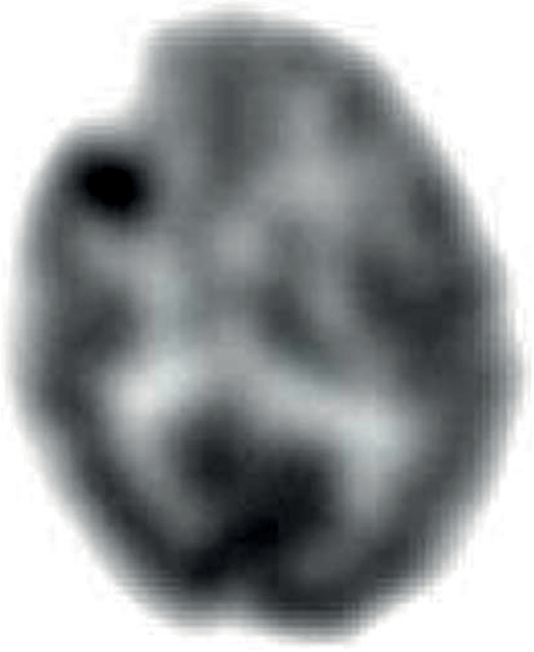


Figure 10 AMT PET scan showing a focal area of increased AMT uptake near the surgical margin in a child with failed epilepsy surgery. All other imaging investigations were noncontributory.

Neonatal seizures

Seizures occur more frequently in the neonatal period than at any other time of life. Population-based studies indicate that seizures occur in approximately 2.5–3.5/1000 live births in North America and may be caused by virtually any condition that affects neonatal brain function.

The most important factor in the therapy of neonatal seizures is accurate diagnosis. In neonates with no obvious metabolic abnormality, structural imaging studies (particularly MRI) may be useful to detect major malformations that are associated with seizures. However, the diagnostic value of MRI in this age group, to identify subtle cortical developmental malformations that are one of the commonest underlying causes of partial seizures, is limited. In fact, in young children less than 2 years of age, even macroscopic cortical malformations may not be apparent on MRI until myelination processes advance and blurring between gray and white matter disappears in normal cortex.

Application of PET can greatly improve the sensitivity of the detection of brain abnormalities causing neonatal seizures. FDG PET has been found to clearly lateralize or localize patterns of abnormal glucose metabolism in the majority of infants, even if CT or MRI fails to show localized structural abnormalities. In newborns (2–5 days of age) suffering from perinatal asphyxia and moderate or severe hypoxic-ischemic encephalopathy, FDG PET has demonstrated focal areas of decreased metabolism in the thalamus and lentiform nucleus with some sparing of the caudate nucleus, and can provide valuable information for the prediction of neurological outcome. However, in the past, neonatal PET studies were scarce, probably because scanners developed for adult studies are not ideal for imaging small babies owing to the limited spatial resolution. Further, transport of sick neonates to the PET scanners outside of the intensive care nursery is always troublesome. These problems can be circumvented by using specifically designed microPET scanners, or adapting animal PET scanners for human use and installing them in or around the neonatal intensive care unit.

Future Directions

PET scanning, along with the development of newer and 'designer' (e.g., targeted to a specific cellular/sub-cellular structure) radiopharmaceuticals, can help in identifying the cellular and molecular mechanisms of epilepsy, and may eventually lead to a more precise identification of the epileptogenic zone – an area that remains elusive and unidentifiable by any other technique (except, perhaps, invasive EEG). Newer PET techniques may also help in developing improved treatments and in the selection of patients for various therapeutic strategies. PET can play a very important role in identifying biomarkers for seizure intractability, information that may radically change our current approach towards epilepsy management. Future studies are also required to establish the role of PET scans in the detection of dual pathology and in identifying any potential secondary epileptic focus.

See also: **Cognitive Dysfunction and Other Comorbidities**: The Relationship of Epilepsy to Other Conditions/Disorders; **Imaging**: PET Neurotransmitter Receptor Imaging in Epilepsy; **Metabolic Changes**: Seizure-Induced Metabolic and Hemodynamic Changes in the Immature Brain.

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Imaging Characterization of Familial Temporal Lobe Epilepsies

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Introduction

Mesial Temporal Lobe Epilepsy

Structural correlates of MTLE include hippocampal sclerosis (HS), glial tumors, vascular malformations, and neurodevelopmental lesions. Gliotic lesions due to trauma or infections can also give rise to MTLE. HS is the major neuropathological substrate in patients with MTLE, and it is present in 60–70% of patients with MTLE who undergo surgery for treatment of medically refractory seizures. HS most likely has different causes in different individuals, and may result from complex interactions among genetic and environmental factors. Histopathological hallmarks include segmental loss of pyramidal neurons, granule cell dispersion, reactive gliosis, and axonal sprouting of surviving neurons. The mechanisms underlying the development of MTLE remain undetermined. It has been hypothesized that developmental malformations or precipitating events in early development, such as febrile seizures, may initiate a lasting and progressive change that leads to a propensity of the hippocampus to generate seizures in later life. Animal models of febrile seizures suggest that, indeed, early life seizures cause lasting changes in the excitability of hippocampal neurons.

The hallmarks of MTLE are typical limbic seizures with or without loss of consciousness. The first recurring seizures usually occur in late childhood or early adolescence. The most common initial ictal symptom is a visceral sensation, described usually as rising epigastric sensation (also refereed to as epigastric aura) that may be followed or accompanied by other features including emotional disturbances such as fear, autonomic symptoms, and olfactory or gustatory sensations. Auras typically occur in isolation and can precede seizures with impairment of consciousness (complex partial seizures). The latter commonly begin with a motionless stare and oroalimentary automatisms (e.g., lip smacking, chewing), during which the patient may be unresponsive. Gestural and reactive automatisms of the limbs or sometimes the whole body are also common. Dystonia of one extremity is usually contralateral to the side of ictal onset. Seizures typically last 1–2 min.

The neurological examination is usually normal. Most atients have deficits in episodic memory. Verbal memory is mostly affected with left MTLE, whereas visuospatial memory is more affected with right MTLE. The interictal electroencephalogram (EEG) of patients with MTLE typically shows unilateral or bilateral independent mesial temporal spikes, best seen with basal (sphenoidal, inferior temporal) derivations. Ictal EEG patterns commonly consist of rhythmic 5–7-Hz discharges seen in the mesial temporal region on the side of onset. High-resolution MRI often demonstrates unilateral and sometimes bilateral hippocampal atrophy associated with hyperintense T2 signal in one or both hippocampi, which is highly specific for HS. More detailed high resolution MRI analyses reveal a network of gray matter atrophy that involves mesial temporal and other structures interconnected with the limbic system, including amygdala, entorhinal, perirhinal, and parahippocampal cortices, and thalamus. Most patients are sporadic, but familial aggregation also occurs.

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Familial Temporal Lobe Epilepsy

Familial MTLE

The best definition of familial MTLE is based on the familial recurrence of MTLE in the absence of any suggestion of other partial (including lateral TLE symptoms) or generalized epilepsy syndromes in other affected family members. The finding of at least two MTLE patients in one family is suggestive of familial MTLE. The observation of an autosomal dominant inheritance pattern with incomplete penetrance implies the presence of asymptomatic carriers of the genetic abnormalities, who can transmit the disease to their offspring. Therefore, we should consider inclusion of not only families with affected first-degree relatives, but also those with affected second and third degree relatives. This criterion has not been employed in some reported series, leading to exclusion of many possible familial MTLE kindreds.

Most affected individuals with familial MTLE have a benign clinical course, and in some families all affected members have good seizure control or remit after a short period of seizures. However, as in patients with nonfamilial MTLE, some affected family members may have poor seizure control and require surgical treatment. Although magnetic resonance imaging (MRI) signs of hippocampal sclerosis, including hippocampal atrophy (HA) and hyperintense T2 signal, are more frequent and more pronounced in patients with refractory seizures, these changes are also observed in patients with good clinical outcome, and even in asymptomatic family members. These are strong indicators that genetic factors play a role in the genesis of hippocampal pathology in patients with familial MTLE. While the pattern of inheritance is autosomal dominant with incomplete penetrance, the genetic background in familial MTLE does not imply a more widespread structural abnormality on MRI.

Familial TLE with auditory auras

Familial TLE with auditory auras (also described as autosomal dominant partial epilepsy with auditory features (ADPEAF)) is a benign epilepsy syndrome, characterized by auras described as buzzing, roaring, radio or motor like sounds, distortions in sounds and words. Although other manifestations such as psychic, cephalic, and other sensory and motor phenomena can occur, the auditory auras are a hallmark of this syndrome. Sometimes ictal aphasia and visual misperceptions can occur and, in some families, secondarily generalized tonic–clonic seizures (GTCS) are frequent before treatment. The EEG may show posterior temporal epileptiform discharges, but is frequently normal.

The pattern of inheritance observed is autosomal dominant with incomplete penetrance. Molecular studies identified linkage to chromosome 10q, and mutations in the *leucine-rich, glioma-inactivated 1* gene *(LGI-1)* have been identified. However, only 50% of families presenting the typical phenotype have LGI-1 mutations suggesting genetic heterogeneity.

Age of onset is variable, usually in the second or third decade of life, and seizures are easily controlled by AEDs. No signs of HA or HS-related signal changes on MRI have been described in different series: however, we have found subtle abnormalities in the neocortical aspects of the temporal lobes.

The prevalence and incidence of these two forms of familial TLE are unknown. However, familial MTLE is apparently more common than familial TLE with auditory auras. There is no predominance in any particular ethnic group, and families with MTLE and with TLE with auditory auras have been described in several countries. There is probably an underestimation of the real prevalence of both forms of familial TLE worldwide, especially in families with predominantly good outcomes.

Ascertainment of these families requires detailed questioning of patients and family members, a practice that has only been emphasized recently, as MTLE, as well as other partial epilepsies, has generally been considered to be symptomatic and largely due to environmental factors.

There is no evidence to suggest that familial partial epilepsy with variable foci, partial epilepsy with auditory features, or temporal lobe variants of benign childhood epilepsy with centrotemporal spikes ever evolve into MTLE with HS. This observation suggests a clear distinction between familial MTLE and these other familial epilepsy syndromes.

Background

Neuroimaging in Familial MTLE

MRI evaluation in familial MTLE has shown a high frequency of HA and other signs of HS (**Figure 1**), even in individuals with seizure remission and asymptomatic family members (**Figure 2**). The identification of MRI signs of HS in familial MTLE patients with a benign clinical course supports the view that the presence of HA is not always associated with refractory epilepsy. Available data indicate that MRI abnormalities, determined by visual analyses, are similar in familial and sporadic MTLE with HS.

Patients with intractable familial MTLE present a clinical profile and most histological findings comparable to patients with sporadic MTLE. Interestingly, mossy fiber sprouting may be more pronounced in patients with sporadic MTLE. This finding suggests that, when compared with sporadic MTLE, patients with familial MTLE may respond differently (i.e., display different plastic changes) to cell loss, neuronal deafferentation or epileptic seizures. This finding is in keeping with our recent MRI studies with voxel-based morphometry

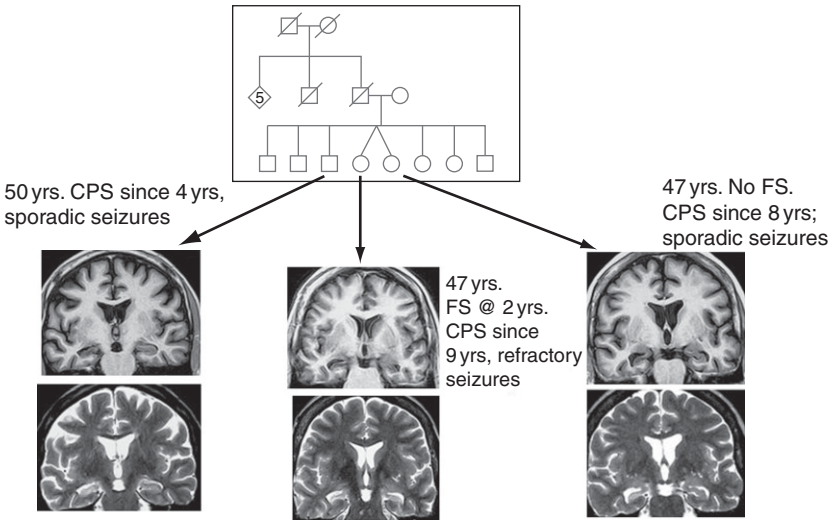


Figure 1 Reduced pedigree from one family with familial MTLE, showing coronal T1 and T2 MRIs showing signs of hippocampal sclerosis: atrophy, hyperintense T2 signal, abnormal shape and abnormal internal structure of right hippocampus in three sibs. CPS: complex partial seizures; FS: febrile seizures.

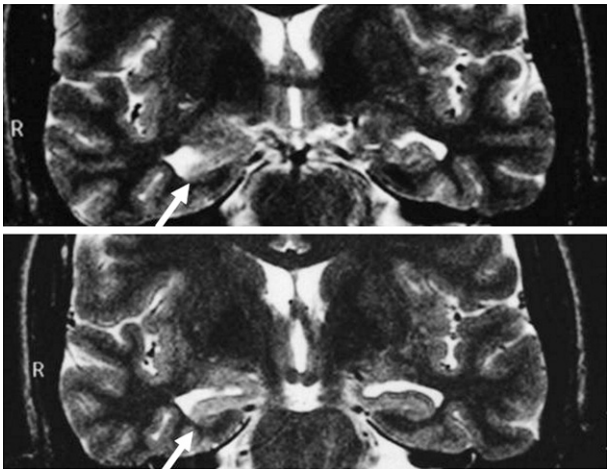


Figure 2 Coronal T2-weighted MR images in a 39 years old man from a family with MTLE. He had his first seizure at 17 years of age and a few other seizures during life. He has been seizure free without medication for several years. Observe the right hippocampal atrophy with hyperintense T2 signal and abnormal shape (arrows).

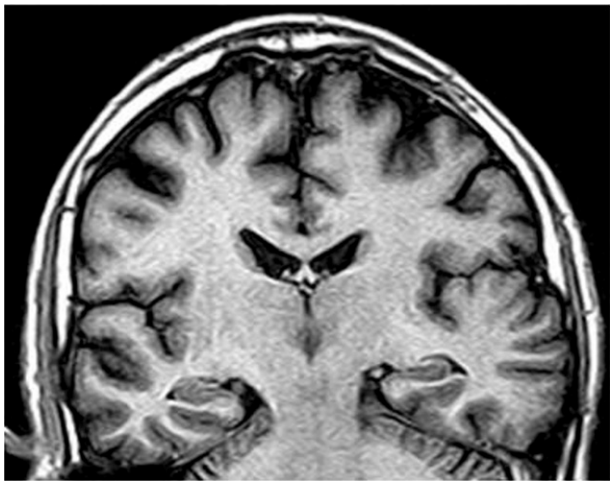


Figure 3 Coronal T1-invertion recovery MRI showing enlargement of left temporal lobe in a patient with familial TLE with auditory auras. Observe also an abnormal pattern of the first and second temporal sulci as compared to the right side.

(VBM), demonstrating more widespread gray matter atrophy in patients with sporadic MTLE compared to familial MTLE as discussed below.

Neuroimaging in Familial TLE with auditory auras

No signs of HA are found on MRI studies, but we have observed a lateral temporal malformation pattern in 45% of affected individuals, including one asymptomatic carrier of the mutation. The left temporal lobes of these individuals seemed enlarged (Figure 3), and these patients sometimes show a protrusion of the brain parenchyma laterally, with an 'encephalocele-like' appearance. Anterior temporal lobe volumetry showed a significant global increase in only two individuals. The epileptogenic significance of these structural abnormalities is unknown.

Methods

Familial MTLE

Our group has performed a genome-wide scan with 332 microsatellite markers at ~12 cM intervals to identify the region harboring the main gene associated with HA

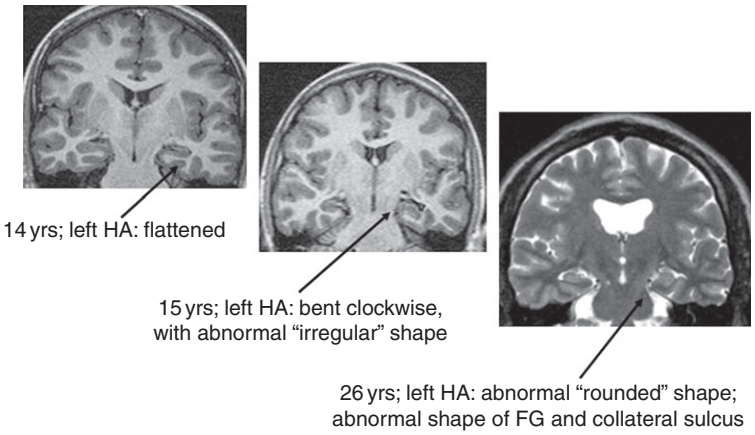


Figure 4 Coronal MRIs from asymptomatic family members of families with MTLE showing different patterns of hippocampal malformation (arrows). HA: hippocampal atrophy. FG: fusiform gyrus.

in a large kindred with familial MTLE. Additional 14 markers were genotyped in the candidate region. Two-point and multipoint LOD scores were calculated with the LINKAGE computer package. All genotyped individuals underwent MRI for evaluation of hippocampal abnormalities. Previous pedigree and complex segregation analysis provided evidence for the presence of a major gene predisposing to HA in familial MTLE.

In addition, we performed visual and volumetric analysis of all MRIs in 12 patients from this kindred who had seizures. Hippocampal measurements were made on 3 mm coronal T1-weighted images. Hippocampal malformation was defined according to previous publications (see Further Reading), and included hippocampi that were abnormally round, pyramidal in shape, with abnormal rotation or vertically orientated (**Figure 4**). All patients had clinical and EEG diagnosis of MTLE, except one patient who had had two generalized tonic–clonic seizures.

In a different study, we used an MRI automatic segmentation protocol and VBM to investigate 92 consecutive patients with benign MTLE (55 women and 37 men; mean age 42 years). Benign MTLE was defined as no disabling seizures reported by the patients, with only mild simple partial seizures occurring once a month or less. All patients and 40 controls (22 women, 18 men; mean age 32 years) were submitted to a 2T MRI scan. The volumetric 3D T1 *gradient echo* (GRE) sequence was used for the analysis. MRIs were normalized using linear and nonlinear transformations, and were automatically segmented using SPM5 (Wellcome Trust Centre for Neuroimaging, London, England; www.fil.ion.ucl.ac.uk) built-in routines. For VBM analysis, the images were smoothed further with an 8 mm Gaussian Kernel. Images were also normalized based on the total intracranial volumes of the controls. We compared differences between sporadic and familiar MTLE versus controls. For these comparisons, images were horizontally flipped according to the smaller hippocampus to avoid side to side cancellation. ANOVA was used, and a $p < 0.05$ false discovery rate correction was selected.

We also followed prospectively 64 individuals with familial MTLE from 28 families. Patients who fulfilled clinical criteria for MTLE (according to the ILAE classification scheme) were divided into three groups. (i) *Remission*: seizure-free for at least 2 years; (ii) *Benign*: less than six complex partial seizures (CPS)/year and no more than two secondary generalized tonic–clonic seizures per year; and (iii) *Refractory*: more than six partial seizures per year despite adequate antiepileptic drugs (AEDs). Thirty-five of these individuals had hippocampal volumetric measurements in two MRI scans, performed with a minimum of 1 year interval, which did not show progression of atrophy.

Familial TLE with Auditory Auras

We acquired MRIs in a 2T scanner (Elscint Prestige, Haifa, Israel) using a spoiled GRE sequence (TR = 22 ms, TE = 9 ms, flip angle = 35°, matrix = 256 × 220). These T1-weighted images were acquired with 1 mm isotropic voxels, and were subsequently transformed into ANALYZE format using MRIcro software (Chris Rorden, www.mricro.com). VBM analysis was performed using SPM99 (www.fil.ion.ucl.ac.uk). Images were then normalized to the standard space using 12 linear parameters and 7 × 8 × 7 nonlinear basis functions, using a brain mask. Spatially normalized images were resliced to an isotropic 1.5 mm and underwent segmentation of gray matter and modulation of the estimated concentration of tissue, based on the spatial deformations encountered during normalization. This technique preserves the quantity of gray matter and ensures a good spatial alignment between patients and controls. Finally, the images were convolved with an Isotropic Gaussian Kernel of 10 mm to minimize gyral interindividual variability. The resulting images were then compared using *t*-tests, to

determine differences in grey matter concentration (GMC) between controls and subjects with the LGI1 mutation from one large family. Contrasts were defined to estimate the probability of each voxel being gray matter. This analysis included grand mean scaling and proportional threshold masking (set to 0.8) and implicit masking. The results from the analysis are given in a parametric map of t-statistic (SPM(t)), and the SPM(t) is corrected for normal distribution (SPM(z)). Considering the previously observed subtle structural abnormalities in the temporal lobes of these same individuals, and the relative small number of participants, we examined clusters with at least 32 voxels that exceed $p < 0.01$, uncorrected threshold ($T = 2.39$). Group differences for age were assessed using one-way ANOVA, and the gender distribution was evaluated with χ^2 test.

Recent Results

Familial MTLE

We have identified linkage to chromosome 18p in a single family with MTLE. Multipoint and haplotype analysis localized the locus within a 13 cM interval. From the 12 patients in this family with seizures, one patient – who had had two generalized tonic–clonic seizures – did not have the haplotype, and MRI was normal. The remaining 11 patients with diagnosis of MTLE had MRI signs of hippocampal malformation, including hippocampal atrophy in 8 of them. All except one of these 11 patients had the haplotype. Further evaluation including asymptomatic family members would be helpful for clarifying the genetic basis of hippocampal malformation and hippocampal sclerosis in familial MTLE. However, our success in this linkage analysis provides clear evidence that HA may be caused by genetic factors, which can have major implications in the study of the pathophysiological mechanisms underlying MTS and its relationship with TLE.

For the MRI VBM study of benign MTLE, there were 52 patients in the sporadic MTLE group and 40 patients in the familial MTLE group. Our investigation evidenced a high frequency of structural abnormalities in patients with benign MTLE. VBM showed areas of gray matter atrophy that were more widespread in patients with sporadic TLE compared to familial MTLE (Figure 5), which

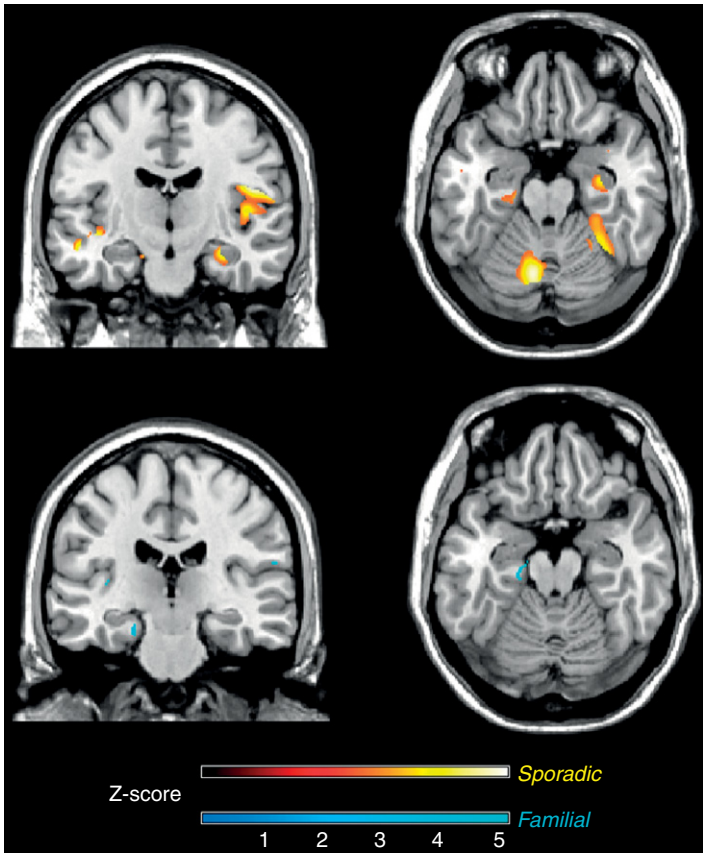


Figure 5 Results of the VBM comparisons among 40 patients with mesial temporal lobe epilepsy and positive family history (TLE, top row – hot colors), 52 patients with sporadic TLE (negative family history, bottom row – cold colors) and 40 controls showing areas of gray matter atrophy. The results are overlaid with an anatomical T1 template and displayed in two orthogonal planes (coronal and axial). The color bar in the inferior portion of the figure represents the number of standard deviations as compared to controls.

is in agreement with another study comparing postoperative histopathology studies in refractory sporadic and familial MTLE. Familial and sporadic TLE showed different patterns of atrophy, as observed in the VBM group comparisons. Multiple mechanisms involved in the pathogenesis of TLE are the probable explanation for this diversity. These findings indicate that environmental factors and modifier genes play an important role in the outcome of TLE.

We followed prospectively 64 individuals, from 28 families, with familial MTLE. Patients who fulfilled clinical criteria for MTLE were divided into three groups, as described above. Mean follow up was 93.4 ± 15.8 months (ranging from 45 to 121.9 months). At baseline they were divided as follows: benign – $n = 29$; remission – $n = 28$; and refractory – $n = 7$. At the last follow-up visit, 12 (41.4%) patients with benign FMTLE remained classified as benign, 6 (20.7%) became refractory, and 11 (37.9%) were in remission. In the subgroup of familial MTLE in remission, 21 (75%) remained without seizures; 6 (21.4%) were classified as benign familial MTLE, and one died (3.6%) from causes unrelated to epilepsy. All refractory patients remained refractory. Prospective follow-up of more than 7 years in patients with familial MTLE revealed that those with refractory seizures are unlikely to achieve seizure control with AEDs. In contrast, patients with benign familial MTLE for more than one year are likely to remit or remain under good seizure control. The majority of patients who had achieved seizure remission remained seizure-free and none became refractory.

Familial Temporal Lobe Epilepsy with Auditory Auras

Studies have reported no signs of HS in MRIs of patients from families with LGI-1 mutation. Enlargement and abnormal gyration, suggesting developmental abnormalities in the lateral cortex of the temporal lobe, were described in 53% of affected individuals in one family with LGI-1 mutation (**Figure 3**), a finding that requires confirmation. The MRI findings in that family are clearly distinct from the MRI findings in familial MTLE, and are consistent with the distinct seizure semiology in these two forms of familial TLE.

We performed a VBM study in 17 family members from a large kindred with familial TLE with auditory auras (three of them asymptomatic), all being carriers of the LGI1 mutation. We observed areas of increased GMC (compared to normal controls) in the left cerebellum, right parietooccipital region, and left hippocampus. In addition, these individuals presented with decreased GMC in the anterior portion of both temporal lobes, in the left precentral area, and in the right cerebellum.

The functional properties of the LGI1 gene remain unknown. This gene was cloned from the breakpoints of a glioblastoma cell line and its expression is reduced or absent in many high-grade gliomas. This evidence suggests a possible function related to cellular proliferation and/or tumor suppression. Furthermore, this gene is characterized by a central leucine-rich repeat region, which is involved in regulation of cell growth, adhesion, and migration. Therefore, LGI1 mutations could, possibly, be leading to subtle abnormalities of neuronal proliferation or migration that would result in dysgenetic lesions not easily detected by MRI.

Future Goals

Further structural and functional imaging and histopathological studies will shed light on the processes involved in genesis and progression of HS, and on other underlying mechanisms of sporadic and familial TLE epilepsies. The finding of a major gene mutation will be crucial for a better understanding of these functional and anatomic changes related to epileptogenesis in MTLE. The presence of structural brain abnormalities, e.g., in the form of GMC excess in patients with familial TLE with auditory auras, could be a consequence of the LGI-1 mutation and requires further investigation.

See also: **Classification**: Classification of Seizures and Syndromes; **Imaging**: Structural Imaging in Epilepsy; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy.

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Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments

B Weber, K Fliessbach, and C E Elger, University Hospital of Bonn, Bonn, Germany

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Introduction

Magnetic resonance imaging (MRI) of the brain has become an indispensable tool in the diagnosis and treatment of epilepsy patients. Since its introduction to the clinical routine in the early 1990s, the detection and characterization of epileptogenic lesions by MRI has improved substantially. This imaging progress has opened up the possibility of epilepsy surgery to more patients, and improved the prognosis for those who are finally undergoing surgery.

Major recent advances lie in the improvement of magnetic resonance (MR)-acquisition techniques, which include improvements in the scanner hardware (e.g., more powerful gradient systems and scanner magnets with increased field strengths) as well as the development of new MR sequences with better contrasts. Besides these developments, the postprocessing of structural MRI data has yielded increasingly insightful results in the detection of cortical and subcortical lesions. Finally, functional MRI (fMRI) has shown its powerful potential to predict postsurgical outcome or complications and to contribute to focus detection.

Developments in MR-Acquisition Techniques

High Field Strengths

During the early years of clinical MRI, the field strength used was rather low (<0.5 T). The majority of clinical magnets today are operated at a field strength of 1.5 T, but the improved signal-to-noise ratio (SNR) with stronger magnets (3 T and above), and the growing experiences with the pitfalls and problems of higher field strengths, have lead to a growing number of these stronger clinical magnets. The drawbacks of higher field strengths, such as increased radiofrequency-energy (applied to the patients) and larger field inhomogenieties and susceptibility artifacts, as well as differences in relaxation rates, have been overshadowed by the ability for faster imaging and/or increased resolutions. Although the use of 3-T magnets becomes more and more routine (at least in larger hospitals and special centers), even higher field strengths of 7 or 9.4 T are now constructed for use in patients by all the vendors. Although these high field-strength magnets are currently viewed rather critically (as were the first 3-T magnets when they were introduced), the increased SNR provided by this enhancement can be used for even more rapid imaging or increased resolution. Exemplary images show promising results (**Figure 1**), but the applicability of these magnets in routine clinical function is debatable – not only due to the much higher costs but also due to an increased ratio of side-effects (e.g., nausea or vertigo) experienced by initial subjects.

Multichannel Coils and Parallel Imaging

Besides the increase in field strength, the development of multichannel coils was one of the cornerstones that improved neuroimaging by enabling parallel data reconstruction, and thereby increasing SNR, allowing decreased scanning time and artifacts. The decrease in acquisition times (by a factor of 4 or even more) alleviates movement artifacts and reduces the influence of field inhomogenieties, which is especially important for fast echo planar imaging (EPI) acquisitions which are used for fMRI to measure the blood oxygen level dependent (BOLD) contrast and diffusion weighted imaging, and also provides higher resolution images (if the same

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acquisition time is maintained). Currently, 8- or 12-channel head coils are most commonly used in routine scanners, but even higher numbers of array coils are being produced and are finding their way into the clinical setting. Preliminary data with 32 (or even more) receiver coils show promising results, with increased SNR up to 3.5 fold (Figure 2).

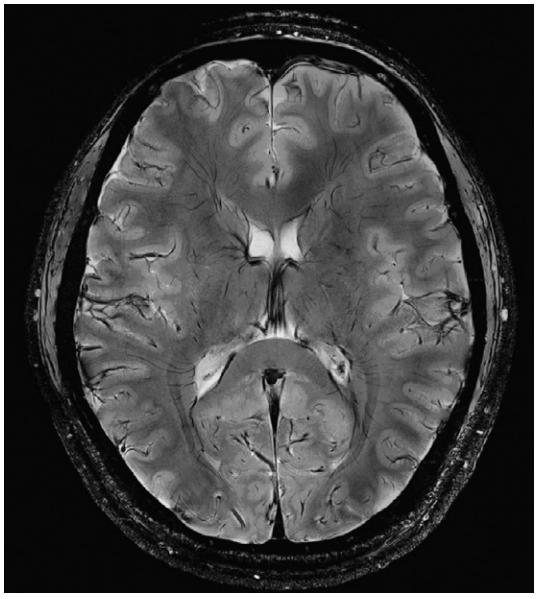


Figure 1 Example image at 7 T. T2-FLASH transversal, 32-channel head coil, slice thickness 1 mm, 1024 × 1024 matrix. Provided by Siemens Medical Solution ©, Erlangen, Germany.

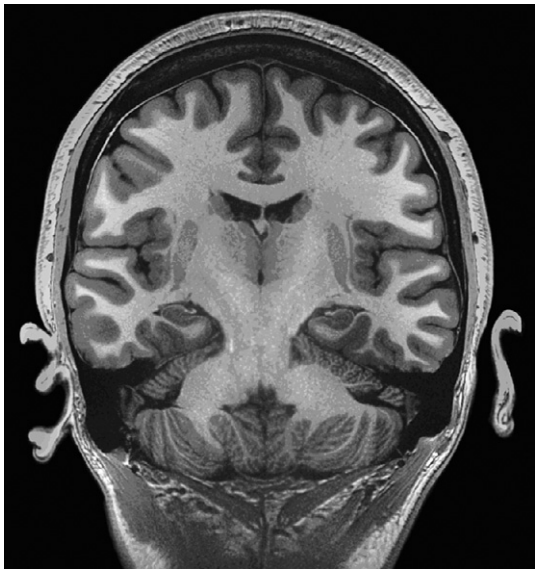


Figure 2 T1 3D turbo FLASH inversion recovery, 32-channel head coil, with GRAPPA PAT Factor 2, 512 × 512 matrix.

New Methods for k-Space Data Sampling

Besides the development of parallel data acquisition, considerable effort has been put into the creation of new data sampling procedures in the spatial-frequency domain (k-space). Images of patients, especially in pediatric context, are frequently disturbed by movement artifacts, rendering their interpretation difficult if not impossible. Based on the different impact of specific parts of the k-space on the contrast and signal intensities in MR images, methods have been invented that are much less susceptible to the movement of patients inside the scanner (e.g., ROTATOR – periodically rotated overlapping parallel lines with enhanced reconstruction – also called BLADE). This method samples the k-space via multiple echo trains, rotating around the center of the k-space (in contrast to a regular rectangular method). Hence, the center of the k-space is sampled during every train, allowing for the correction of spatial shifts by the comparison of the repeated acquisitions of the k-space center (Figure 3). Other trajectories, as spiral k-space acquisition, have not yet proven additional performance increases but have potentially an application in fMRI.

The Role of MR Postprocessing

Voxel-Based Morphometry

Probably the most challenging aspect of MRI in epilepsy is the investigation of patients classified as having a 'cryptogenic' focal epilepsy. A significant amount of 'cryptogenic' epilepsies is thought to be due to focal cortical dysplasias (FCDs). The difficulty of detecting these FCDs on MRI scans lies in the subtlety of their appearance on conventional images, and the complexity and variability of cortical convolutions concealing these abnormalities of development. Much effort by different research groups has been put forward to enhance the visualization of conventional MR images by means of automated postprocessing of the images. The basic idea behind most of the studies is to build a normative database, and to compare individual patients to this database to identify significantly differing structures. While some studies have been focused on rather simple voxel-based comparisons of gray- or white-matter distributions, other approaches have tried to highlight the characteristics of FCDs by means of texture analyses – i.e., blurred gray–white-matter line or more intense T1-weighted signal within the dysplastic cortex (Figure 4).

These approaches show very promising results, but the available studies on their specificity and sensitivity are mostly retrospective. Thus, they should be considered as heuristic tools that can complement findings from other sources. For routine clinical use, they need to be

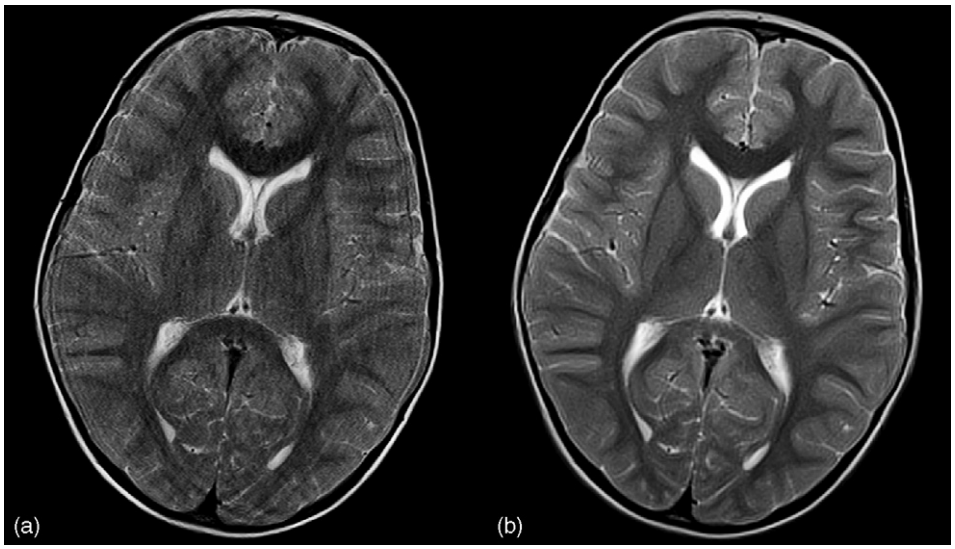


Figure 3 T2 TSE transversal (a) without BLADE (matrix 448 × 448; TA, 2:06 min) and (b) with BLADE (matrix 384 × 384; TA, 1:57 min). Provided by Siemens Medical, Erlangen, Germany.

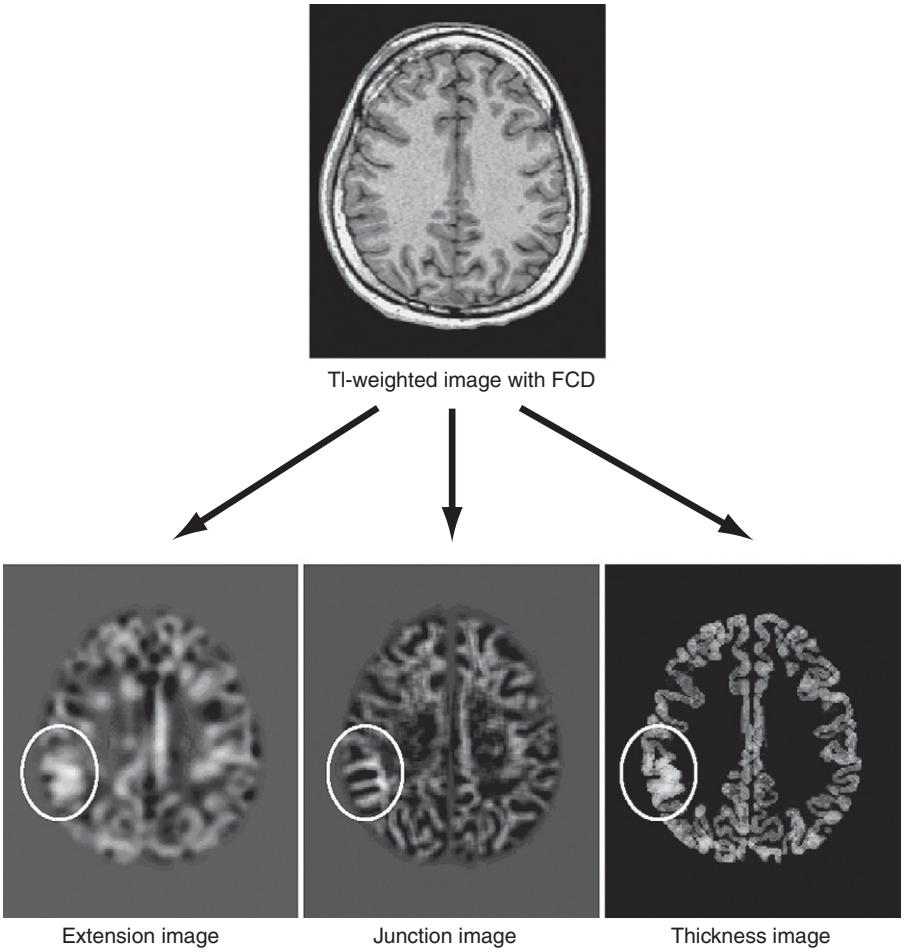


Figure 4 Example of a texture-based postprocessing of a cortical dysplasia not visible on a conventional T1-weighted MRI. Provided by Hans-Juergen Huppertz.

prospectively validated in larger number of patients, to prove their utility for the presurgical evaluation of epilepsy patients.

Multimodal Segmentation and Quantitative Imaging

One major problem of the approaches mentioned earlier is the dependency on the segmentation and delineation of the gray–white-matter boundary. Most segmentation procedures are based on signal intensities and (with more modern approaches) prior probabilities of specific tissue types. Recent approaches have tried to enhance the segmentation process by adding additional information by means of different MR contrasts (e.g., fluid-attenuated inversion recovery (FLAIR), T2-weighted, proton density, T1-weighted) and applying a cluster analysis. This approach promises a more precise segmentation, and might prove useful to apply to the investigation of cryptogenic epilepsy. In this context, the application of quantitative MRI data, such as T2-relaxation times or water content, might additionally enhance the segmentation process. Quantitative MRI approaches have the strong advantage of being less sensitive to perturbing factors (such as gradient drifts or field inhomogenieties) and hence may be more sensitive when used for individual patient diagnostics.

Cortex Reconstructions and Fractal Dimension

A different approach to investigate pathological differences in cortical structures is to generate three-dimensional models of the cortex, which can be parameterized and then entered into an analysis. Different procedures have been developed for the parameterization of cortical surfaces and applied to different neurological or psychiatric diseases (e.g., schizophrenia and multiple sclerosis) as well as to medial temporal lobe epilepsies (e.g., describing extratemporal changes in gyral complexity). To what extent these methods may be used in individual diagnostics remains to be evaluated.

Diffusion Tensor Imaging and Tractography

Diffusion tensor imaging (DTI) is an MRI technique that measures water molecule diffusion and its directionality. The most commonly used outcome measures are fractional anisotropy (FA) and (rotationally indifferent) mean diffusivity (MD). Tractography is an additional DTI-based technique for the mapping of discrete fiber tracks, using defined originating regions ('seeds') and statistical procedures that can follow the trajectories of white-matter tracks from this region. Because water diffusion in the brain is mainly restricted by fiber tracts, DTI has been suggested as an indirect marker for white-matter integrity. In several types of brain lesions, including tumors, multiple sclerosis plaques, or ischemic lesions, DTI has been found to complement other clinical data in the characterization of these lesions.

In epilepsy, DTI might help identify and characterize epileptogenic lesions such as hippocampal sclerosis or FCD. In proven hippocampal sclerosis, the epileptogenic hippocampus demonstrates increased MD and decreased FA. Prospective studies are needed to investigate the potential of DTI to detect the epileptogenic side in medial temporal lobe epilepsy *without* clear MRI abnormalities. Besides changes in the mesial temporal lobe structures themselves, other white-matter abnormalities (e.g., in the corpus callosum and internal capsule) have been found to be associated with hippocampal sclerosis. At present, no clear relation between the extension of extrahippocampal abnormalities and success of surgery has been found, but comprehensive studies on this interesting topic are missing.

In FCD and other types of cortical malformations, DTI and tractography have been used to characterize changes in subcortical connectivity. In many cases, diffusion changes are found not only close to the cortical lesion but also in adjacent subcortical regions that appear unremarkable in conventional MRI. This finding again raises the question of whether the extent of subcortical involvement can be used to predict surgery outcome and improve surgical planning. DTI might also prove useful in the detection of lesions undetected by conventional MRI. A recent study by Rugg-Gunn and colleagues found a significant increase in diffusivity in 8 of 30 patients with cryptogenic focal epilepsy; in 6 of these patients, the locus of the increase overlapped with the localization of EEG abnormalities, suggesting that DTI might be an additional tool for the detection of MRI-negative epileptogenic foci.

Besides the potential of DTI in characterizing epileptogenic regions, it is a promising tool for investigating the association between structural connectivity and functional changes associated with epilepsy and epilepsy surgery. Powell and colleagues found, e.g., a reduced left and enhanced right-hemispheric interconnectivity in patients with left temporal lobe epilepsy (TLE) than in healthy controls and patients with right TLE. These structural changes might be related to language dominance. Because language dominance shows interindividual fluctuations (e.g., during the menstrual cycle in women, or after successful epilepsy surgery), longitudinal studies that correlate functional imaging changes with DTI seem warranted.

New Developments of fMRI in Epilepsy Patients

fMRI of Cognitive Functions

fMRI in the domain of epilepsy has widely been applied for research purposes, but has also entered into the

clinical evaluation process of individual patients. The most established application of fMRI in clinical routine is the investigation of language dominance. Many studies have shown a high correlation with the gold standard of the Wada (amobarbital) test, and thereby its applicability as a less-invasive method. If language fMRI shows a good signal and a clear lateralization, the Wada test can be regarded as dispensable. Limitations of fMRI are its use in children and patients with severe cognitive deficits. Besides the use in language dominance, other cognitive domains are being investigated with fMRI in an effort to predict (and thereby potentially improve) postsurgical neuropsychological outcomes. On a group level, it has been shown that postsurgical memory function can be predicted by presurgical memory fMRI. Again, the method first has to be prospectively evaluated in larger patient groups, to determine the value of the method for the evaluation of individual patients.

Pharmaco-fMRI

The application of pharmacological challenges in fMRI studies has been performed mainly in the domain of psychiatric diseases such as schizophrenia or major depression, with the aim to obtain surrogate markers of successful drug treatment. The use of this method as a prognostic tool for the evaluation of drug success for seizure control is rather unlikely. Pharmaco-fMRI has, however, been applied to study the patient's susceptibility to cognitive side-effects of antiepileptic drugs (AEDs). A recent study by Jansen and colleagues has investigated the cognitive effects of topiramate and found decreased prefrontal cortex activation in patients treated with topiramate in contrast to a patient group without this drug. This finding raises the possibility that fMRI might predict longstanding cognitive side-effects, which are otherwise difficult to monitor. The investigation of other drugs, with their special side-effect profiles (e.g., levetiracetam), is being performed in preliminary studies.

Combined fMRI-EEG

By overcoming several technical difficulties, the combined acquisition of fMRI and EEG data has shown very promising results in recent years. By the improvement of artifact-reduction algorithms, both on EEG as well as on fMRI time series, several epilepsy centers are integrating spike-triggered fMRI into the presurgical workup program of their patients. When interpreting these data, one must keep in mind the technical limitations and basis of the acquired BOLD signal used in fMRI. The signal does not originate from neural activity directly, but rather from differences in blood oxygenation (i.e., from the blood vessels). The coupling of these two signals – especially in pathological tissue – is still not completely understood, and is complicated by spatial incongruence of the signals. Larger studies are still needed, and because of safety and technical issues, combined invasive EEG-fMRI recordings, which would shed light on some of these issues, are not yet performed. Hence, the usefulness of combined fMRI-EEG remains to be evaluated further, before epilepsy-surgical recommendations can be drawn from the observed activations.

Outlook for the Future

The future of MRI in the domain of epilepsy is very promising. The continuing development in scanner hardware, MR sequences, and postprocessing tools will improve the presurgical evaluation of patients with symptomatic epilepsy even further. While some applications mentioned in this article are already close to routine clinical use, others still have to prove their utility in larger studies. Without doubt, the further development in this field will prove very valuable for the patients, with advances anticipated in improved surgical outcome to better counselling in regard to drug treatment.

See also: **Imaging**: Functional MRI in Basic Epilepsy Research; SPECT in Epilepsy Research; Structural Imaging in Epilepsy; Structure and Function: Imaging Connectivity Using Tractography.

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MRS in Epilepsy

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Introduction

Unlike conventional MRI studies, which detect the concentration of water and are sensitive to its cellular environment, MR spectroscopy provides specific biochemical information. For example, ^{1}H spectroscopic measurements have utilized decreases in *N*-acetyl aspartate (NAA), a compound synthesized only in neuronal mitochondria, to identify regions of neuronal loss and injury. Increases in total creatine (both phosphorylated and nonphosphorylated forms) and choline have been interpreted as reflecting gliosis and altered membrane turnover. In temporal lobe epilepsy, where neuronal loss and reactive gliosis are common findings, decreases in NAA and or increases in creatine and choline have been shown to have lateralization sensitivities as high as 97%. Using ^{31}P spectroscopy, in vivo concentrations of phosphocreatine, ATP, and inorganic phosphate can be imaged noninvasively along with intracellular pH to provide a measure of bio-energetic status. In this article, we review the role that MR spectroscopy has had as a clinical tool in identifying epileptic foci and monitoring the effects of various antiepileptic medications. We then discuss the biological significance of these findings, and particularly the role that bioenergetic impairment may play in the pathophysiology of seizure disorders. We also identify and discuss some of the technical hurdles that must be overcome in acquiring and accurately interpreting in vivo MR spectroscopy data.

Background

^{1}H NMR Spectroscopy and Spectroscopic Imaging

Nuclear magnetic resonance (NMR) spectroscopy allows the measurement of compounds with nonzero nuclear spin. A detailed description of the theory behind NMR is beyond the scope of this review. Briefly, in the presence of a magnetic field, these compounds absorb and emit radiofrequency signals. These signals have varying frequencies, depending upon the nucleus (^{1}H, ^{31}P, ^{13}C, etc.) and the type of chemical bonds present. NMR spectroscopy allows different chemical compounds to be identified by their unique fingerprint of frequencies. In the field of organic chemistry, NMR spectroscopy has been widely used to determine the presence, purity, and structure of the sample of interest. In biochemistry, the interaction of different resonances has been used to help identify the structure of different proteins. With the development of wide-bore magnets capable of accommodating human beings, NMR spectroscopy has been extended to studies of a variety of different organs including the brain. In the human brain the most widely investigated resonances include NAA (a compound synthesized only in neuronal mitochondria), creatine (both phosphorylated and nonphosphorylated compounds), and choline (elevated in high grade tumors and cells with increased membrane turnover) (**Figure 1**). Additional compounds of interest include lactate (a product of anaerobic glycolysis), the neurotransmitters glutamate and GABA. Unlike NMR spectroscopy studies of small aqueous samples as carried out by biochemists and chemists, in vivo measurements of the human brain require some form of spatial localization so as to link a metabolic profile with a specific anatomical structure. Depending upon the methods and hardware used, the spatial resolution of these measurements range from a few tens of cubic centimeters to a few hundred microliters. To achieve spatial localization of the signal, a variety of techniques based on the concepts used for MRI have been developed to select specific regions of the brain. More recently, spectroscopic imaging methods have been developed which allow spectra from

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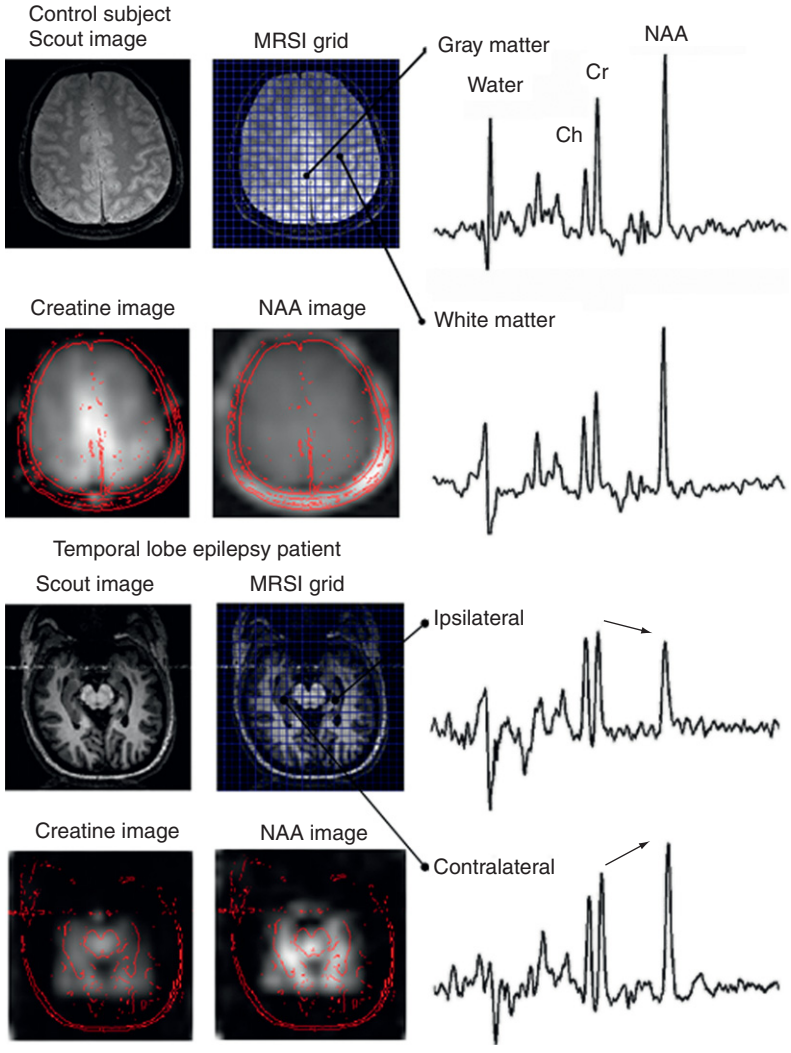


Figure 1 Displays a set of images including a scout image (upper left), a scout image with the sampling grid for the spectroscopic imaging data superimposed (upper right). This data was acquired using a whole plane acquisition with suppression of the lipid resonances provided by an inversion recovery sequence. Displayed to the left are two spectra from the spectroscopic image (gray matter upper, white matter lower). The labeled resonances are *N*-acetyl aspartate (NAA), creatine (Cr), choline (Ch) and the residual water signal (H_2O). Integration of the creatine and NAA resonances provides a metabolic image of creatine (lower left) and NAA (lower right). The increased creatine content from gray matter is easily visualized as increased intensity in the Cr image along the intrahemispheric gray matter. The bright ring surrounding the brain in the NAA image is due to residual lipid surrounding the brain from skeletal muscle and fat. Displayed below are the equivalent images from a patient with unilateral temporal lobe epilepsy. This data was acquired using two dimensions of in-plane localization, restricting the sampled data to a rectangle (80 mm $\times$ 100 mm) centered about the medial temporal lobe. The spectra shown to the left demonstrate a large decrease in NAA/Cr in the ipsilateral hippocampus in comparison to the contralateral hippocampus.

multiple locations to be acquired simultaneously (i.e., imaged) which allows the formation of metabolic images (Figure 1). These methods provide a highly efficient method for probing for focal changes in metabolism characteristic of pathology.

Methods

Although a large number of methods have been developed for acquiring ^{1}H spectroscopic imaging data from the human brain, all sequences carry the following common elements: (1) some form of water suppression; (2) some form of slice or slab selection to restrict the data collection in the cranial–caudal direction; (3) some form of lipid or in-plane selection to eliminate extracerebral lipids; and (4) two or three dimensions of spatial phase encoding to generate images. Due to the high concentration of water in the body (40–55 M, depending upon tissue type), some form of water suppression is required for adequate digitization and visualization of the resonances from ^{1}H metabolites (1–10 mM). Fortunately, the lipids signals intrinsic to brain tissue are relatively immobile, which results in extremely broad lines that are

typically not visible in ^{1}H spectra. However, extracerebral lipids, which are molar in content, must be suppressed. Due to large differences in their physical properties in comparison with most ^{1}H metabolite resonances, the extracerebral lipids can be suppressed by spin echo sequences and inversion recovery sequences. Additionally, based on their location outside of the brain, they can also be excluded when spatially selective pulses are used to restrict the acquired signal to being entirely within the brain (in-plane selection). Once a slice or slab of brain tissue has been selected for imaging, two (slice selection) or three dimensions (slab selection) of phase encoding are used to further subdivide the region into an array of pixels, each containing its own spectrum. Depending upon the size of coil used and field strength, typical volume sizes for individual pixels range from 0.25 to 1.0 cm^3 for the human brain. Examples of inversion recovery based lipid suppression for whole slice coverage and in-plane selection where only the medial temporal lobes were of interest are presented in **Figure 1**. These studies were acquired with 0.5–0.64 cm^3 resolution, with a volume head coil at 4T, and required 19 (0.64 cm^3) or 34 (0.5 cm^3) min.

In contrast, due to the lack of interfering unwanted resonances (i.e., lipids and water for ^{1}H measurements), ^{31}P data are typically acquired using three dimensions of phase encoding without any other form of spatial or spectra suppression. However, due to the low sensitivity of the ^{31}P nucleus, the spatial resolution is much lower than in ^{1}H measurements, with spatial resolutions of ~6 cm^3 for volume head coils (achievable for acquisitions of ~40 min at 4T). Finally, it should be noted that these resolutions are typical for human brain studies. In rodent models where the radiofrequency coils used for detection are much smaller (e.g., ~1–2 cm for rat brain), spatial resolutions of ~1–2 ul (^{1}H spectroscopic images) and 6–12 ul (^{31}P spectroscopic images) are readily achievable with acquisition times of ~40 min.

Results

^{1}H Spectroscopy and Temporal Lobe Epilepsy

In the mid 1990s, a number of investigators from different laboratories demonstrated the sensitivity of magnetic resonance spectroscopy (MRS) measurements of NAA to the lateralization of the epileptogenic region in patients with temporal lobe epilepsy. Specifically, changes in NAA content, and its ratio to other metabolites such as creatine, (NAA/Cr), choline (NAA/Ch), and the sum of creatine and choline (NAA/(Cr + Ch)) were used to lateralize the seizure focus in patients with intractable temporal lobe seizures (**Figure 1**). As a lateralization and localization tool, the reported sensitivities have ranged from 60% to 97%, somewhat higher than the 70–80% typically reported for conventional MRI studies. For example, one study reported that MR spectroscopic imaging, when combined with tissue segmentation, correctly lateralized the epileptogenic hippocampus in patients without MRI detectable volumetric loss. The ability of MR spectroscopy to attain higher sensitivities in comparison to standard imaging is most likely the result of the following factors: (1) that the metabolic perturbation seen in temporal lobe epilepsy is widespread, affecting not only the hippocampus (3–4 cm^3 in volume) but also surrounding temporal gray and white matter and (2) detectable decreases in hippocampal volume by conventional MRI may require neuronal losses of up to 50%. Thus, for temporal lobe epilepsy where the effects of the disease are macroscopic and submillimeter spatial resolution is not required, the biochemical specificity afforded by spectroscopy provides significant advantages over higher spatial resolution measurements of tissue water (as obtained with MRI).

In addition to providing a highly sensitive method for lateralizing the seizure focus, spectroscopic imaging studies may also provide some predictive insight into surgical outcome. Notably, among the patients classified as having unilateral temporal lobe epilepsy based on EEG measurements, some 40–50% have significant decreases in NAA in the contralateral hemisphere. These data indicate that although the seizures may emanate from one hippocampus, the effects are clearly manifesting themselves in the contralateral hemisphere. One study has correlated the severity of NAA/Cr alterations in the ipsilateral and contralateral hippocampi with surgical outcome; although the severity of NAA/Cr abnormality in the ipsilateral hippocampus did not appear to have a significant impact on surgical outcome, more severe alterations in the contralateral hippocampus were associated with poorer surgical outcomes (i.e., continuing seizures). Similarly, investigators have found that in MRI-negative patients, the presence of more severe reductions in NAA in the contralateral hippocampus also correlated with poorer outcomes following surgery. Further, in patients with bilateral disease, poorer surgical outcomes were associated with the presence of more severe reductions in the contralateral hippocampus. Thus, the presence of MRS defects in the contralateral lobe (in the presence or absence of MRI defects) is predictive of poorer surgical outcomes.

The significant decreases in NAA in patients with temporal lobe epilepsy is not restricted to the temporal lobe; reductions in NAA have also been reported in the frontal and parietal lobes. Further, using FDG PET, a number of investigators have reported that the thalamus is also affected in temporal lobe epilepsy. This latter work suggested the possibility of a network of metabolic defects involving the thalamus. To evaluate the relationship between various components of the macroscopic network,

we carried out an MRS study in which individual loci within the hippocampus, thalamus, and basal ganglia were averaged; a correlation analysis was then performed to evaluate the relationships between the ipsilateral and contralateral hippocampi with the other structures. This analysis identified highly significant correlations in decrements in NAA/Cr between the ipsilateral hippocampus (i.e., the side from which seizures arose) and the bilateral thalami and basal ganglia. An exceptionally strong correlation was detected between the ipsilateral hippocampus and the anterior thalamus ($R = +0.76$, $p < 1 \times 10^{-5}$). The contralateral hippocampus showed generally weaker correlations, and primarily with structures ipsilateral to the epileptic focus (hippocampus, anterior thalamus, and putamen ($p < 10^{-3}$–0.04). In contrast to the very strong correlations between the hippocampi, thalami and putamen in patients, no significant correlations were observed in controls between the hippocampi and thalamus within the same hemisphere or across hemispheres. Thus, it is clear that decreases in NAA not only reflect neuronal injury and the processes that give rise to epileptogenic foci, but also reflect injury/dysfunction in regions which are involved in the spread and or propagation of seizures.

NAA and Neocortical Epilepsy

The finding of decreased NAA is not solely restricted to temporal lobe epilepsy. Reduced NAA levels have been reported in a variety of other forms of epilepsy, including frontal lobe epilepsy and malformations of cortical development. Unlike temporal lobe epilepsy where the seizure focus is relatively well defined, in patients with neocortical epilepsy, the epileptogenic regions are often poorly defined anatomically, and there may be no accompanying MRI abnormality. This situation poses significant additional demands for MRS studies with regard to data acquisition and analysis. In addition to evaluating the observed ratios of NAA, Cr, and Ch in hundreds, if not thousands, of locations, such studies must also consider natural tissue-specific alterations. For example, as seen in Figure 1, white matter concentrations of Cr have been reported to be ~6 mM, while gray matter values are ~8–9 mM – and variations in the content of these same compounds have been reported between different lobes. Thus, depending upon the location of the region of interest and its mixture of gray and white matter, the 'normal' levels of metabolites will vary, complicating detection of 'abnormal' regions.

To overcome the effects of tissue heterogeneity, some investigators have employed linear regression analyses to compensate for the inclusion of varying levels of gray and white matter. Although these methods are most commonly used in global pathologies to identify gray or white matter specific alterations in comparison with control subjects, they can also be employed on a voxel by voxel basis within subjects. Using segmented anatomical images, the gray and white matter content of each spectroscopic imaging voxel can be determined. From these data, a regression analysis can be performed in healthy volunteers to obtain the 'pure' gray and white matter metabolite contents and ratios (Figure 2). With these population statistics, one can calculate the probability that any voxel of specific gray and white matter content in a patient is abnormal. Voxels that are statistically abnormal ($p < 0.05$) can then be color-coded according to p-value and superimposed on the anatomical images for easy evaluation and interpretation (Figure 2). When these regression based statistical methods are applied in patients with temporal lobe epilepsy, false negatives and incorrect lateralizations are reduced in comparison with simple left right voxel comparisons.

Interpretation of Decreased NAA levels

Despite the obvious linkage between neuronal loss in epilepsy and decreases in NAA, there have been few correlative in vivo studies. In epilepsy patients with malformations of cortical development, significant reductions were also observed in NAA/Cr in the epileptogenic region – but in the absence of neuronal loss. Resected tissue from these patients showed normal or elevated numbers of neurons, although morphologically the neurons were abnormal. Subsequently, Kuzniecky and colleagues demonstrated that NAA losses in patients with temporal lobe epilepsy were also not significantly correlated with neuronal densities. To further probe the histological basis for the alterations in NAA, we correlated in vivo measurements of NAA from the hippocampi of patients with intractable TLE with quantitative measurement of neuronal loss and reactive astrocyte proliferation in the resected tissue. Regression analyses of NAA/Cr measured in vivo with quantitative neuronal counting across CA1–CA4 and the dentate gyrus, revealed statistically significant correlations only in the CA2 sector. Interestingly, GFAP staining was also correlated with neuronal number only in the CA2 sector of the hippocampus. However, statistically significant correlations were found between NAA/Cr and GFAP staining across all four hippocampal sectors, suggesting that reductions in NAA/Cr are more closely correlated with the presence of reactive astrocytes than alterations in neuronal density in TLE. The presence of reactive astrocytes is largely interpreted as a marker for recent or ongoing neuronal injury. Interestingly, neuronal loss in the CA1, CA3, and dentate gyrus is typically believed to be largely due to the initial precipitating insult in temporal lobe epilepsy, while neuronal loss in the CA2 is more generally believed to correlate with the progression of the disease.

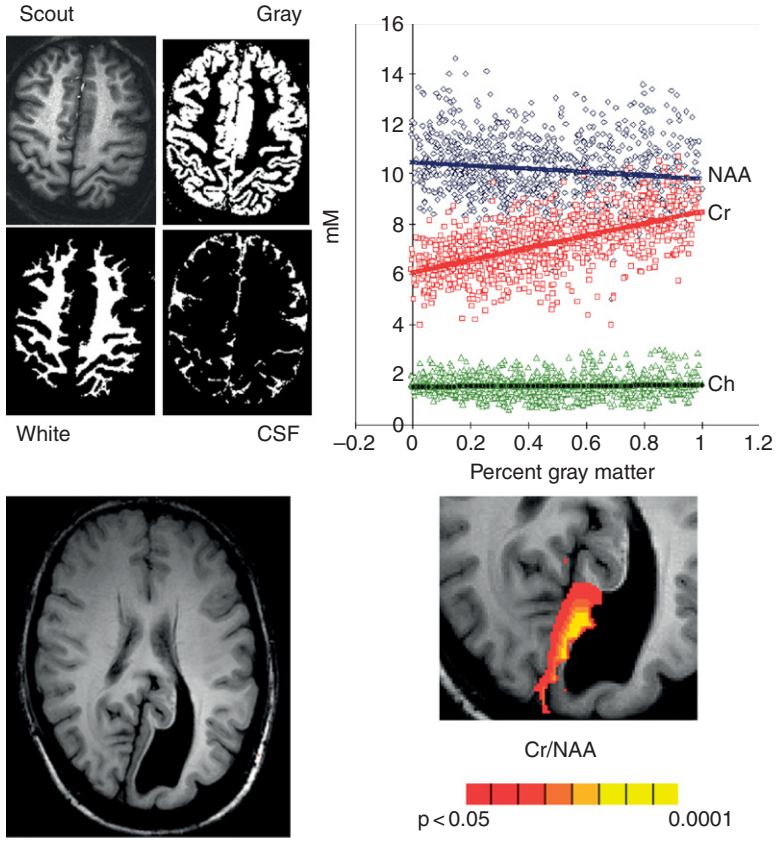


Figure 2 Displays a scout image and tissue segmented images of gray matter, white matter and CSF (upper left). Using the tissue composition data the concentrations of *N*-acetyl aspartate (NAA), creatine (Cr) and choline (Ch) for 10 volunteers (96 locations each) are plotted as a function of fraction gray matter. Displayed in the lower panel (left) is the scout image of an epilepsy patient with a cortical malformation. Using the regression data displayed in the upper right panel, the pixels from this patient which were metabolically abnormal ($p < 0.05$) based on an increased ratio of Cr/NAA (equivalent to a decrease in NAA and or increase in Cr) were color-coded according to significance and overlaid on the anatomical image. As can be seen, there is an excellent correspondence between the anatomical location of the cortical malformation and the metabolically abnormal region.

Given the lack of correlation between NAA levels and degree of neuronal loss in temporal lobe epilepsy and malformations of cortical development, the biochemical changes underlying the reductions in NAA are of significant interest. NAA has been reported to act as a source of acetate transfer from the mitochondria to the cytoplasm for lipid synthesis. Additionally, NAA has been proposed to act as a transporter of amino nitrogen from the mitochondria to the cytoplasm. NAA has also been proposed to act as an osmotic agent, based on its redistribution between extracellular/intracellular compartments in response to osmotic stress. Investigations of agents that perturb mitochondrial function have been demonstrated to affect NAA levels and production rates, and investigators have shown that with application of mitochondrial poisons (in isolated rat brain mitochondria) there is a significant decrease in ATP production, oxygen consumption, and NAA production. Such in vitro findings, identifying a linkage between NAA and bioenergetics, have also been found support in vivo studies. In a study of healthy control subjects, there was a highly significant correlation between hippocampal NAA levels and ADP. This relationship displayed a positive slope (with higher NAA levels associated with higher ADP levels), suggesting that NAA levels are responsive to demand for energy as reflected by increased ADP. This result is consistent with the finding that in epilepsy patients, who are well controlled (<4 seizures year), with cortical malformations of development, NAA levels are not altered despite the presence of structural abnormalities. Thus, in epilepsy patients with intractable seizures, the decreased NAA levels may reflect an inability to respond to energetic demand due to mitochondrial injury. This interpretation of NAA decreases would suggest that bio-energetic deficits (decreased PCr/ATP) should also be present in the hippocampi of patients with temporal lobe epilepsy – and that upon successful treatment, the decrement in NAA from affected loci should normalize.

This reversibility of the NAA changes in temporal lobe epilepsy has indeed been reported; NAA recovered in the

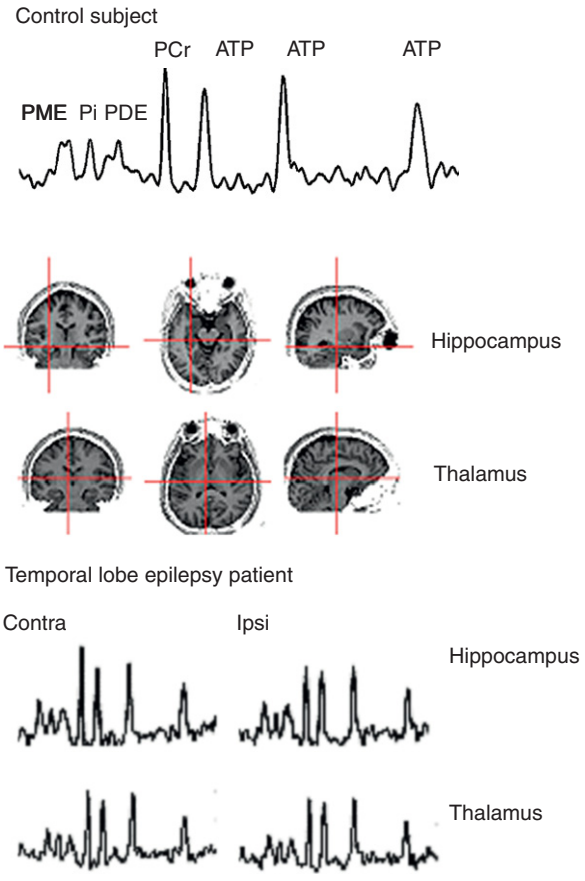


Figure 3 Displays a ^{31}P spectrum ~6 cm^3, acquired at 4 T. The labeled resonances from left to right are: phosphomonoesters (PME), inorganic phosphate (Pi), phosphodiesters (PDE), phosphocreatine (PCr), and ATP (γ, α, β). Displayed in the middle panel are anatomical scout images showing the locations of the ^{31}P spectra acquired from the hippocampus and thalamus. In the lower panel are ^{31}P spectra from the contralateral and ipsilateral hippocampus and thalamus from a patient with temporal lobe epilepsy. Note the reduction in PCr relative to ATP in the ipsilateral lobe in comparison to the contralateral lobe.

contralateral hippocampus in patients with bilateral abnormalities 1 year after successful surgery. In a follow up study, Arnold and colleagues found that, on average, 50% of the metabolic recovery occurred over a period of less than 6 months, demonstrating a slow but responsive nature of neuronal NAA levels. The reversibility of decreased NAA in the contralateral hippocampus with successful surgical outcome (i.e., cessation of seizures) suggests that the recovering NAA levels reflect a 'healing' or 'normalization' of cellular process in the contralateral hippocampus. The fact that NAA normalization is associated with 'normalization' of neuronal function suggests that the processes associated with NAA decrease directly reflect the dysfunction seen in epilepsy. However, it is not clear if the impairment in bioenergetics, as reflected by decreased NAA, is merely a secondary effect of the presence (reduced NAA) or absence (normalization of NAA) of seizures.

^{31}P Spectroscopic Measurements of Bioenergetics in Human Epilepsy

Prior to the widespread availability of ^{1}H spectroscopy on clinical MR systems, Weiner and colleagues evaluated the use of ^{31}P spectroscopy (for the lateralization of epileptogenic regions). In these initial studies at 2.0 T, significant increases in inorganic phosphate were reported in the temporal lobe ipsilateral to the focus, in comparison with the contralateral temporal lobe, in patients with temporal lobe epilepsy. Subsequent studies at 1.5 T showed that PCr/Pi was reduced by 50% in the ipsilateral (to the focus) temporal lobe and by 24% in the contralateral lobe in comparison with healthy controls – a result generally confirmed by a 4.1 T study (decreased ipsilaterally (32%) and contralaterally (19%)). Within this group, 73% of the patients were correctly lateralized using the PCr/Pi ratio. Similar to results with ^{1}H MRS, these effects were also seen throughout the epileptic network, including the thalamus (Figure 3). As patients with recent seizures (<24 h previous to the study) were excluded, these effects are believed to reflect a chronic impairment of energy metabolism (not an acute effect of seizures), consistent with a mitochondria-based deficit leading to NAA reductions.

Declines in bioenergetics are also seen in a variety of other forms of epilepsy, including children with Lenox–Gasteau syndrome and absence seizures, and in adults with frontal lobe epilepsy. In children with Lenox–Gasteau and absence seizures, a nonfocal form of epilepsy, PCr/ATP was decreased in the cortical gray matter in comparison with control subjects. Notably, no such change was seen in white matter, reflecting a specificity for neuronal cell bodies/dendrites and their high energetic demands. In patients with frontal lobe epilepsy, investigators reported elevations of inorganic phosphate, consistent with a decrease in overall energy charge. Thus, the finding of bioenergetic impairment is not restricted solely to temporal lobe epilepsy, but may reflect a basic mechanism underlying many forms of epilepsy.

Factors Affecting the Interpretation of ^{31}P Data

Although ^{31}P studies have shown that abnormalities in bioenergetics are present, their use to date has been limited. Limited use of ^{31}P spectroscopy can be attributed, in part, to the large range of normal values for cerebral PCr (3–5 mM) and ATP (2–4 mM) in low field studies, and the resulting perception that ^{31}P spectroscopy is highly inaccurate. This view was due to the fact that initial reports used large volumes (25–100 cm^3) and did not attempt to account for tissue or regional heterogeneity. Although spatial resolution for ^{31}P studies is limited by the signal to noise ratio, recent studies at high field strength have identified clear regional and tissue specific heterogeneities in ^{31}P metabolites using regression

analyses. Depending upon the content of gray matter in the tissue, the ratio of PCr/ATP in cerebral structures varied by ~40% Thus, failure to account for these differences can result in substantial variability that is not reflective of underlying pathology, but rather normal heterogeneity based on tissue composition.

Interpretation of Alterations in ^{31}P Metabolism in Epilepsy

As seen in the ^{1}H studies, ^{31}P spectroscopic imaging suggests that the energetic deficits seen in TLE patients are reversible with successful surgery. In pediatric epilepsy patients being treated with the ketogenic diet, bioenergetic impairments present prior to initiation of the diet were reversed as the seizures were brought under control. Specifically, after 4 months of the ketogenic diet therapy, the PCr/Pi and PCr/ATP ratios had improved by 22% and 14% respectively. Thus, both reductions in NAA and impairments in bioenergetics appear to be tightly linked with seizure activity and can be reversed with successful treatment. This finding also indicates that the alterations in ^{31}P metabolites are not solely due to neuronal death, but reflect a dynamic and reversible process. Although the exact mechanism for the ketogenic diet remains controversial, it is known that ketones can supplement or replace glucose as a fuel source for oxidation, thus potentially modulating bioenergetics. While it appears clear that there is a significant bioenergetic component to the etiology of seizure disorders, it remains unclear as to whether this association reflects general damage associated with seizures or is a causative factor.

Glutamate and GABA: Intracellular Alterations in Neurotransmitters

As described, most measurements using MR spectroscopy and spectroscopic imaging have focused on measurements of NAA, creatine, choline, and to a lesser extent, ^{31}P metabolites. Together, these measurements have suggested a strong role for metabolism and bioenergetics in the pathophysiology of epilepsy. However, these measurements do not directly assess the neurotransmitter changes which occur in epilepsy. Both glutamate and GABA can be measured in vivo, using ^{1}H MRS and other specialized methods.

Glutamate is the primary excitatory neurotransmitter in human brain. The finding of elevated extracellular glutamate and decreased rates of glutamate uptake in epileptogenic hippocampi have sparked substantial interest in measuring in vivo glutamate levels in patients with epilepsy. However, unlike the main resonances of NAA, Cr, and choline, which are singlet resonances, glutamate's primary resonances are triplets or higher order multiplets (**Figure 4**). This feature substantially reduces their detection, because the signal intensity is distributed over a broader range of frequencies. Further, unlike NAA and other singlet resonances, the appearance of these multiplets is strongly dependent upon the exact timings of the acquisition method used. As such, specialized sequences and timings are required for their detection, making their measurement more demanding. Due to spectral overlap with glutamine, low field measurements (1.5 T) are exceptionally difficult to interpret. However, with increasing field strength (3 T, 4 T and most recently 7 T), these hurdles can be overcome.

Although much less common than measurements of NAA, a number of investigators have evaluated glutamate content in patients with temporal lobe epilepsy. For example, one study reported that the combined resonance of glutamate and glutamine (the signals were not resolvable at 1.5 T) was decreased in EEG-defined neocortical epileptogenic regions (in comparison with the corresponding contralateral location). Others have reported that the combined resonance of glutamate and glutamine was alternately elevated in ipsilateral or contralateral hippocampus of MRI negative TLE patients. Using 4 T (where the resonances of glutamate and glutamine can be better resolved), Pan and colleagues evaluated a group of temporal lobe epilepsy patients, and showed that ipsilateral glutamate and NAA were significantly decreased (5.1 ± 1.2 and 8.0 ± 1.1 mM) in comparison with controls (6.7 ± 1.5 and 9.5 ± 1.0 mM), respectively, yielding declines of ~25% in glutamate and ~16% in NAA (**Figure 4**). Notably, the MRS measurement of glutamate reflects total tissue glutamate levels including both intracellular levels of glutamate (5–10 mM) and extracellular levels (1–10 μM). Thus, even moderate losses in cytosolic glutamate, or of glutamatergic neurons, can dominate significant elevations in the extracellular concentration of glutamate (as reported from microdialysis studies). As such, the reductions in total glutamate level reported by Pan are consistent with glutamatergic neuron loss. The greater fractional decline in glutamate, as opposed to NAA, suggests that measurements of glutamate may be more sensitive than measurements of NAA in identifying epileptogenic regions. However, this advantage is likely to be realized only at very high field strengths (7 T) where SNR and spectral resolution are less limiting.

As the primary inhibitory neurotransmitter for mammalian brain, GABA and its receptor systems have been an area of intense study. Due to its low concentration (~1 mM) and spectral overlap with glutamate, glutamine, and creatine, the measurement of GABA requires the use of specialized methods, commonly referred to as 'spectral editing.' These methods typically utilize the unique frequencies of the ^{1}H resonances of GABA to selectively perturb the GABA resonance in alternating scans, and through that interaction modulate the appearance of the

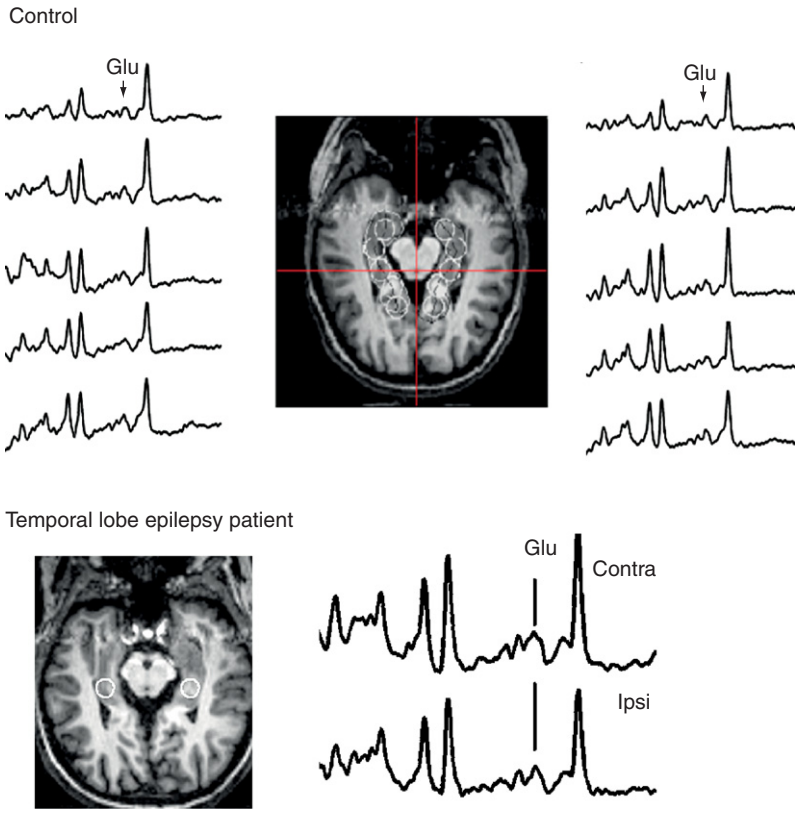


Figure 4 The upper panel displays ^{1}H spectra of glutamate (glu) from the right and left hippocampus of a control subject. The locations of the spectra are identified with white circles on the anatomical image. Displayed in the lower panel are data acquired from a patient with temporal lobe epilepsy. Note that that the glutamate (glu) and NAA resonances are significantly decreased in the ipsilateral hippocampus in comparison to the contralateral hippocampus.

adjacent ^{1}H resonance on the GABA molecule. As other resonances are not affected by the selective perturbation, subtraction of the two measurements results in an 'edited' spectrum, displaying the GABA resonance free of spectral overlap (**Figure 5**).

Given the difficulties of low SNR, the majority of MR studies have evaluated the occipital lobe, where small highly sensitive surface coils can be placed in close proximity to the tissue. For example, one study examined the resting levels of occipital GABA in patients with complex partial seizures (CPS) and juvenile myoclonic epilepsy (JME). In the CPS group, low GABA was associated with poor seizure control, while JME patients showed low GABA independent of seizure control. These findings of reduced in vivo GABA are similar to those reported by Petroff in a variety of other forms of epilepsy. Notably, the occipital lobe represents a site distant from the presumed epileptogenic region. To evaluate the role of GABA in the seizure focus, another study acquired data from patients with occipital lobe seizures, but failed to identify a decrease in GABA. This result may be due to the fact that the study used a volume coil (lower sensitivity) and the test–retest accuracy for GABA in controls was only 38%. Thus, whether GABA is increased or decreased in the epileptogenic region remains and open issue.

Despite this uncertainty of the status of intracellular GABA levels in the seizure focus, in vivo measurements of GABA have become highly useful in assessing the response of various antieplipetics. For example, Petroff and colleagues established an in vivo dose–response curve for vigabatrin, an inhibitor of GABA transaminase. Dose escalation revealed increases in cerebral GABA as a function of vigabatrin level up to 3 g day^{-1}. Above 3 g day^{-1}, brain GABA levels were constant (did not rise further), indicating a maximal effect on brain GABA. Although the mechanism for the elevation of GABA in response to vigabatrin is clear, other common antiepileptic drugs (AEDs) have also been shown to result in elevation of GABA. For example, Kuzniecky and colleagues demonstrated that topiramate, gabapentin, and lamotrigine can all raise brain GABA levels in control subjects under acute (topiramate and gabapentin) and chronic (lamotrigine) conditions. Conversely, other AEDs such as levetiracetam have been shown to have no effect on brain GABA. One limitation of this work is that due to the low sensitivity of the GABA measurements (arising from its low concentration in human brain – about 1 mM), the majority of studies of brain GABA have been performed in the occipital lobe, where the measurements reflect a global response of the brain to seizures and pharmaceutical interventions.

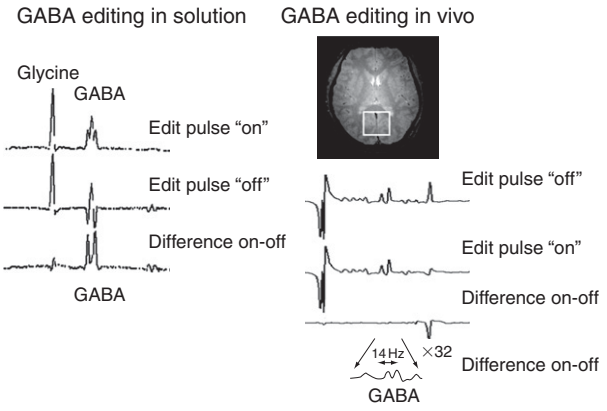


Figure 5 Displays spectral editing data acquired from a solution of GABA and glycine (left panel) and from the occipital lobe of a healthy control subject (right panel). In the solution (left panel), the triplet resonance of GABA is upright with the editing pulse 'on' while the outer lines invert when the editing pulse is 'off.' Differencing the two spectra results in: (a) elimination of the glycine resonance (not affected by the editing pulse); and (b) preservation of the outer two lines of the GABA triplet which are separated by ~14 Hz. In the panel on the right, the GABA resonance is overlapped by the creatine resonance and only visible after editing (difference on-off). Note the elimination of the creatine and choline resonances in the difference spectrum. Displayed at the bottom of the panel is an expanded region about the creatine resonance where the vertical scale has been increased by a factor of 32 to allow the two outer lines of the GABA resonance (separated by 14 Hz) to be visualized.

Finally it is important to note that the findings of increased GABA in response to gabapentin and topiramate were contrary to those described in the rodent literature. In subsequent studies, Petroff and colleagues demonstrated that the differential effect of gabapentin was due to a species difference. Specifically, gabapentin had no effect on GABA levels in the rat brain slices, but significantly increased GABA levels in the slices from human brain. Similarly, no effects of gabapentin on GABA levels were seen in the in vivo rat brain as assessed by tissue extracts. Thus, while investigating the effects of various drugs or interventions with the potential to affect the GABAergic system, it is important to take into account the species difference between humans and rodents.

Directions for Future Research

Despite the ability of MR spectroscopic imaging to noninvasively localize epileptogenic regions, determine the status of bioenergetics, and monitor change in brain GABA levels, its use remains relatively limited to research studies. This fact is primarily due to limitations in the signal to noise ratio and spectral resolution achievable in conventional (1.5 T) clinical studies. Over the past several years, new ultra-high field (7 T) MR imaging systems have become available from major vendors of clinical MRI systems. In addition to dramatic improvements in sensitivity, more than fivefold greater than standard 1.5 T clinical systems, the systems also offer dramatic improvements in spectral resolution, substantially enhancing the accuracy of MRS measurements of GABA and glutamate. With these newer systems becoming more common, the use of MRS in the localization of epileptogenic regions in neocortical epilepsy and in monitoring the response to therapy should be significantly enhanced. However, at 7 T, interactions between the radiofrequency pulses used to generate the data and the tissue become complex. These interactions result in complex spatial inhomogeneities in detector sensitivity and significant increases in tissue heating for conventional head coil designs and pulse sequences. Such effects are sufficiently severe that standard MRS spatial localization methods used at 1.5 T can easily exceed FDA guidelines for power deposition when applied at 7 T. Thus, to achieve the substantial benefits of this new generation of MR systems for spectroscopic imaging studies, new methods which incorporate novel detector designs and pulse sequences need to be developed.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Imaging**: Physiologic Correlates of Metabolic Imaging in Human Epilepsy; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy.

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PET Neurotransmitter Receptor Imaging in Epilepsy

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Introduction

Positron emission tomography (PET) is a technique that offers the opportunity to build a bridge from epilepsy investigations in cellular and animal models to clinical research. Hypotheses developed in experimental animals can be tested in man using radiolabelled neurotransmitter receptor ligands as PET tracers. Questions about the mechanisms of action of antiepileptic drugs can be addressed. Investigation of depression in people with epilepsy is an excellent example of the value of PET. Depression is one of the most common co-morbidities of the disorder, occurring in more than 30% of patients in some studies, and contributing to reduced quality of life. A wide range of etiological factors could lead to depression. Social and psychological mechanisms include stigma, inability of work with reduced income, and social isolation. Some studies have suggested that patients with temporal lobe epilepsy, or a left temporal focus, or mesial temporal sclerosis, may be more likely to be depressed. PET receptor imaging offers an opportunity to assess the role of neurotransmitter systems that have been implicated in the etiology of major depressive disorders in preclinical investigations.

Background/Methodologic Issues

Several methodological issues affect PET investigations. PET depends on radiochemistry, and successful ligand development can take years of work. For interpretation of neurotransmitter receptor ligand imaging, a mathematical model is needed that relates injected tracer to radioactive decay counts (measured by the scanner), and takes into account tracer protein binding and metabolites. The 'input function' can be derived from arterial blood samples, or from radioactivity in a structure thought to have no specific binding (and thus to represent activity from blood flow alone). Although fluorodeoxyglucose PET has become a routine tool, for cardiac and oncologic as well as neurologic applications, the visual interpretation often used in these studies never is appropriate for receptor binding investigations (and may be misleading in clinical contexts).

^{18}F or ^{11}C-labeled neuroreceptor ligands or precursors are important for epilepsy. The half-life of ^{11}C is 20 min, so an on-site cyclotron is needed to produce it; in contrast, ^{18}F (half-life 110 min) can be brought in from outside the scanning facility. Current PET scanners produce sets of 30–50 images that can be reconstructed with true 3-D resolution. The spatial resolution of the newest PET scanners is 2–3 mm.

Given the limitations of human radiation exposure, as well as the difficulty and expense of performing multiple studies, it generally is not possible to obtain the multiple data points needed to distinguish between changes in receptor number or dissociation constant, either of which could explain a change in binding potential. However, the former is much more likely on theoretical grounds.

In patients with epilepsy, and particularly those with focal lesions such as mesial temporal sclerosis or malformation of cortical development, alterations in tissue structure (including gray-white ratio due to volume loss) may affect neurotransmitter ligand binding. MRI-based partial volume correction programs should be used to correct for this effect.

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Once the data are obtained, a number of statistical approaches (such as the widely used program 'statistical parametric mapping (SPM),' developed by the Wellcome Institute) can be used to compare patient results with control databases. Each of the available statistical methods has underlying assumptions, advantages and disadvantages that are important to keep in mind.

In standard region of interest (ROI) analysis, anatomic regions are drawn, preferably on co-registered MRI images, and compared with contralateral or control regions. Here, there usually is an a priori hypothesis to be tested. Increasing the number of regions necessitates correction for multiple comparisons. This approach has important advantages when patients with focal dysfunction or lesions are being studied.

SPM uses spatial normalization of individual images to a standard spatial template, which facilitates group comparisons, but may mask some inter-patient differences. It is, however, observer independent, and avoids ROI drawing errors, which can be particularly serious for small structures or regions with indistinct borders. Correction for multiple comparisons is built into the program. Since all brain regions are compared, unexpected findings may emerge.

Single photon emission tomography (SPECT) also can be used for receptor imaging. Compared with PET, spatial resolution is lower, and quantitation is much more difficult, reducing SPECT's value for research.

Recent Results of Potential Clinical and Physiologic Importance

One of the recurring themes of PET receptor imaging in epilepsy has been transient enthusiasm for a receptor system, often due to availability of a particular ligand, followed by sudden abandonment of the research direction. Studies of opiate receptors in patients with temporal lobe epilepsy are a good example. Several investigators used a reference region method (with occipital cortex as a measure of non-specific binding) to show that μ opiate-receptors were increased in inferior temporal lateral neocortex ipsilateral to epileptic foci, and decreased in amygdala, compared with the contralateral structures. δ receptors were increased in more widespread ipsilateral temporal regions and κ receptors reduced. An interesting study using arterial blood sampling to obtain voxel-based parametric images showed reduced ^{11}C-diprenorphine (a ligand with little receptor subtype specificity) binding during reading-induced seizures, suggesting ictal opiate release in left parieto-temporo-occipital cortex, possibly associated with seizure termination.

However, theses studies have not been pursued in recent work. One important reason was that their clinical relevance and utility remained unclear. Instead, focus has shifted to other receptor systems, including benzodiazepines, and dopaminergic and serotoninergic ligands. These studies will be discussed below to illustrate potential implications of the results and approaches to interpretation.

[^{11}C]flumazenil (FMZ) PET benzodiazepine receptor binding has been the focus of greatest interest; since loss of receptors is presumed to be associated with increased cortical excitability. Binding is reduced ipsilateral to temporal and frontal lobe epileptic foci. In patients with hippocampal sclerosis, reduction often is confined to mesial temporal regions, even when hypometabolism on FDG-PET scans is widespread. Ocassionally, reduced benzodiazepine receptor binding can be detected in patients with intractable localization-related epilepsy even when MRI and FDG-PET are normal. In patients with cortical dysplasia, benzodiazepine receptor binding may correlate well with the ictal onset zone (determined by invasive electrode recording within the dysplastic region). In some studies, white matter binding increases for flumazenil have been reported, possibly due to the presence of heterotopic gray matter.

Receptor binding could be affected by various physiologic processes. In one study, patients with drug-resistant epilepsy underwent two FMZ-PET scans at a 1 week interval, in order to investigate seizure-related short-term receptor plasticity. Five of ten patients, but no controls, had variability in the binding ratio between mesial temporal structures ipsilateral and contralateral to the epileptic focus. For two patients with normal structural MRI, significant binding reduction ipsilateral to the focus was found in only one of the two PET scans. In a third patient, reversal of the asymmetry occurred, and one of the scans was falsely lateralizing. A shorter interval between the last seizure and the FMZ PET scan was associated with closer agreement between imaging and EEG seizure focus localization. The shorter the time since the last seizure, the lower the benzodiazepine receptor binding in the epileptogenic region. This study used a partial saturation approach with combined injection of labeled and cold FMZ, allowing calculation of parametric binding images without arterial blood sampling. Several issues may affect interpretation of the results. For example, interictal discharges, which were not analyzed in this study, could have led to fluctuations in receptor availability and FMZ binding. In addition, the PET scan with the shorter interval since the ictal event would also have had a shorter interval since the pre-ictal events that preceded that seizure. Thus, it is uncertain whether the decrease in receptor availability is attributable to ictal or pre-ictal events. Recent EEG studies show that increased subclinical epileptiform discharges may precede clinical seizures by hours or even days, suggesting a potential correlate of pre-ictal alterations in

GABA-benzodiazepine receptor binding. The FMZ results parallel earlier results with fluorodeoxyglucose, showing metabolic variability in the peri-ictal period. However, these results suggest that PET studies cannot be assumed to reflect steady-state conditions in people with epilepsy, and that careful clinical observation is crucial for their interpretation.

Antiepileptic drugs (AEDs) might also affect receptor binding, especially when the AED is thought to have a mechanism of action directly related to the system in question. Vigabatrin (VGB) is an antiepileptic drug that acts by elevating brain gamma-aminobutyric acid (GABA) levels, and thus might affect ^{11}C-FMZ binding at the GABA-benzodiazepine receptor complex. FMZ binding was significantly lower in children with intractable epilepsy who had been taking VGB for prolonged periods than those on other AEDS. VGB increases synaptic GABA availability, and long-term exposure to supra-physiologic levels might have led to decreased receptor availability, or possibly changes in maturational patterns, and altered FMZ binding. The investigators suggested that their data showed how PET could be used to pose questions about the long-term developmental effects of AEDs.

Almost all epilepsy receptor studies have been performed in patients with long-term uncontrolled seizures. One study used the parallel SPECT benzodiazepine receptor ligand ^{123}I-iomazenil tracer in patients with new onset temporal lobe epilepsy (median duration seven months) and normal MRI. Nineteen of 40 patients (including some with infrequent seizures) had reduced tracer activity in mesial temporal regions. Since these patients had only had seizures for a short time, and MRI was normal, it is possible that the reduced BZP receptor binding found here preceded seizure onset, and was not a consequence of persistent epilepsy. PET studies of glucose metabolism in children with new-onset seizures have shown that the presence of focal hypometabolism at initial evaluation predicts development of intractable epilepsy.

Recent studies have shown reduced basal ganglia ^{18}F-fluoro-L-DOPA binding in patients with several epilepsy syndromes, suggesting that these regions may be involved in secondary seizure spread. Occipital cortex activity was used as the input function to calculate relative striatal isotope uptake using multiple-time graphical analysis. Patients with three epilepsy syndromes (ring chromsome 20 epilepsy, drug-resistant generalized 'absence-like' epilepsy, and drug-resistant temporal lobe epilepsy with hippocampal sclerosis) were studied. Comparing all epilepsy patients to normal controls, binding decreases were found bilaterally in caudate and putamen. Reduced substantia nigra tracer uptake was found only on analysis with SPM, but not with a region of interest approach. SPM, but not ROI analysis, also detected reduced binding ipsilateral to hippocampal sclerosis in the TLE patients. The difference in results using alternative analytic approaches in these studies illustrates the problems inherent in the small sample sizes typical for receptor PET investigations. Other potential confounders include the effects of antiepileptic drugs and structural atrophy. ^{18}F-fluoro-L-DOPA is a relatively non-specific ligand, and cannot discriminate among alterations in receptor subtype binding; an important change in one subtype but not others might be difficult to detect. Moreover, as with other receptors, it may be hard to determine whether reduced dopaminergic receptor binding is a cause or consequence of epilepsy.

Several studies by a number of research groups have shown reduction of 5HT1A receptor binding in patients with TLE. Regions affected include hippocampus, amygdala, parahippocampal gyrus, entorhinal cortex, and varying neocortical regions (both ipsilateral to the epileptic foci, as well as sometimes contralaterally). In addition, the midbrain raphe, where serotoninergic fibers projecting to cortex originate, show reduced presynaptic autoreceptor binding. Similar results have been found with three different ligands ([Carbonyl-^{11}C]WAY-100635, ([^{18}F]FCWAY, and ^{18}F-MPPF), and using region of interest as well as SPM analysis. Each of the three ligands has advantages and disadvantages. The long half-life of ^{18}F allows longer imaging time and higher decay counts. WAY-100635 has higher specific binding than MPPF, but FCWAY has a fluoride metabolite that has to be measured and corrected for in image analysis. The magnitude of reduction appears greatest in the zone of seizure onset, usually in hippocampus, with receding effects in zones of seizure spread or regions where only interictal spikes are seen. After MRI-based partial volume correction, only about 20% of the reduced binding could be attributed to volume loss (**Figure 1**). Moreover, reduced binding occurs in patients with mesial temporal lobe foci but normal MRI. It is interesting that patients with juvenile myoclonic epilepsy, a generalized rather than localization-related epilepsy syndrome, showed reduced 5HT1A receptor binding in the dorsolateral prefrontal cortex, raphe nuclei, and hippocampus.

PET studies using the serotonin precursor, alpha methyl tryptophan (AMT) labeled with ^{11}C, using ROI-based asymmetry indices, showed increased uptake in epileptogenic tubers in patients with tuberous sclerosis, as well as in malformations of cortical development in children. In patients with partial epilepsy, AMT uptake was increased in patients with normal MRI (including several with negative FDG-PET results) but not those with hippocampal volume loss; partial volume correction was not performed in these studies.

These PET findings are consistent with the antiseizure effects of 5HT1A receptor activation in experimental models. Although it is uncertain if reduced binding occurs at the onset of epilepsy, or is related to progression of the epileptic process, 5HT1A receptor loss

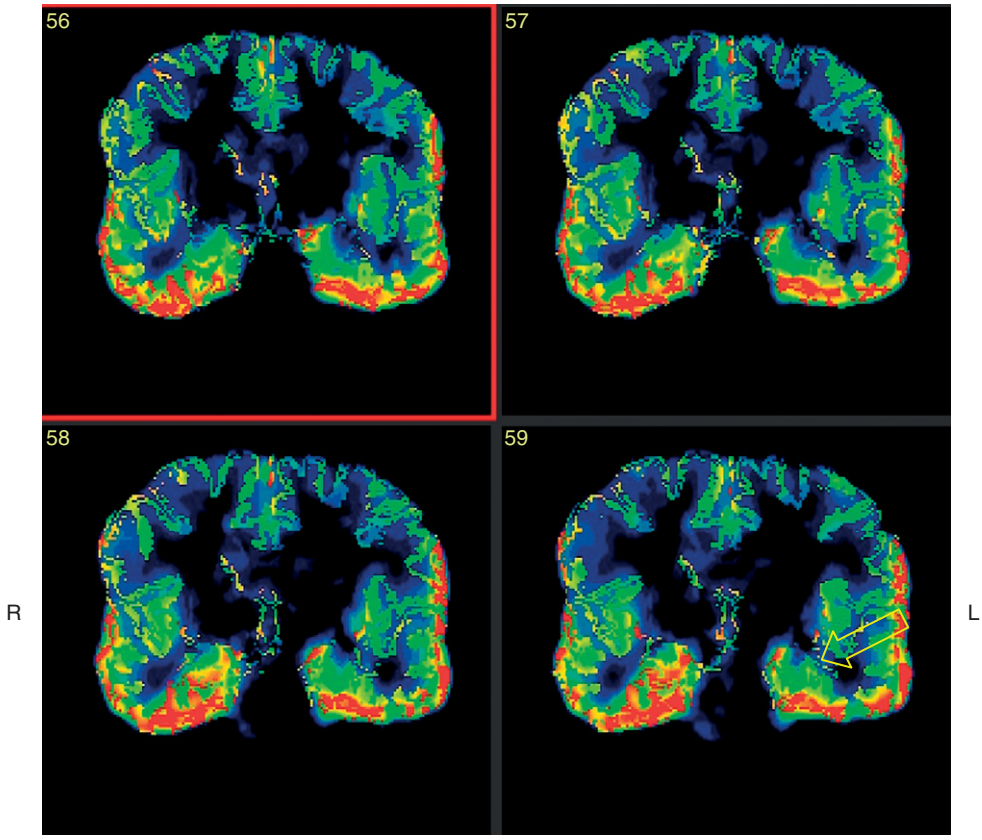


Figure 1 ^{18}F-FCWAY PET scan showing reduced tracer volume of distribution, after partial volume correction, in left hippocampus and parahippocampal gyrus in a patient with left mesial onset temporal lobe epilepsy.

might occur in an early stage in the development of mesial temporal sclerosis. 5HT1A receptor activation promotes cell proliferation in hippocampus by a direct post-synaptic effect. Reduced receptor binding is found in patients with depression, in association with hippocampal atrophy (although not the gliosis seen in TLE). It has been suggested that the increased precursor uptake seen in AMT PET studies might be due to diversion of the serotonin precursor AMT into alternate, excitatory pathways, as well as to increased serotonin synthesis in response to receptor loss.

Patients with depression as well as epilepsy have greater 5HT1A binding reduction than those with epilepsy alone (Figure 2). Reduced binding in regions beyond the focus, such as the cingulate gyrus, have been shown to be associated with depression in epilepsy as well as primary major depressive disorders. In these studies, focus side and the presence of mesial temporal sclerosis were not associated with the presence of co-morbid depression.

One PET study targeted to a known physiologic abnormality in a specific epilepsy syndromes was performed in patients with autosomal dominant nocturnal frontal lobe epilepsy (who have mutations in the nAChR alpha4 or beta2 subunit). PET studies using [^{18}F]-F-A-85380, a high affinity agonist at the alpha4beta2 nAChRs, showed increased binding in mesencephalon, pons and cerebellum, and decreases in the dorsolateral prefrontal region when compared to control subjects. The nAChR density increase in mesencephalon may reflect altered ascending cholinergic arousal mechanisms.

Challenges for the Future

PET receptor studies depend on the radiochemical synthesis of interesting ligands. One system that would be of particular interest is the excitatory amino acid system. Several attempts to produce a useful tracer have been unsuccessful so far. Attempts to develop useful excitatory amino acid receptor ligands have been unsuccessful, in part due to high non-specific binding. In an older study, the *N*-methyl-*D*-aspartate (NMDA) receptor ligand ^{11}C-labeled (*S*)-[*N*-methyl]ketamine, showed reduction of tracer radioactivity in epileptogenic temporal lobes, possibly due to focal atrophy. Some recently g-protein coupled metabotropic gluR5 receptor ligands may be promising.

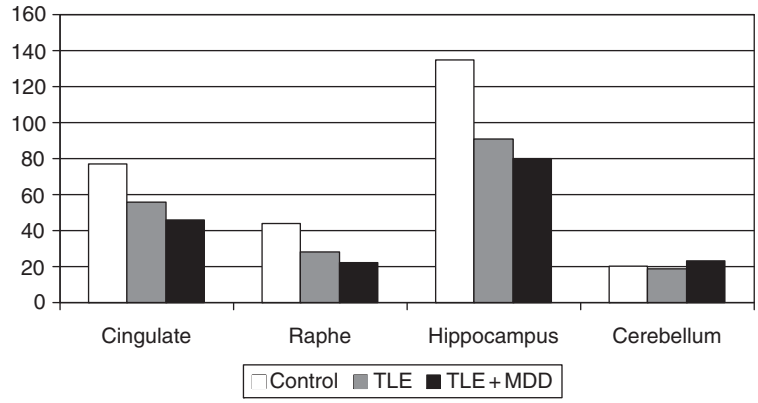


Figure 2 Comparison of 5HT1A receptor binding potential in patients with temporal lobe epilepsy with and without major depressive disorder as assessed by the standardized clinical interview for depression, and normal volunteers. Reduced binding was found in patients with both epilepsy and depression, compared to patients with epilepsy alone and controls. Overall binding is greater for all groups in the hippocampus, a structure wit the greatest concentration of 5HT1A receptors. No differences were found in cerebellum, a structure with a very low receptor concentration.

It would also be very useful to have a way to investigate binding at the GABA receptor site itself (in addition to the linked benzodiazepine receptor), and to have a means to study possible receptor conformation changes. For serotonin, other receptor subtypes in addition to 5HT1A are of interest.

Finally, ligands that can be used to image brain inflammatory changes are under development. These ligands will be helpful in testing recent hypotheses, e.g., regarding the viral and immunologic mechanisms for epilepsy.

See also: **Cognitive Dysfunction and Other Comorbidities**: The Relationship of Epilepsy to Other Conditions/Disorders; **Hormones and Gender**: Neurosteroid Modulation of $GABA_A$ Receptor-Mediated Synaptic Transmission in an Animal Model of Temporal Lobe Epilepsy; **Imaging**: Imaging Approaches to Seizure Analysis: PET Analysis.

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Physiologic Correlates of Metabolic Imaging in Human Epilepsy

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Introduction

There is extensive evidence that metabolic dysfunction is common in human epilepsy. The regional localization of such dysfunction is frequently specific enough to identify the seizure onset zone in many patients. As a measure of total glucose consumption, ^{18}fluorodeoxyglucose PET is commonly used to identify hypometabolic areas. The success rate of FDG PET in identifying the seizure onset zone depends very much on the nature of the epilepsy, with the best localization rates of ~80–90% in temporal lobe epilepsy. FDG PET is clearly less successful in the nonlesional neocortical epilepsy patients. In the mid-1990s, a number of investigators demonstrated the high sensitivity (70–100%) of magnetic resonance spectroscopy (MRS) and MRS imaging (MRSI) based methods using decrements in *N*-acetyl aspartate (NAA) to localize the seizure onset zone in mTLE patients. As NAA is synthesized only in neuronal mitochondria, and is strongly correlated with oxidative metabolism, it provides a complementary approach to PET, which evaluates total glucose consumption. More recent work in healthy volunteers has also shown a tight positive relationship between NAA and ADP, a key regulator of metabolic flux. Thus, NAA and its ratio to creatine may well be expected to sensitively reflect the mitochondrial capacity to meet metabolic demand.

Background

Studies in epilepsy patients with cortical malformations have revealed that NAA changes are specific to the epileptogenic zone, and are 'normal' in patients whose seizures are well controlled. Furthermore, in patients with on-going seizures, the regions that are anatomically abnormal but nonepileptogenic also displayed normal NAA levels. An important aspect of the MRSI approach has been its ability to provide more quantitative evaluations – i.e., without the use of asymmetry indices and without being biased by tissue loss. As a result, many of the studies using spectroscopy have shown the frequent occurrence of bilateral involvement in temporal lobe epilepsy (**Figure 1**), which has since also been reported by conventional and diffusion weighted imaging.

In addition to the role of metabolic imaging for seizure localization and lateralization, an important aspect of MRSI has been its contribution to our understanding of how metabolic dysfunction may contribute to the pathophysiology and pathology of human epilepsy. Using data obtained from intracranial monitoring, pathology, and electrophysiology obtained from resected specimens, we may better define the clinical and pathophysiologic meaning of aberrant NAA/Cr, high energy phosphates and glutamate. In turn, the dynamic range of MRSI-determined bioenergetic and mitochondrial dysfunction (as reflected across different patients) may help to explain how energetic state and capacity influence key parameters of hyperexcitability. In this article, we discuss how metabolic imaging may relate to neural activity and neurotransmitter levels.

Methods

MRS (magnetic resonance spectroscopy) and MRSI (MR spectroscopic imaging) are closely related MR approaches to the acquisition of spectroscopic data. MRS acquires spectroscopic data from a single voxel – that is, a measure of the chemical compounds within a voxel (e.g., $2 \times 2 \times 2\ \text{cm}^3$ or $8\ \text{cm}^3$), with the data typically presented as a single spectrum. MRSI acquires spectroscopic data from many contiguous voxels simultaneously; that is, many spectra are acquired from a region of interest, thus allowing construction of an image showing a metabolite's distribution within that region (e.g., see the spectroscopic image (SI) in **Figure 1**). While many clinical radiology MR facilities allow single voxel MRS studies, the acquisition and analysis of MRSI data is more complex. However, as the intrinsic volume averaging that is present in the single voxel MRS acquisition can clearly dilute abnormalities, many recent research studies in human spectroscopy use MRSI. The data that are discussed in this article are MRSI data, acquired at 4T using a Varian Inova whole body MR system.

Depending on the type of spectroscopic data to be obtained (e.g., ^{1}H data of creatine, NAA, etc., or ^{31}P data of ATP or phosphocreatine), different acquisition protocols are implemented. In this article, the ^{1}H data are acquired along the temporal plane to include bilateral medial temporal lobes, with the planar region of interest (ROI) being 10 mm thick × 8 cm (anterior–posterior) × 10 cm (left-right) (**Figure 1**). 24 × 24 phase encoding is applied over this ROI, resulting in a nominal voxel size of

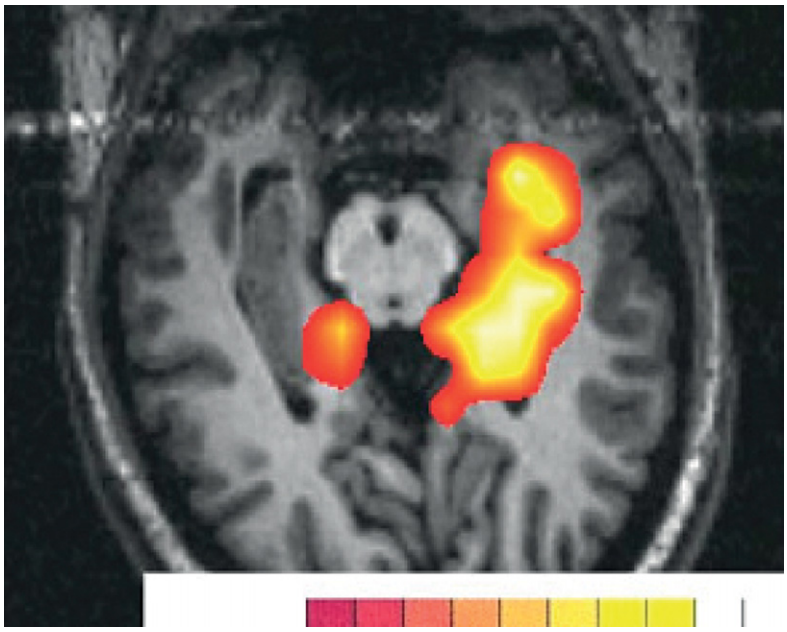


Figure 1 MRSI statistical map showing the bilateral, but dominant left, hippocampal abnormalities in NAA/Cr. The data are corrected for tissue content variability and are independent of CSF and parenchymal tissue volume. The color bar indicates level of statistical significance, starting at $p < 0.05$ (red), going to $p < 0.0001$ (white).

0.64 cm^3 (acquisition time of 20 min). ^{31}P data are acquired in whole brain spectroscopic imaging format, using a 3D spherical encoding strategy to provide a voxel size of ~12 cm^3 (acquisition time of 46 min).

Results

Hyperexcitability and Metabolic Imaging

While ATP is the cell's fundamental energy source, phosphocreatine (PCr) is the major alternative high-energy phosphate compound. Its role is to ensure the continual availability of ATP. ATP is produced in the mitochondrion, but is consumed at varying cytoplasmic sites (e.g., the membrane based Na–K ATPase, which is a large consumer of ATP). PCr serves to shuttle and buffer ATP, keeping the ATP concentration in healthy brain at ~3 mM (depending on tissue type and CSF content). In situations where production and/or consumption of ATP may be abnormal, the concentrations of PCr may be much more dynamic than the concentrations of ATP. The ratio of PCr/ATP is a measure of how well PCr is maintained per unit of ATP, and has been experimentally shown to be highly sensitive to abnormalities in epilepsy. Evidence for a link between energetics and physiologic function comes from studies of temporal lobe epilepsy (MTLE), where lowered PCr/ATP levels are found, and where the ratio is worse at the site of seizure onset. Moreover, following temporal lobectomy, there is an improvement in contralateral temporal metabolism as measured by NAA/Cr.

Thus, alterations in bioenergetics are part of the spectrum of changes that characterize MTLE, along with cell loss and synaptic reorganization. We have found that in MTLE patients, the pre-operative measures of mesial temporal lobe PCr/ATP correlate well with several parameters as determined from electrophysiologic study of the resected hippocampal dentate granule cells. As membrane repolarization is known to be an energetically consumptive process, we anticipated and verified that the rate of recovery of the granule cell membrane potential after a suprathreshold orthodromic stimulus (applied at the outer molecular layer at twice the intensity needed to evoke a single action potential) train (10 Hz, 10 s duration) correlated with PCr/ATP ($R = +0.85$, $p < 0.01$) (**Figure 2(a)**). The higher PCr/ATP was associated with a faster membrane potential recovery rate, consistent with a functional relation between energetics and Na–K ATPase flow. This relationship was not seen with milder stimuli, and most likely reflects the substantive demand on energetic function with the more intense, 10 s stimulus train paradigm. We also determined that hyperexcitability, as measured by synaptic bursting, correlates significantly with PCr/ATP ($R = -0.62$, $p < 0.02$). Synaptic bursting (multiple action potentials fired in response to a single suprathreshold stimulus) is an abnormal response of granule cells (in normal tissue, such a stimulus evokes only a synaptic response with no or perhaps one action potential), and is much more commonly seen in tissue with extensive cell loss and synaptic reorganization. As shown in **Figure 2(b)**, more bursting was associated with lower PCr/ATP.

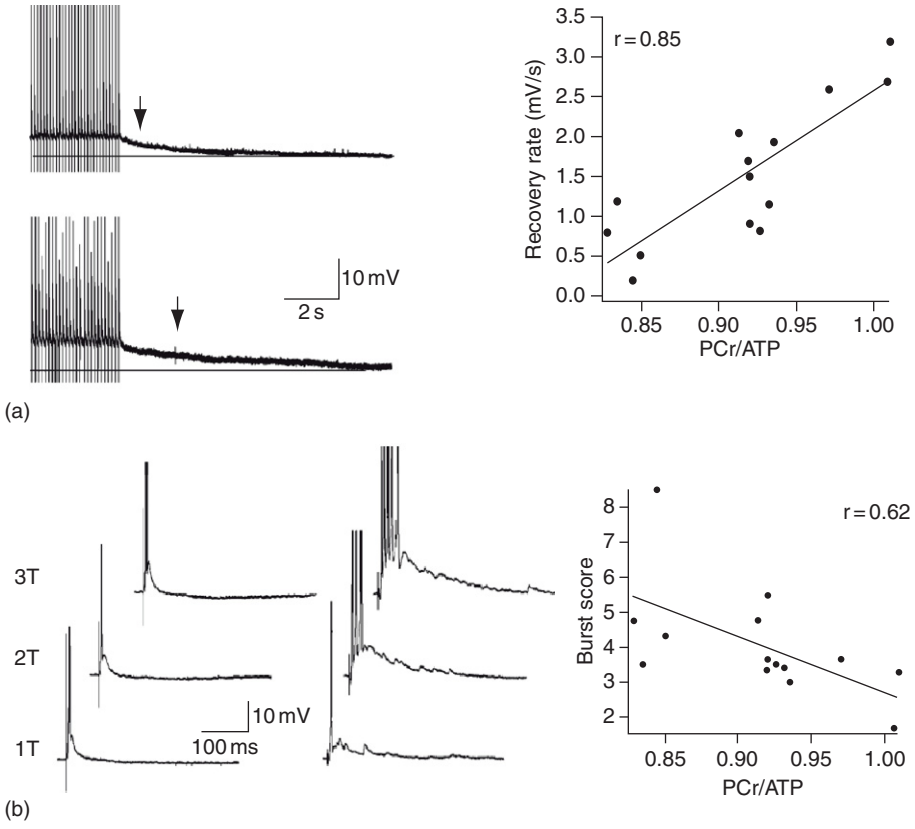


Figure 2 Electrophysiological function relates to bioenergetic status of PCr/ATP. (a) Left: Membrane potential recovery after a 10-Hz 10-s stimulus train obtained from two different MTLE patients. Right: The rate of membrane repolarization after this suprathreshold train of stimulation is significantly related to PCr/ATP, $R = +0.85$, $p < 0.01$. (b) Left: Action potential data are shown in response to three different levels of stimulation (measured relative to the action potential threshold T that provokes a single action potential). In patient #1 (far left), the increasing stimulation intensity to 3T elicited a single spike. In patient #2 (middle), the increasing stimulation intensity elicited a three and then five spike burst of action potentials. The extent of abnormally increased synaptic bursting is also related to PCr/ATP, $R = +0.62$, $p < 0.02$.

It is notable that in these relationships, contributing data were obtained from patients with sclerotic ($n = 11$) and nonsclerotic ($n = 3$) hippocampi, with no major discrepancies seen between them. While the number of patients is clearly limited, the consistency of the data argues that these findings reflect a common characteristic of electrophysiologically determined hyperexcitability, and may be independent of specific disease etiology.

In vivo microdialysis has proved to be another key approach to studying in vivo human epilepsy. This procedure has provided an opportunity to study more carefully the physiology of the extracellular inhibitory and excitatory neurotransmitters, and serves as a useful complement to intracranial EEG monitoring, which has been demonstrated to be a highly sensitive and successful approach for identifying the seizure onset zone. We have linked MRSI with microdialysis studies to evaluate how glutamate may be influenced by the bioenergetic state. These measurements have been made most consistently within the hippocampus, because of the frequency of the pathology and identifiably consistent location of dysfunction. The full range of metabolic abnormalities associated with extracellular glutamate becomes clear under these conditions. In these in vivo studies electrophysiologic function was measured as 'Teager energy' – which is believed to represent a greater degree of abnormality in electrical activity. Within the ipsilateral hippocampus of MTLE patients, increasing Teager energy is well linked with increased extracellular glutamate as shown in **Figure 3(a)**. In this same population of patients, significant correlation between the bioenergetic state with extracellular glutamate is seen (**Figure 3(b)**), with lower PCr/ATP linked with higher glutamate. Thus, if we view PCr/ATP as a measure of the local energetic state, it is clear that those processes that cause energetic perturbation (e.g., changing cerebral demand, fuel availability or stress) alter the ambient extracellular glutamate concentrations – which in turn contribute to altered tissue excitability.

Neuropsychologic Performance and Metabolic Imaging

The abnormalities seen with metabolic imaging are also associated with clinical functional parameters – for example,

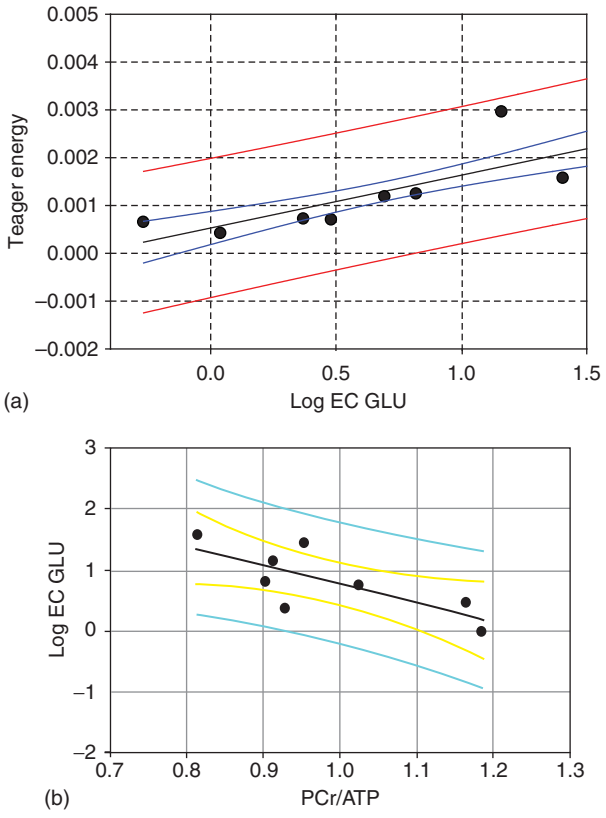


Figure 3 (a) In vivo measures of extracellular glutamate increases with increasing Teager energy ($R = +0.75$, $p < 0.001$); 95% confidence interval and prediction bands are shown. (b) Relationship between preoperative PCr/ATP and microdialysis measurements of log extracellular glutamate ($R = -0.76$, $p < 0.03$); 95% confidence intervals and prediction bands are shown.

with neuropsychologic performance. As part of the epilepsy evaluation, neuropsychologic function is often assessed as an important reflection of local injury, and these measures are commonly used to suggest hemispheric and/or lobar dysfunction. Thus, several groups have found that NAA regional measurements parallel neuropsychologic performance. We have also accumulated data from clinical tests of memory function – using the verbal selective reminding test (vSRT, dominant hippocampal function) and visuomotor selective reminding test (vmSRT, nondominant hippocampal function). As anticipated, the SRT relates significantly to the mitochondrial function, as measured from the epileptogenic mesial temporal lobe ($R = +0.56$, $p < 0.0001$, Figure 4(a), $n = 24$ patients). What is also notable is the similar relationship between the same SRT score with contralateral mesial temporal lobe ($R = +0.57$, $p < 0.0001$, Figure 4(b)). These relationships may reflect the bilateral injury that is frequently present in temporal lobe epilepsy patients, and/or shared memory function between the hippocampi. As discussed by other investigators, both of the mesial temporal lobes contribute to visual memory. Consequently, performance on the vmSRT is likely to reflect both right and left temporal lobe contributions, as suggested from the present data, showing a significant relationship between performance with the vSRT and vmSRT ($R = 0.55$, $p < 0.0005$, Figure 4(c)).

Summary and Directions for Future Research

The overall integration of these data argues for the view that metabolic and energetic states are closely related to experimental and clinical measures of cerebral dysfunction. It is notable that bioenergetics (as measured by PCr/ATP) appear to link well with measures of hyperexcitability (from ex vivo and in vivo electrophysiology and microdialysis studies), while similar relationships have not been seen with the mitochondrial measures of NAA and NAA/Cr. This dissociation may be a consequence of the distinct aspects of metabolism that are evaluated in these different studies. For example, as a direct measure of energetics, interictal PCr/ATP appears to be only moderately reduced even in the face of very abnormal NAA/Cr, most likely as a result of both depressed tissue energy demand and energy production. NAA/Cr may best be viewed as a capacitive measure of mitochondrial function. To date, NAA/Cr has been used by a number of groups to identify the region of seizure focus; it has also been linked with a range of measures including neuropathology (measures of injury, in particular as seen with GFAP immunocytochemistry and glial counts) and cognitive performance.

The methods and issues briefly described in this article open several avenues of clinical and scientific investigation in human epilepsy. Given the large body of evidence showing metabolic abnormalities in the seizure focus, and given that many of the spectroscopically detected compounds (we have only mentioned a few in this article; e.g., NAA, glutamate, glutamine, GABA, glutathione, lactate, ATP, and phosphocreatine) are important for metabolic and neurotransmission function, MR spectroscopy can make significant contributions in the clinical localization and lateralization of epilepsy. Beyond this contribution, the studies of both animals and humans have provided evidence that mitochondrial and metabolic dysfunctions make key pathophysiologic contributions toward determining susceptibility to epilepsy. For example, animal studies have shown that conditions of deteriorating redox and mitochondrial function can induce seizures. Conversely, studies in seizure models have shown that injected fructose diphosphate (which is believed to increase glutathione, a key reducing agent) reduces seizure activity and severity significantly. In vivo MR spectroscopy can help ascertain the dynamics of such compounds, and – based on such measures – may be

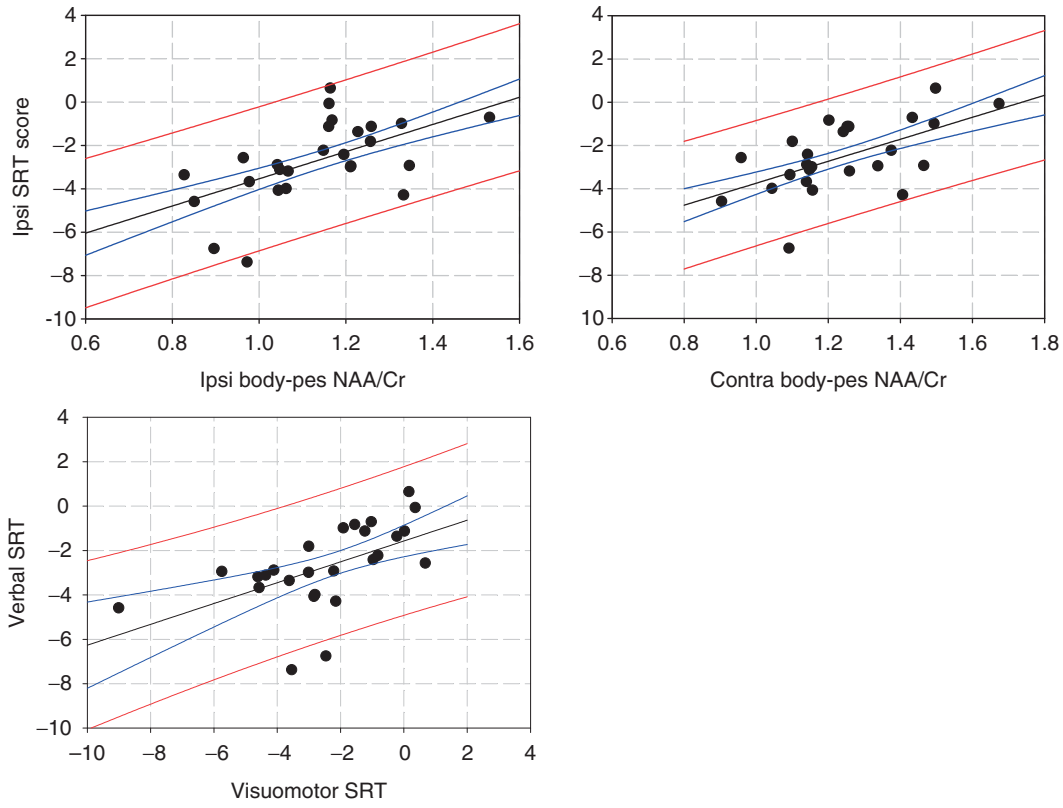


Figure 4 NAA/Cr correlations with ipsilateral selective reminding tests. (a) Ipsilateral hippocampal NAA/Cr value is correlated with the matching ipsilateral SRT score ($R = +0.56$, $p < 0.0001$). (b) Contralateral body-pes NAA/Cr value is correlated with the ipsilateral SRT score ($R = +0.57$, $p < 0.0001$). For both (a) and (b), hemispheric dominance, as determined from Wada testing, was used to determine whether the verbal or visuomotor SRT score was used. (c) Verbal and visuomotor SRTs are linked in mTLE patients ($R = +0.55$, $p < 0.0005$).

able to predict the epileptic outcome. With both clinical and investigative roles, the integration of MRSI with other monitoring modalities (e.g., electrophysiology and microdialysis) will be fruitful in better defining the interdependence of abnormal metabolism with icto- and epileptogenesis.

See also: **Cell Damage/Excitotoxicity**: Neuronal Injury from Seizures: Influence of Models, Development and Genetics; **Imaging**: MRS in Epilepsy; **Metabolic Changes**: The Emerging Role of Mitochondrial Dysfunction and Oxidative Stress in Epilepsy.

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Seizure-Related Acute MRI Changes

A J Cole, Massachusetts General Hospital and Harvard Medical School, Boston, MA, USA

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Introduction

Brain imaging is a mainstay in the approach to patients with epilepsy. With the advent of magnetic resonance imaging it is possible to identify the cause, and sometimes the consequences, of epileptic seizures in perhaps 50% of patients with epilepsy. Over the last decade, it has become increasingly apparent that, in addition to identifying genetic, congenital, and acquired lesions that lead to seizures, MRI also has the ability to reveal acute brain changes related to epileptic activity that we have termed 'peri-ictal' imaging abnormalities. Peri-ictal MRI abnormalities may be reversible, evolving, or permanent – and may occur in the region of the epileptic discharge or remote from it. Their occurrence seems highly variable but frequently correlates with the duration and severity of ictal activity, the so-called duration–severity integral. Here, we propose a classification of peri-ictal imaging findings, provide examples, and comment on potential pathophysiological mechanisms for their occurrence.

MRI Techniques

Modern anatomic MR imaging relies on a series of pulse sequences designed to highlight differing aspects of tissue composition – such as lipid content, water content and its microenvironment, diffusivity, and anisotropy. The major MRI modalities emphasize the T1 and T2 relaxation times of the tissue being imaged. Specific sequences are chosen to highlight either T1 or T2 characteristics (T1- or T2-weighted images), although no specific pulse sequence is solely sensitive to T1 or T2 content. Diffusivity is measured by applying a pulsed gradient magnetic field across a subject of interest and calculating diffusivity based on the signal loss that occurs due to Brownian motion between pulses in the gradient. As the distance that a molecule will diffuse is a function of the structure of the tissue in which it is located, diffusivity varies between differing brain compartments such as fluid, myelin, and gray matter. Moreover, diffusivity is sensitive to certain pathological states, such as edema, ischemia, and neuronal loss. Anisotropy is a measure of the coherence or directionality of diffusion. In a fully anisotopic material, diffusion occurs in all directions equally anisotropic, whereas in a tissue with a low fractional anisotropy (FA), diffusion occurs in a highly coherent fashion. FA maps are increasingly used to evaluate connectivity between neuronal structures.

Diffusion imaging changes are prominent among both local and remote peri-ictal findings described in the following sections and are frequently reversible. It is, therefore, important to consider the origin of these findings and their significance. Diffusivity is an intrinsic property of tissue. The apparent diffusion coefficient (ADC) is independent of both T1 and T2 relaxation times. T2 relaxation time is largely reflective of local brain water content. Whereas increased T2 signal suggests edema or gliosis, restricted diffusion is usually associated with metabolic dysfunction or energy deficiency. Some of the hypothesized causes of restricted diffusion are listed in **Table 1**.

Table 1 Causes of restricted diffusion

Na+-K+-ATP-ase pump failure
Cytotoxic edema
Increased intracellular viscosity due to cytoskeletal fragmentation
Increased tortuosity of intracellular space
Decreased extracellular volume
Microvacuolization of myelin

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Restricted diffusion has commonly been identified as a marker of irreversible ischemic injury, but in some situations decreased diffusion associated with seizures is reversible without the subsequent appearance of tissue injury. The temporal relationship between the appearance of restricted diffusion and T2 signal change in association with seizures may be variable. Whereas in acute ischemia DWI images reveal restricted diffusion before any abnormality of T2 signal becomes apparent, in ongoing status epilepticus DWI and T2 signal changes appear to occur roughly synchronously. These differences in the timing of DWI and T2 signal change suggest a difference in the underlying pathophysiology. Whereas in ischemia there is early energy failure, in ongoing status epilepticus activity-induced injury with cytotoxic edema may precede overt energy deficiency.

Peri-Ictal Imaging Changes

Peri-ictal imaging changes may occur in the region of the epileptic discharge (local) or in distant structures (remote). Some of the findings that have been described are listed in Table 2. Each of these findings may be reversible or irreversible.

Local Findings

The pathophysiological basis of local peri-ictal imaging findings, which include localized increases in volume of specific structures (swelling), increased T2 signal, reduced ADC, and vascular changes, is seemingly related to increased neuronal activity and its associated metabolic and vascular responses.

Local Anatomic Abnormalities

Local swelling of hippocampus has been described in children with prolonged febrile convulsions and in adults with seizures related to paraneoplastic or nonparaneoplastic limbic encephalitis. In both conditions there is suggestive evidence that there is involution over time of the hippocampus, with residual sclerosis and atrophy. Effacement of gyri in the region of epileptic discharge may be seen in all age groups. Increased T2 signal intensity, indicating an increase in brain water, occurs in areas of cortex and subcortical white matter in association with some prolonged or intense seizures (Figure 1). Many of these lesions show restricted diffusion (bright on DWI, dark on ADC maps). While restricted diffusion suggests the presence of cytotoxic (arising from neuronal injury) rather than vasogenic (arising from abnormally leaky blood vessels) edema, these lesions are reversible, at least initially, raising a question about the actual cause – and indicating that cell death is not an inevitable sequel.

In several studies, localized reduction in ADC has been observed soon after a single seizure. Diehl *et al.* examined patients 45–150 min after a single seizure and found significantly decreased ADC in 1 of 6 patients studied, while Hufnagel *et al.* found similar changes in 5 of 9 patients studied. In these cases, the site of change in ADC appeared to correlate well with the presumed site of maximal seizure activity; however, the variability in occurrence of this finding makes its current utility in routine diagnostic evaluation uncertain. Diffusivity appears to evolve over time. Several studies indicate that in patients with chronic epilepsy, at least during the interictal period the ADC is modestly increased. This finding may be the consequence of progressive neuronal loss with increased extracellular space.

Migratory Anatomic Abnormalities

In occasional patients with multifocal epilepsy, T2 and DWI lesions appear to be migratory (see Figure 2). These lesions appear to colocalize with regions of focal hypermetabolism as illustrated by ^{18}FDG-PET or ^{99}Tc-SPECT scanning (Figure 3) – and these hypermetabolic regions, in turn, corresponded to areas of maximal EEG abnormality. These features, in conjunction with the timing with which T2 and DWI abnormalities appear, suggest that the lesions are the consequence of the epileptic activity, and not its cause.

Abnormalities of Blood Flow and Perfusion

Special MR techniques can be utilized to measure perfusion of a local brain region. Perfusion-weighted MR imaging may show initial hyperperfusion in the area of ictal activity, with subsequent relative hypoperfusion. This complex temporal evolution of postictal imaging points to the need for careful electroclinical correlation when assessing the significance of findings on postictal scans. MR angiography in patients in status epilepticus may demonstrate increased flow-related enhancement, and this finding corresponds well to the observation of arterialization of venous blood reported by surgeons examining the cortex during provoked or spontaneous

Table 2 Classification of peri-ictal imaging abnormalities

Remote	*Local*
Unilateral/bilateral diencephalic lesions	Mass effect
Cerebellar diaschesis	Hippocampal swelling
Splenium abnormalities	Focal cortical edema
Reversible posterior leukoencephalopathy	Migratory lesions
	Blood-brain barrier breakdown
	Increased vessel caliber/flow
	Increased perfusion

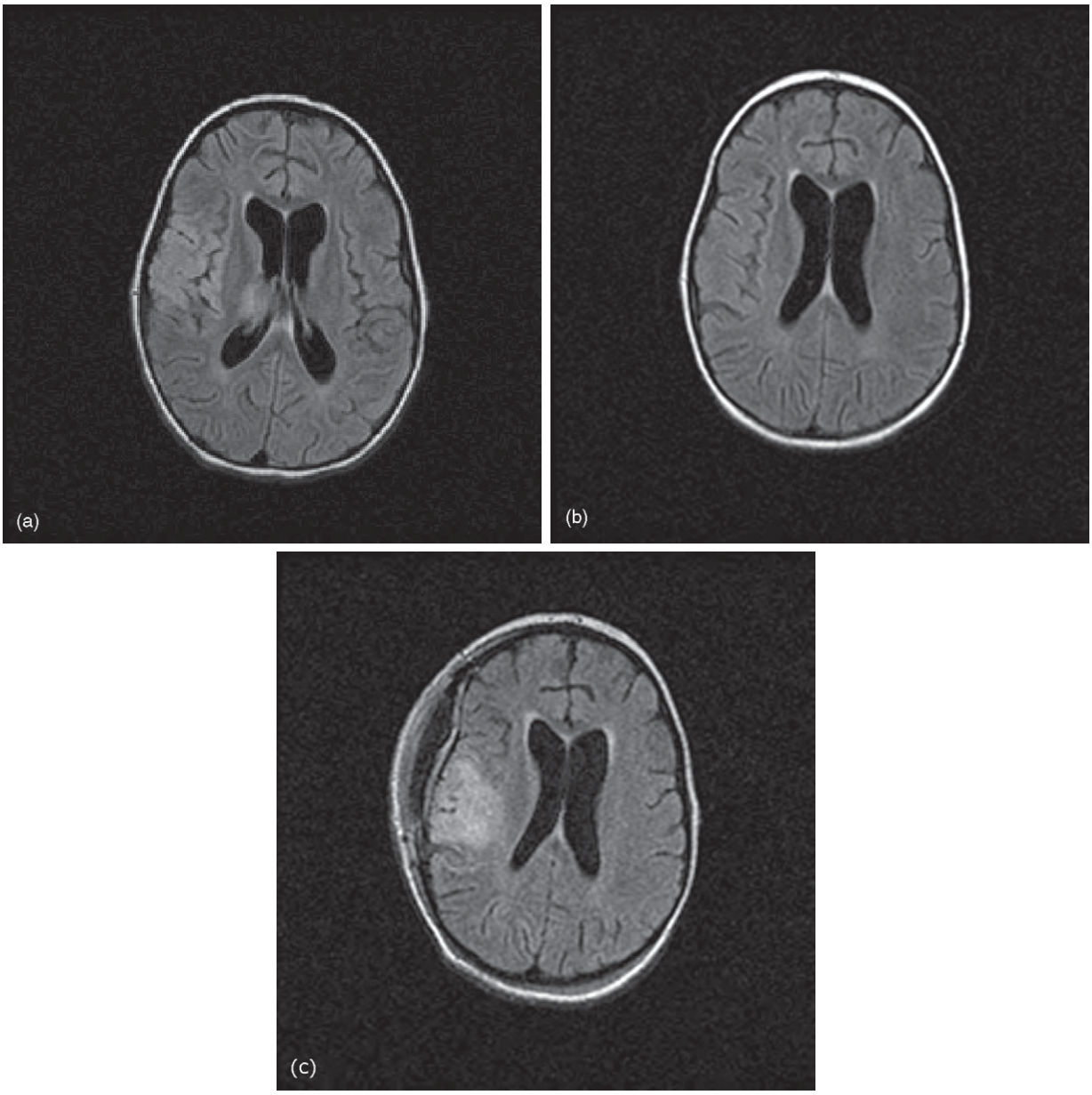


Figure 1 Resolution and recurrence of increased T2 signal associated with focal status epilepticus after 7 days of pentobarbital anesthesia. The patient was a 12-year-old girl who presented 14 days before with epilepsia partialis continua arising from the right hemisphere. (a) Axial FLAIR MR image obtained on day 14 prior to anesthetic treatment. (b) Axial FLAIR MR image obtained after 7 days of pentobarbital anesthesia titrated to produce burst suppression on continuous EEG monitoring. Note that T2 signal changes have largely resolved. (c) Axial FLAIR MR image obtained 7 days after anesthetics were discontinued and epilepsia partialis had recurred. Reproduced from Doherty CP, Cole AJ, Grant PE, *et al*. (2004) Multimodal longitudinal imaging of focal status epilepticus. *Canadian Journal of Neurological Sciences* 31: 276.

epileptic seizures (Figure 4). SPECT studies in this circumstance may not demonstrate evidence of increased perfusion at the microvascular level, suggesting that some portion of the locally increased blood flow represents arteriovenous shunting. That is, if there is increased flow at the macrovascular level, but no increased perfusion at the microvascular level, then the increased flow must be shunted to bypass the microvascular circulation.

Remote Findings

The pathophysiological bases of lesions that occur remote from the site of ictal activity are not understood.

Diencephalic and Cerebellar Diaschesis

The term 'diaschesis' refers to a change in function and electrical activity in an area of the brain due to a lesion in

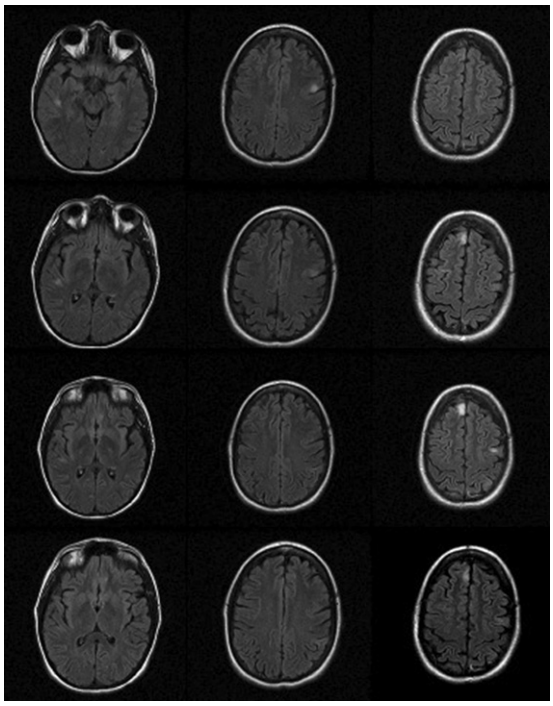


Figure 2 Migratory peri-ictal imaging changes. FLAIR images obtained at multiple levels at time of presentation (top row), on day 14 (second row), on day 28 (third row), and on day 35 (fourth row) from a 28-year-old woman with recurrent partial seizures associated with a mitochondrial disease. Reproduced from Cole AJ (2004) Status epilepticus and periictal imaging. *Epilepsia* 45(supplement 4): 72, with permission from Wiley-Blackwell.

a remote area that is connected with it. Classically, this term was applied to the observation that frontal lesions were associated with altered function of the contralateral cerebellar hemisphere. In patients with focal epilepsy, we have commonly observed hypometabolism – using ^{18}FDG-PET scanning – in the cerebellar hemisphere contralteral to the epileptic focus. Ipsilateral diencephalic and contralateral cerebellar lesions, including restricted diffusion and increased T2 signal, may be a striking finding in status epilepticus (**Figure 5**). These lesions appear to resolve over days to weeks when seizures are controlled, but may presage the appearance of local atrophy of the affected structures in some cases. The pathophysiology of these lesions is unknown, but they presumably result from the recruitment and driving of thalamic and cerebellar circuits in the expression of epileptic activity.

Lesions of the Splenium of the Corpus Callosum

Transient lesions of the splenium of the corpus callosum have been described in patients with epilepsy. Splenial lesions are typically ovoid and well-circumscribed regions of increased T2 signal with restricted diffusion (**Figure 6**), and may reflect abnormal activity in white matter tracts driven by the epileptic focus. Neither the mechanism nor the precise nature of the white matter change responsible for these imaging alterations is understood. One study suggested that corpus callosum lesions

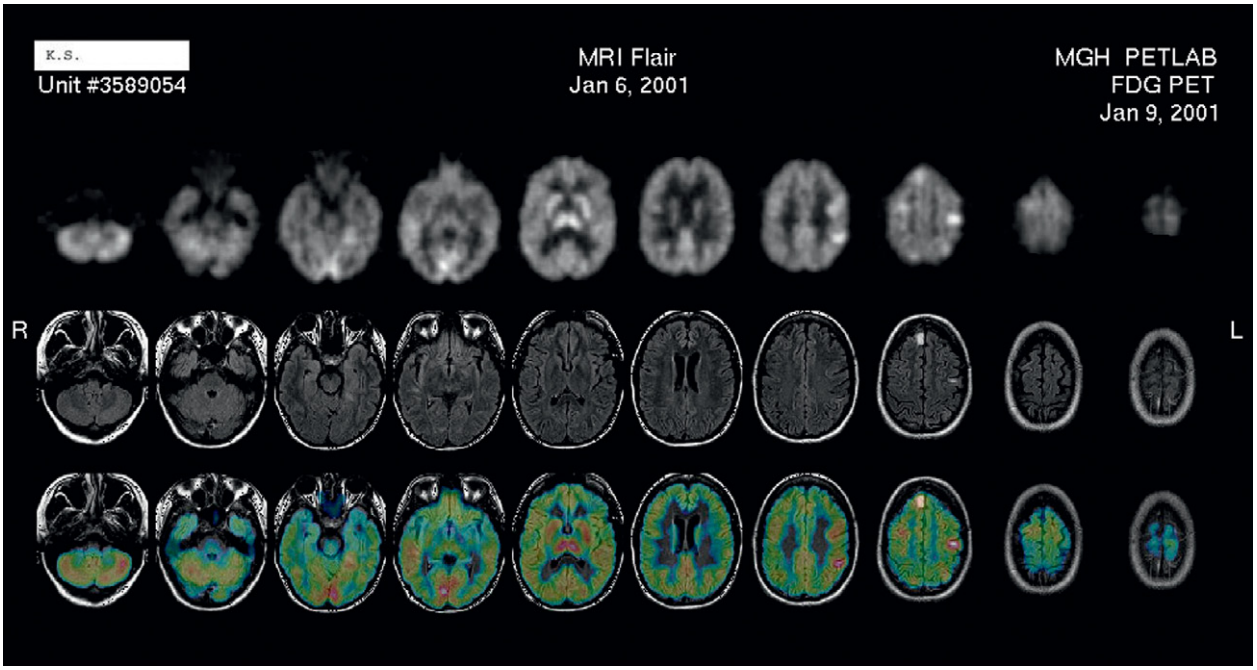


Figure 3 Multimodal imaging using MRI and PET. Upper panel: ^{18}FDG-PET images from patient described in **Figure 4** obtained on day 25 showing multiple focal areas of hypermetabolism. Middle panel: FLAIR images obtained on day 28. Lower panel: Coregistration showing colocalization of areas of hypermetabolism and areas with FLAIR abnormalities. EEG recording (not shown) demonstrated a similar localization of ictal activity involving right frontal pole and left central region. Reproduced from Cole AJ (2004) Status epilepticus and periictal imaging. *Epilepsia* 45(supplement 4): 72, with permission from Wiley-Blackwell.

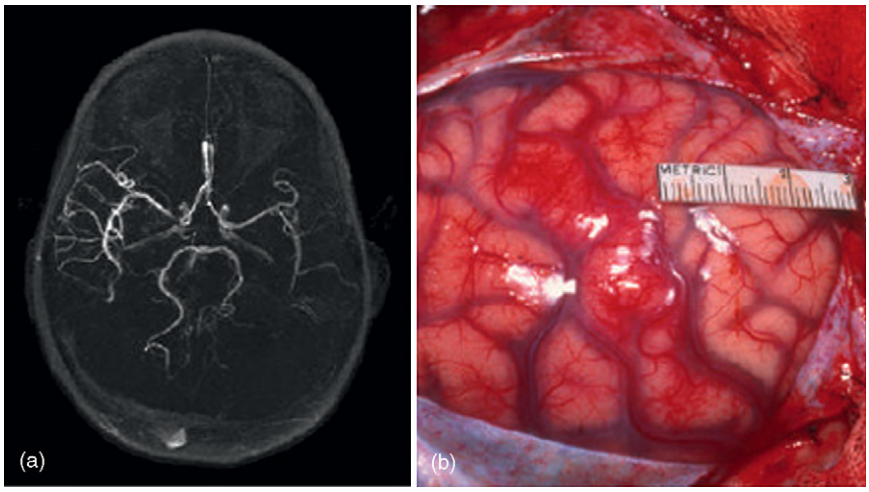


Figure 4 Cortical hyperemia associated with increased flow-related enhancement in a patient in focal status epilepticus. (a) MR angiogram showing dilatation of the right middle cerebral artery (MCA) branches presumably reflecting increased blood flow to the active cortex. (b) Intraoperative photograph showing right motor strip exposed beneath the dura (right and panel). Reproduced from Doherty CP, Cole AJ, Grant PE, *et al*. (2004) Multimodal longitudinal imaging of focal status epilepticus. *Canadian Journal of Neurological Sciences* 31: 276.

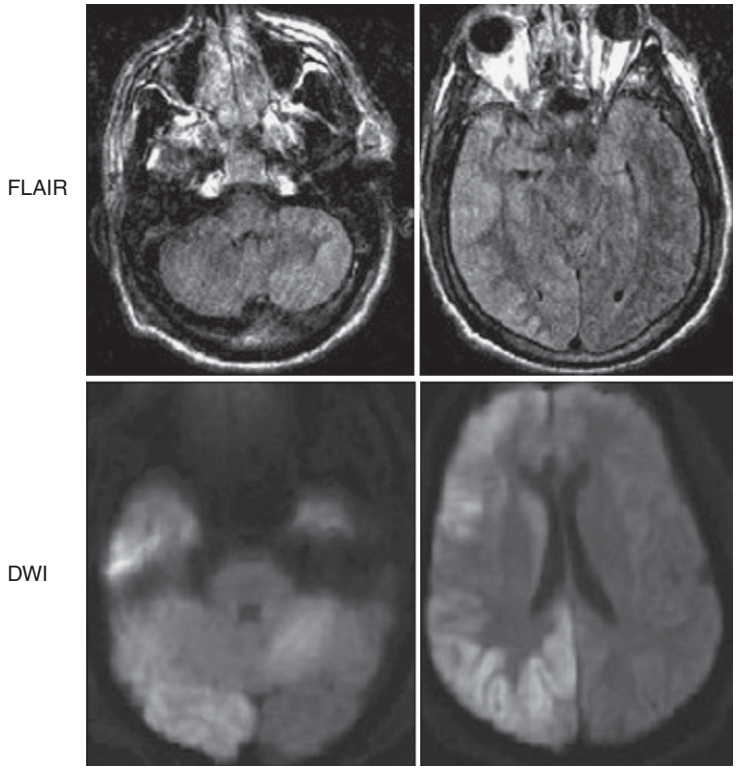


Figure 5 Crossed cerebellar diaschesis. Images obtained from a 38-year-old patient who presented with recurrent right hemispheric seizures. Upper panels show abnormal signal on fluid-attenuated inversion recovery (FLAIR) images in the ipsilateral cortical ribbon and in the contralateral cerebellar hemisphere. Lower panels show reduced diffusion on diffusion-weighted images in corresponding regions . Reproduced from Cole AJ (2004) Status epilepticus and periictal imaging. *Epilepsia* 45(supplement 4): 72, with permission from Wiley-Blackwell.

were due to anticonvulsant drug toxicity, but this explanation seems unlikely given the large number of patients on anticonvulsants who undergo MR imaging and do not show such lesions. Moreover, several cases have been reported in patients off anticonvulsants who had flurries of seizures. Polster *et al.* suggested that drug withdrawal might underlie this splenial phenomenon, but their argument rested on the notion that the splenium lesion was

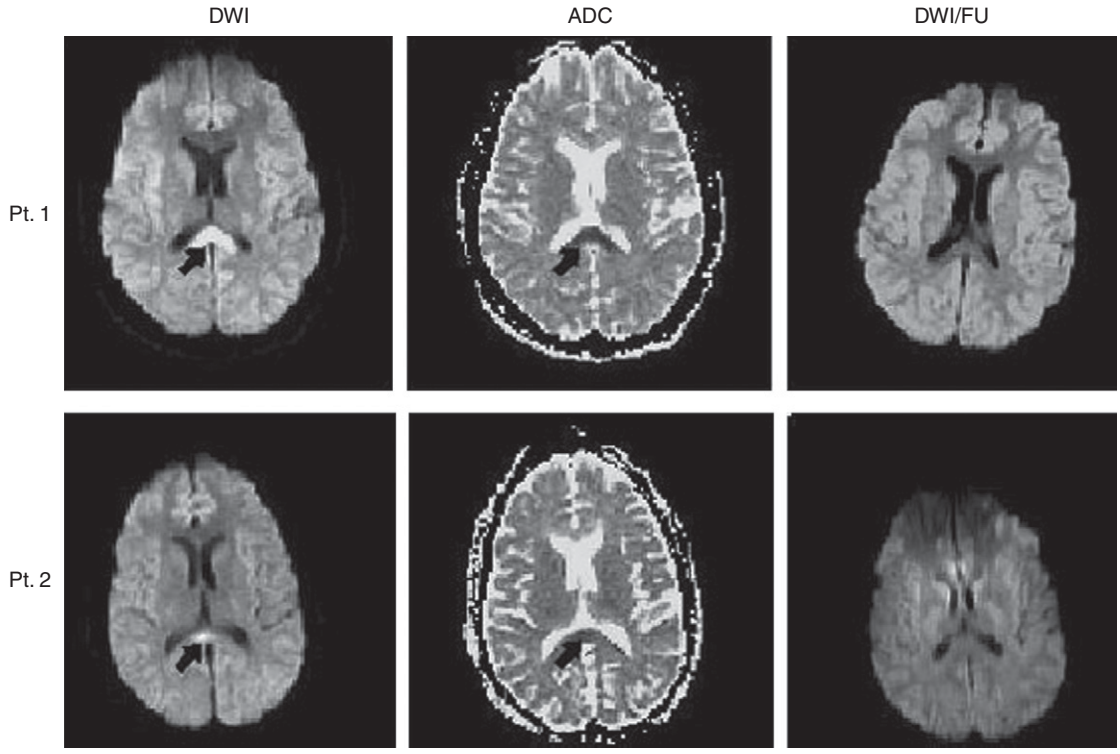


Figure 6 Transient diffusion-weighted imaging changes in the splenium after seizures. Representative images from two patients (upper and lower panels) demonstrate restricted diffusion (left hand images) with associated decreased apparent diffusion coefficients (center images) that resolved in 4–6 weeks (right hand images). Reproduced from Oster J, Doherty C, Grant PE, *et al*. (2003) Diffusion-weighted imaging abnormalities in the splenium after seizures. *Epilepsia* 44: 852.

vasogenic edema. Our studies indicate that the lesion demonstrates a decreased apparent diffusion coefficient (ADC), a finding that is not consistent with their hypothesis but rather supports the idea that in most cases the lesion is related to the ictal activity per se. As shown in Table 1 (which lists potential causes of restricted diffusion), microvacuolization of the myelin is one pathological substrate that could account for the increased T2 signal, the decreased ADC, and the reversibility of lesions of the splenium. Similar lesions have also been reported in association with encephalitis, osmotic disturbances, or altitude sickness. It remains unclear whether the pathophysiology of callosal lesions is similar in each of these situations.

Posterior Reversible Leukoencephalopathy

Reversible posterior leukoencephalopathy (RPLE), described in patients with hypertension, eclampsia, and sometimes in association with certain immunosuppressive drugs, may be associated with repeated seizures. Some patients with seizures, but without another obvious cause for RPLE, have been described – suggesting that seizures per se may contribute to the occurrence of this finding. However, it is difficult to exclude moderate changes in systemic blood pressure with respect to the patient's baseline as a cause in most cases.

Issues for Discussion and Future Research

Variability in Occurrence of Peri-Ictal Imaging Changes

An important and unresolved issue is why local acute peri-ictal changes on MRI occur in some, but not all patients after focal seizures. The fact that such changes are sometimes first apparent days or weeks after the start of focal status epilepticus suggests that there is a threshold of seizure duration and/or severity below which changes will not occur. Most reports of acute imaging changes on MRI describe patients experiencing prolonged seizures or status epilepticus; however, current knowledge does not allow us to predict where that threshold might lie. Importantly, as with most threshold phenomena in biology, it is likely that there are differences between individuals with respect to when imaging findings will become apparent,

Table 3 Critical Variables that may determine occurrence or severity of local peri-ictal imaging changes

Seizure type/location
Duration/severity of ictal discharge
Host characteristics
Age
Preexisting conditions
Cardiovascular/Metabolic reserve
Pharmacological intervention

and when they will become irreversible. Some critical variables that might determine whether seizures in a specific individual are sufficient to trigger acute imaging changes are listed in Table 3.

Impact of Seizure-Related Acute MRI Findings on Clinical Management

Local and remote peri-ictal imaging findings – including T2 signal change, restricted diffusion, local swelling, and altered perfusion – must be interpreted with caution in the setting of acute or ongoing seizures or status epilepticus. It is important to recognize that these changes can occur as the consequence of seizure activity per se, and therefore may not be helpful in establishing the cause of the seizures. In extreme cases, acute findings may mimic tumor or stroke, but if misinterpreted these would lead to inappropriate diagnostic or therapeutic interventions. As peri-ictal imaging changes evolve over time, it may be possible to use serial imaging to assess the efficacy of antiseizure treatment. For example, in focal status epilepticus, we have shown that anesthesia sufficient to suppress seizure activity also results in reversal of imaging abnormalities. Serial studies of brain perfusion after seizures suggest that replacement of hyperperfusion with hypoperfusion may be a useful index of therapeutic efficacy in some patients. Similarly, it may be possible to use careful analysis of the degree of T2 signal change and local volumetric measurements to better assess the efficacy of therapy. As some imaging findings after seizures are remote from the site of maximal ictal activity, it is important to evaluate imaging abnormalities in conjunction with clinical and electrographic data that provide information about the localization of ictal activity. Particularly when these modalities are convergent, the imaging findings may support a plan for early surgical intervention and help in the process of surgical planning.

See also: **Imaging**: Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; SPECT in Epilepsy Research; Structural Magnetic Resonance Imaging of Epilepsy; **Pediatric Epilepsy**: Seizure-Induced Injury and the Two-Hit Hypothesis.

Further Reading

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Structural Imaging in Epilepsy

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Introduction

Structural neuroimaging has greatly contributed to the overall understanding of epilepsy pathogenesis, and currently plays a valuable role in the diagnosis and management of epilepsy patients. Improvement in image quality and resolution over the past several decades has translated clinically into greater sensitivity, and as a result has increased the detection of focal brain lesions. This technical progress ultimately impacts treatment and clinical outcome, particularly when patients are offered the chance of cure through lesionectomy.

The further advancement of structural imaging is essential. Currently, many patients must undergo intracranial EEG (IEEG) monitoring because the location of epileptogenic origin is unclear. In the analysis of pathology specimens following surgical resection, it is not uncommon to find small focal cortical dysplasias (FCDs). Should structural neuroimaging resolution continue to improve to the point of detecting FCDs, IEEG could be used in a more limited fashion, requiring the implantation of fewer electrodes that are more precisely placed. It is also possible that highly sensitive structural neuroimaging, combined with functional neuroimaging studies, will in some cases obviate the need for invasive monitoring altogether.

The following section discusses the two most commonly applied modalities in structural neuroimaging, computed tomography, and magnetic resonance imaging. Each modality's current application to the field of epilepsy, as well as its short-comings, is reviewed. This section is followed by a detailed discussion of recent developments and improvements in the field of structural neuroimaging, and the potential impact such advancements might have on the clinical management of patients.

Background

Presently, the two primary modalities of structural neuroimaging are computed tomography (CT) and magnetic resonance imaging (MRI). Each modality serves a different purpose in the clinical evaluation of a patient with epilepsy. The basic physiologic principles of these two imaging techniques are explained, followed by a discussion of the general utility of, and indication for, each type of study.

Computed Tomography

Computed tomography (CT) uses ionizing radiation, which passes through the tissue being studied. The radiation is absorbed at varying levels, depending upon tissue composition. Different tissue compositions will produce different absorption patterns and images. As a result, CT imaging of the brain is able to delineate bone, CSF, and soft tissue. Overall, imaging contrast is excellent with regard to bone, but soft tissue structures are less clearly distinguishable. Despite this short-coming, CT offers certain advantages over other imaging modalities such as MRI. For example, its relatively low cost, broad accessibility, and speed of image acquisition are features which make CT an appealing imaging modality. In the emergent setting, CT tends to be the first line of diagnostic imaging due to its aforementioned rapidity. Especially when the patient has an altered mental status or focal findings on neurologic exam, rapid imaging is the most sensible course of action to rule out acute pathology. For this reason CT is also the imaging study of choice in the perioperative state, when the quick detection of recent hemorrhage, hydrocephalus, and major structural changes is paramount.

However, CT is less sensitive in the detection of small focal cortical and white matter abnormalities when compared to MRI. Studies have shown that CT does not reliably detect small tumors or hippocampal sclerosis (HS), which is a major disadvantage. HS is one of the more common abnormal findings in patients with temporal lobe epilepsy, and often serves as an indication of localization-related epilepsy, potentially amenable to surgical treatment. The failure to detect such a lesion could negatively impact the patient's clinical course, deviating from a path optimal treatment. As a result, when it comes to the evaluation of patients with epilepsy, the utility of head CT is limited.

In 2007, the International League Against Epilepsy (ILAE) published guidelines on performing a head CT in the emergent setting of a patient presenting with a first time seizure. The ILAE study group concluded that 'immediate noncontrast CT is possibly useful for emergency patients presenting with seizure, to guide appropriate acute management especially where there is an abnormal neurologic examination, predisposing history, or focal seizure onset.' This statement speaks to the three primary attributes of CT – speed, accessibility, and affordability – as well as the ability of CT to quickly detect brain hemorrhage, ischemia, or large structural

abnormalities, all of which are potentially life-threatening and epileptogenic. The ILAE also recommends that an MRI be performed if an initial head CT is normal in patients with intractable epilepsy. Overall, MRI is preferable to CT in the nonemergent, outpatient setting. CT becomes the imaging modality of choice when MRI is not available or not possible secondary to patient characteristics (implanted devices, obesity), or not suitable due to an emergent clinical situation.

Magnetic Resonance Imaging

When performing a routine evaluation of a patient with epilepsy, magnetic resonance imaging (MRI) is the imaging procedure of choice as it offers multiple advantages over CT, including its excellent soft tissue contrast and high anatomical resolution.

MRI does not employ ionizing radiation. Rather MRI utilizes the natural magnetic properties of hydrogen atoms, which are present in human tissue to varying degrees depending upon the water component. The hydrogen nucleus possesses a single proton which rotates haphazardly on its own axis, producing a small magnetic field. The MRI magnet produces its own intense field, which aligns all of the hydrogen nuclei in the tissue being imaged. Energy in the form of a pulsed radiofrequency is then emitted by MRI coils, which collides with the protons, pushing them out of alignment. As the protons relax back to their original energy state, they emit a signal which is detected by the MRI coils. The variation of water composition between different tissues results in the investigator's ability to visually delineate structures; the higher the water content in an area, the more hydrogen protons there will be emitting signals. These signals are translated into shades of gray which vary depending upon signal strength. Through this process, the radiologist is able to differentiate between bone, CSF, white matter, and gray matter.

The strengths of MRI, particularly its superior resolution and delineation of structures, make this imaging modality well suited to evaluate epilepsy patients. One of the most common pathologic findings in patients with temporal lobe epilepsy (TLE) is mesial temporal sclerosis (MTS). MTS is pathologically characterized by hippocampal sclerosis (HS), with neuronal loss and gliosis seen on histology. This pattern is readily detected on MRI by hippocampal atrophy, increased signal on T2-weighted images or fluid-attenuated inversion recovery (FLAIR) sequences, and decreased signal on inversion recovery sequences. Part of a typical 'epilepsy MRI protocol,' which includes angulated coronal sections perpendicular to the long axis of the hippocampal structures, increases the likelihood of detecting MTS. Similarly, prior studies have illustrated the importance of the imager's level and area of training and expertise. HS detection rates are higher when the interpreting physician is experienced in reading epilepsy protocol MRIs. MRI is currently felt to be the most effective imaging modality for the detection of MTS, with over 90% of cases being successfully identified.

MRI is also useful in the detection of malformations of cortical development (MCDs) which are common in children with epilepsy. MCDs are characterized as being either diffuse or focal in nature. Diffuse malformations such as lissencephaly, band heterotopia, and periventricular nodular heterotopia, as well as focal malformations consisting of hemimegalencephaly, schizencephaly, and focal cortical dysplasias (FCDs), can be defined accurately through structural imaging. The latter tend to be the most common developmental abnormalities observed in children with extratemporal lobe epilepsy. An abnormal cortical mantle with altered gray–white matter architecture and thickened cortex are characteristic features of FCDs on MRI. However, these lesions are often small. The likelihood of their being detected increases if one targets a clinically suspected region, using three-dimensional (3D) volume techniques, surface coils, and inversion recovery sequences. Recent advances in this area, and application of the technology to the neuroimaging of tuberous sclerosis (TS), are discussed in greater detail in the following section.

Imaging Indications

Not all patients with epilepsy require neuroimaging. Patients with uncomplicated febrile convulsions and a normal neurological exam may not require imaging studies. In addition, patients with well-defined idiopathic generalized epilepsy, or with typical idiopathic benign partial epilepsy with centro-temporal spikes (Rolandic epilepsy), usually do not require imaging. However, structural MRI abnormalities have been reported in each of the latter two cases, making it difficult to dictate absolutely when imaging should and should not be performed. While further studies are needed, in general the presence of atypical clinical features, or the development of an atypical clinical course in patients with seemingly benign or nonfocal epilepsy, should raise a red flag and prompt an imaging study.

As opposed to the primary generalized epilepsies, all patients with symptomatic generalized or focal seizures should have an MRI. An MRI should be performed if a patient's seizures do not fit into any of the recognized epilepsy syndromes. When seizures persist in the presence of a previously normal CT scan, or when there are progressive neurologic changes on the clinical exam of a patient with epilepsy, an MRI is indicated. MR imaging should be routinely repeated 2–5 years following a previously normal MRI in any patient whose seizures remain refractory. Furthermore, an MRI should be repeated if the seizure semiology or pattern changes. Again, an epilepsy-specific protocol with fine cuts through the hippocampus should be performed, and preferably using a high strength magnet.

Limitations

Despite the obvious utility of MRI in the evaluation and treatment of patients with epilepsy, the technology is not perfect. While MRI is far superior to CT with regard to sensitivity and delineation of gray–white structures, there is still room for improvement in the differentiation of structures and cortical layers. In addition MRIs can be laden with artifact caused by implanted devices, and it is not uncommon that patient movement distorts images, rendering studies uninterpretable. The sections which follow explore some of these issues by focusing on current research and new developments in the field of structural neuroimaging.

Methods and Results – Recent Advances in Structural Neuroimaging Techniques

The utility of structural neuroimaging in the management of patients with epilepsy will likely continue to increase as technology advances. In that context, it is useful to address three major limitations of structural neuroimaging: (1) suboptimal sensitivity and limited detection of small lesions; (2) unidimensional representation of cortical structures – which limits the investigator's ability to accurately localize lesions; and (3) unavoidable introduction of artifact, which degrades image quality and limits interpretation. These issues are discussed separately in the following sections by focusing on some of the latest research developments in neuroimaging. There is special focus on a study currently underway at the New York University Comprehensive Epilepsy Center, where voxel image analysis is being used to study relationships between epilepsy and psychiatric symptoms. A final section of this article explores the application of neuroimaging to the diagnosis and treatment of tuberous sclerosis (TS), a genetically determined abnormality that often presents with seizures.

Refining Sensitivity and Delineation of Structures: High Field MRI

The quest for better imaging has resulted in the development of high field magnets such as 3–7 Tesla (T) units. Three Tesla (3 T) MRI was first approved in 2001 for clinical use. The theoretical doubling of signal (compared to the conventional 1.5 T MRI magnet) affords more detailed imaging, particularly important when small focal lesions are sought. However, despite the increased magnet strength, there are some drawbacks to 3T MRI, chiefly due to the limitation of individual specific absorption rates (SARs) and the doubling of chemical shift misregistration. The latter problems result in decreased contrast between gray and white matter on T1 weighted images, a major drawback when attempting to identify MCDs in epilepsy patients. However, recent refinements in 3 T imaging have helped to address this issue. Development of the eight-channel coil has led to dramatic improvement of image quality and gray–white contrast. Schmitz and colleagues reduced the refocusing pulse (RF2) of spin echo pulse sequences from 180° to 120°, which similarly improved contrast (see Delano *et al.* in Further Reading). Some investigators have found that using the conventional spin echo technique produces an image superior to one produced using the fast spin echo technique, as the latter can be associated with image blurring, also noted in the Delano et al. article. The use of inversion-prepared sequences, such as inversion-prepared 3D fast spoiled gradient echo (FSPGR), also produces images with excellent gray–white contrast, as do fluid-attenuated inversion recovery (FLAIR) sequences.

Unlike T1-weighted 3 T imaging, which has been fraught with problems concerning gray–white contrast, T2-weighted images generated on a 3 T MRI are of very high quality. T2-weighted sequences have excellent gray–white differentiation, as well as superior delineation of pathological structures. Through the adjustment of different imaging parameters, T2-FLAIR sequences have even greater gray–white contrast than the latter. This trend will undoubtedly progress as magnet strengths continue to increase and imaging parameters, in response, are refined.

Improving Dimensionality: Volumetric MRI

When viewed in isolation, the images produced by conventional MRI are 2D representations of a patient's neuroanatomy. By incorporating the third dimension, voxel-based morphometry (VBM) more accurately represents the location of a proposed epileptogenic focus or lesion, and its relation to other surrounding cortical and subcortical structures. The latter can be especially helpful to neurosurgeons performing epilepsy surgery.

VBM typically measures gray matter voxels. A voxel is the smallest measurable box-shaped unit of volume that comprises a larger 3D image. More simply, it can be thought of as a 3D version of a pixel. Thus, gray matter voxels are the smallest measurable volumetric units that together make up the total volume of gray matter on a brain image. Following a series of steps involving image processing and statistical analysis, a 3D surface rendering of the cortical gyri can be produced. Such images can be used to describe cerebral malformations, and to illustrate surface anatomy. Using VBM, one can also assess the thickness and volume of mesial temporal structures such as the hippocampus – particularly useful to epileptologists who often rely on imaging to determine whether HS is present. A recent study by Bonilha *et al.* (see Further Reading section) went one step further, demonstrating

that patients with left mesial temporal lobe epilepsy (MTLE) had a wider area of atrophy related to hippocampal gray matter loss, compared to patients with right MTLE. The authors suggested that this finding may help to explain the larger degree of cognitive impairment usually observed in patients with left versus right hippocampal atrophy.

Currently at the NYU Comprehensive Epilepsy Center, structural neuroimaging research in volumetric analysis is underway. Specifically, VBM is being performed on epilepsy patients who suffer from post-ictal psychosis (PIP), comparing their cortical thickness with that of other epilepsy patients without this history. The proposed hypothesis is that epilepsy patients who experience psychotic episodes post-ictally are anatomically different from those epilepsy patients who do not. It is possible that differences in cortical volume exist between PIP patients and non-PIP controls. This study employs the automated method for measuring cortical thickness as described by Fischl and Dale (see Further Reading secion). The technique generates very accurate models of both the gray/white and pial surfaces of the brain through image segmentation. The segmentation process uses both intensity and continuity information from a 3D high-resolution, high signal-to-noise MR image volume to produce accurate representations of both the gray matter–white matter boundary and the outer cortical surface (Figure 1). As noted in Fischl and Dale, by measuring the distance between gray/white and pial surfaces, the thickness of the cortical gray matter can be determined at any location with submillimeter accuracy. Cortical thickness maps can then be generated on an inflated brain surface, allowing for comparisons of cortical thickness across the whole brain surface between patient groups (Figure 2). Such imaging methods may not only prove to be useful in the analysis of epilepsy patients with PIP, but also may one day serve as a routine method for assessing temporal lobe volume in patients with presumed temporal lobe epilepsy, or the progression of cortical volume loss in patients with neurodegenerative disease.

Decreasing Artifact: The PROPELLER Technique

At times, patient movement in the MRI scanner is unavoidable and generalized anesthesia must be administered. When present, movement artifact can render images largely uninterpretable. In an attempt to resolve this problem, a new method of image acquisition was developed. Termed 'periodically rotated overlapping parallel lines with enhanced reconstruction' ('PROPELLER'), this sequence provides excellent contrast between gray and white matter despite the presence of patient movement.

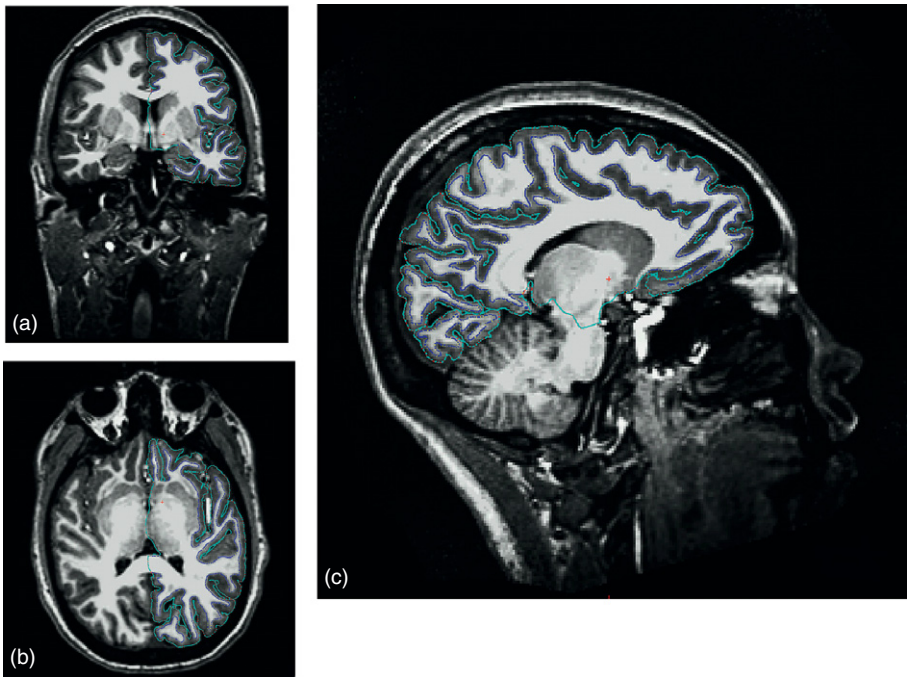


Figure 1 Cortical reconstruction. Example of MR image segmentation in the coronal (a), axial (b), and sagittal planes (c, enlarged view). The segmentation procedure uses both intensity and continuity information from a 3D high-resolution, high signal-to-noise MR image volume to produce accurate representations of both the gray matter/white matter boundary (blue line) and that of the outer surface of the cortex (green line). Thickness measurements are obtained by calculating the distance between the two surfaces across the entire volume in each hemisphere. Images provided by Dr. Thomas Thesen, NYU Comprehensive Epilepsy Center.

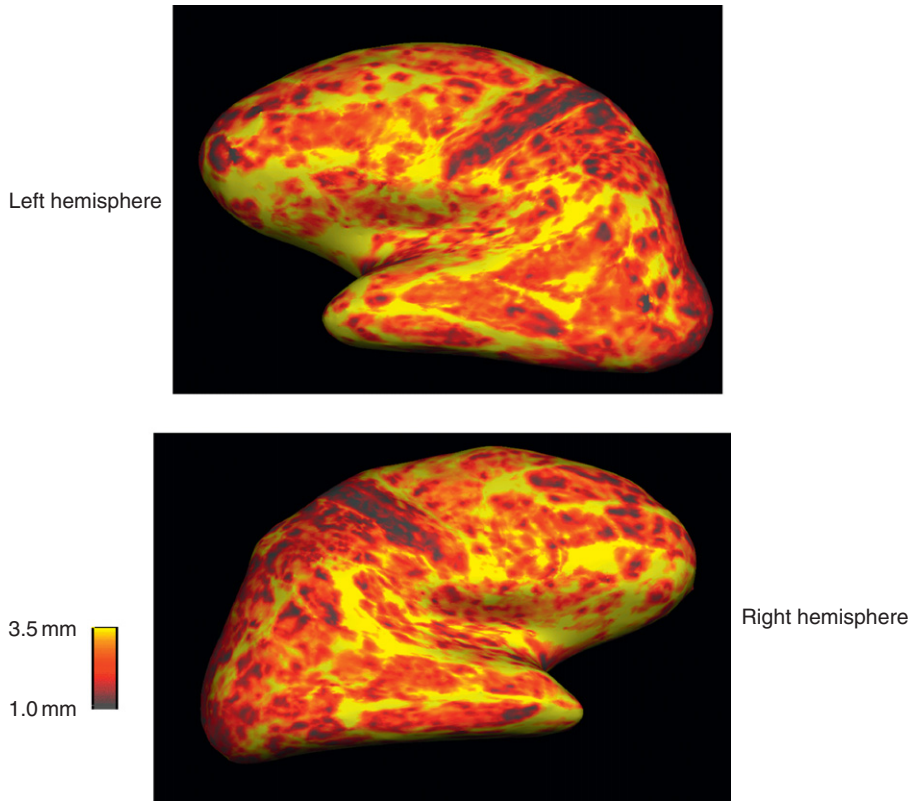


Figure 2 Example of cortical thickness maps of one patient on an inflated brain surface. Yellow indicates greater thickness (scale from 1.0 to 3.5 mm). Averaged maps in a standardized space allows for comparisons of cortical thickness across the whole brain surface between patient groups. Images provided by Dr. Thomas Thesen, NYU Comprehensive Epilepsy Center.

Both translational and rotational head movements are accounted for, greatly reducing motion artifact. This advance is achieved by averaging images and rejecting those data that correlate with through-plane motion. The latter explanation is provided by Eriksson *et al.* (see Further Reading section), who sought to compare imaging of the hippocampus on PROPELLER T2-weighted images with routine T2-weighted images. They concluded that PROPELLER images had superior tissue contrast, showing the internal hippocampal structures and tissue changes associated with hippocampal sclerosis. By refining this imaging technique and making it more readily available to all epilepsy centers, one could avoid the need for sedation, which carries its own set of morbidity and mortality risk factors. This capability would be a great advancement, particularly for the field of pediatric epilepsy where sedation for imaging is fairly commonplace.

Further Applications: The Neuroimaging of Tuberous Sclerosis

The successful treatment of epilepsy in the setting of tuberous sclerosis (TS) is often heavily reliant on invasive EEG monitoring and structural neuroimaging. By clearly identifying the presence of cortical tubers, structural neuroimaging can both define the extent of disease within the central nervous system and help guide intracranial electrode placement in those patients bound for surgery.

Tubers are characterized as well-circumscribed areas of signal hypointensities on T1-weighted MRI and as signal hyperintensities on T2-weighted sequences (see Luat *et al.* in Further Reading section). The reverse pattern is observed in neonates and young infants due to incomplete myelination. The detection of tubers is even better using fluid attenuated inversion recovery (FLAIR) sequences. Complementing these more conventional sequences is the newly developed technique termed magnetization transfer imaging (MTI), which can detect tubers obscured by myelination as noted in the Luat et al. article. Another potentially valuable tool is the magnetization transfer ratio (MTR), which reflects brain lesion histology. For example, Zikou *et al.* (see Further Reading section) found that the MTRs of TS brain lesions were significantly lower compared to controls. They determined that this finding likely correlates with the depleted myelin sheaths and microscopic cystic degeneration typical of TS lesions. In contrast, subependymal nodules – which are composed of more densely packed cells – were associated with a higher MTR.

Another investigational technique is diffusion tensor magnetic resonance imaging (DT-MRI). DT-MRI detects alterations in two primary parameters: apparent diffusion coefficient (ADC) and fractional anisotropy, both of which can be used to characterize TS lesions. Increased ADC values have been noted in epileptogenic hamartomas for instance. In addition, Peng *et al.* (see Further Reading section) found that the fractional anisotropy value of tubers in TS patients was significantly lower versus controls. Lastly, DT-MRI can potentially track white matter fibers and assess their integrity. It is hoped that application of this imaging technique will lead to the identification of any anatomical abnormalities that may exist beyond the borders of a tuber, including fiber networks involved in neuronal recruitment and ictal spread. The ability to delineate such a neuronal network with imaging would have a profoundly positive impact on the treatment of TS-related epilepsy; comprehensive excision of all nonessential tissue with epileptogenic potential is the ultimate goal.

Future Goals

Clearly, many of the imaging techniques described above are in their infancy, and further development and refinement are needed. Once the sensitivity, reproducibility, and efficiency of such techniques are optimized, larger-scale clinical trials studying efficacy and clinical utility of the new technology will be warranted. The impact of these new imaging modalities on the field of epilepsy has yet to be determined, but certainly seems promising.

Acknowledgements

We would like to thank Dr. Thomas Thesen, Ph.D., for providing the images featured in this chapter.

See also: **Imaging**: Imaging Characterization of Familial Temporal Lobe Epilepsies; Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Structural Magnetic Resonance Imaging of Epilepsy.

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Structure and Function: Imaging Connectivity Using Tractography

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Introduction

The anatomy of white matter pathways is crucial to our understanding of both normal and abnormal brain function. To date, these connections have been studied using direct visualization, predominantly in animal brain specimens. Because this work is intrinsically slow, and drawing inferences from animal models is difficult, our knowledge of cerebral white matter connections in humans is still very limited. MRI-based tract mapping techniques offers the prospect of noninvasive, in vivo imaging of white matter tracts for the first time. Furthermore, this technique can be employed synergistically with clinical, fMRI, PET, and electrophysiological data to obtain insights into both the anatomy and the function of these connections. This knowledge has diverse clinical applications to patients with epilepsy.

The goal of structural MRI in epilepsy patients is to identify those patients suitable for surgery, and locate the epileptogenic lesion or zone (by using concurrent clinical, electrophysiological, and other imaging data). The epileptogenic lesion can then be resected with minimal collateral damage to eloquent cortex. In patients with cerebral lesions, the localization of cognitive activation may differ from the pattern in normal subjects, and therefore functional MRI (fMRI) is useful to delineate areas of eloquent cortex such as primary sensorimotor cortex, essential language areas, occipital visual areas, and mesial temporal regions crucial for episodic memory. By noting the anatomical relation of these areas to the planned resection, postoperative complications can be minimized. The use of tractography makes it possible to delineate white matter fiber tracts that connect these eloquent cortical areas, so inadvertent surgical damage can be avoided, thus further reducing postoperative complications. Moreover, this technique also lends itself to explore the reorganization of white matter networks in chronic epilepsy, which may underlie changes in cognitive function.

Background

The Biological and Physical Basis of Diffusion Based Imaging

In a free medium, the molecular diffusion of water refers to the random translational motion (Brownian motion) of molecules resulting from the thermal energy carried by these molecules. In the brain, diffusion is restricted by intra and extracellular boundaries, and represents the effects of several variable, independent factors. These factors include the presence of impermeable or semipermeable membranes, macromolecules that hinder the diffusion of small molecules, and intra and extracellular microcirculatory effects. The measurement of water diffusion, therefore, provides a means of probing cellular integrity and pathology.

The principles of diffusion MRI were first developed in vivo in the mid-1980s. In diffusion-weighted imaging (DWI), images are sensitized to the diffusional properties of water by the incorporation of pulsed magnetic field gradients into a standard spin echo sequence. By taking measurements in at least three directions, the mean diffusion properties could be characterized within a voxel in the image; these properties are expressed by way of a single scalar apparent diffusion coefficient (ADC). Early diffusion studies discovered that ADC measurements depended on a subject's orientation relative to the magnet and gradient coils. White matter tracts parallel to an applied gradient had the greatest ADC whereas those lying oblique or transverse to a gradient had smaller ADC values. This observation gave rise to the concept of asymmetry of diffusion of molecules in three directions, or 'anisotropy.'

Diffusion tensor imaging (DTI) enables not only the quantification of water molecule diffusion, but also the characterization of the degree and direction of anisotropy. The diffusion tensor is a mathematical construct that can be calculated from a nondiffusion-weighted image plus six or more diffusion-weighted measurements along noncollinear directions. The tensor can be diagonalized to give three eigenvectors – ε_1, ε_2, and ε_3 – representing the principal directions of diffusion, and three eigenvalues – λ_1, λ_2, and λ_3 – representing the magnitudes of diffusion (or the corresponding ADC values) along these directions. The eigenvectors are ordered with decreasing values of their eigenvalues, so that ε_1 represents the principal direction of diffusivity, and λ_1 will be the ADC value along ε_1. This value corresponds to the maximum diffusion in each voxel, regardless of the orientation of the subject's head.

Furthermore, several diffusion parameters can be derived in each voxel, which are insensitive to subject positioning and fiber tract alignment within the diffusion gradients of the MRI scanner. Mean diffusivity (MD) is a

summary measure of the average diffusion properties of a voxel and is equivalent to the estimated ADC over three orthogonal directions. Fractional anisotropy (FA), in contrast, is an estimate of what proportion of the magnitude of the diffusion tensor is due to anisotropic diffusion. Anisotropy in white matter results from the organization of tissue as bundles of axons and myelin sheaths run in parallel; the diffusion of water is freer and quicker in the long axis of the fibers than in the perpendicular direction. Malformations or acquired insults cause disruption to the microstructural environment, and more often than not, a reduction in anisotropy. Such abnormalities may also lead to a reduction in cell density and/or expansion of the extra cellular space, resulting in an increase in MD/ADC.

From DTI to Tractography

With conventional MRI, variations in white matter signal are subtle and white matter tracts cannot be accurately parcellated. In most studies, DTI quantitative measures have been assessed using either region of interest (ROI) or voxel-based analysis. The former has limitations in that it is user-dependent, and can lack precision (i.e., other white matter tracts, gray matter or CSF may be included erroneously in the analysis). Voxel-based analysis, although observer-independent, has problems associated with the need for spatial normalization and smoothing due to anatomical variations in ventricular size, gyral patterns etc. Both methods have limited ability to quantify specific white matter pathways along their entire trajectories. Tractography is an extension of DTI whereby the directional information obtained in each voxel is used to generate virtual, three-dimensional white matter maps. These maps are based on similarities between the diffusion properties of neighboring voxels in terms of shape (quantitative diffusion anisotropy measures) and orientation (principal eigenvector map). Tractography does not, therefore, trace fibers in the sense that injected tracers do; rather it demonstrates the path of least resistance to water diffusion.

Methods

Selecting a Seed Region

In most tractography experiments, to delineate the white matter structure of interest, a 'seed' region within or adjacent to the structure needs to be defined. These seed regions, from which tracking will commence, are often defined using anatomical reference points. As a result, they are susceptible to operator bias, and this bias can introduce a source of variability into tractography results. The combination of fMRI and tractography enables the use of operator-independent placement of seed regions. Furthermore, it offers an opportunity to study the relationship between brain structure and function by providing a selective tracing of white matter pathways within a behaviorally-characterized network. In our studies of language networks, therefore, our group has used verbal fluency, verb generation, and reading comprehension paradigms to define functional regions, which were then used to generate start regions for tractography.

Tractography Algorithms

Various algorithms have been devised to generate white matter maps using the information provided by diffusion imaging. Linear or streamline methods use the orientation of maximum diffusion at each voxel, and are performed by following these orientation estimates to reconstruct pathways that, within a coherent bundle, correspond to the underlying fiber pathway. They therefore define a single route of connection for each start point(s) or ROI, from voxel to voxel. These streamline approaches are susceptible to errors in the orientation of ε_1 due both to noise and to instances in which the direction of the underlying tract anatomy is ambiguous or reflects the presence of multiple noncollinear fiber pathways. These streamline approaches also assume absolute knowledge in fiber direction at every point, with no representation of the uncertainty present in the recovered pathway. An erroneous pathway, due to noise or partial volume effects, is therefore assigned as much significance as 'true' pathways.

For this reason, in our laboratory we use a probabilistic tractography approach to exploit the inherent uncertainty in the orientation of ε_1 for each voxel to generate maps of connection probability. Orders of uncertainty based on the anisotropy of the tensor and the relative magnitudes of the eigenvalues are used to generate probability density functions (PDFs) of the result of the diffusion tensor mixture model. These PDFs are a means of describing the local uncertainty in fiber orientation. Each PDF is intended to interpret the information from a diffusion imaging acquisition in terms of the likely underlying fiber structure at each point within the brain. The end result is that for every voxel, a distribution of directions rather than a single principal eigenvector is obtained. Any of these points can then be selected as a starting place for running a streamline propagation process. This process can be repeated a large number of times (N), and the number of times that any voxel in the brain is encountered over N repetitions provides an index of connectivity for that voxel to the start point. This process eventually yields a map of probability of connection, or a Probabalistic Index of Connectivity (PICo), to the chosen seed point. The probability of connection in any experiment generally decreases with distance from the start point due to the cumulative effect of the uncertainties in propagation at each step in the streamline process.

The effects of noise on this model are also explicitly simulated and used to analyze the uncertainty associated with the principal diffusion direction of each diffusion tensor. This uncertainty, and therefore the distribution of the principal diffusion direction, decreases as the FA increases.

Tract Analysis

The tracts generated in individual subjects can be analyzed at a qualitative level. Tracts from different individuals can also be combined to generate group maps, which give an indication of how reproducible a given connection is across a population of subjects.

Finally, the white matter tracts can also be used to derive quantitative information, including volume and anisotropy parameters. These measures can then be compared across groups, or tested for correlations against fMRI or clinical data.

Results

Language – The Superior Longitudinal Fasciculus

Temporal lobe epilepsy (TLE) may be associated with disrupted lateralization of language, and may be further impaired by anterior temporal lobe resections (ATLR). Indeed, selective language deficits have been reported in up to 30% of patients following dominant hemisphere ATLR. The superior longitudinal fasciculus is known to be a crucial part of the language network, connecting anterior (Broca's) and posterior (Wernicke's) language areas. In a study of 14 patients with unilateral TLE and 10 controls, the language paradigms described earlier were used to generate seed regions for tractography in both Broca's and Wernicke's area. Controls and right hemisphere TLE patients were found to have a left-lateralized pattern of both language-related activations and associated white matter organization; measures of FA and volume were statistically greater on the left than those on the right side. Left TLE patients showed more symmetrical language activations (in comparison with both controls and right TLE patients), along with reduced left hemisphere and increased right hemisphere white matter pathways (**Figure 1**).

Correlations between measures of structure (FA) and function in both patient groups were found, with subjects with more lateralized functional activation having more lateralized white matter pathways. The findings of this study, therefore, demonstrate that tractography is able to identify the structural network underpinning the lateralization of language function elucidated by fMRI studies.

Memory – The Parahippocampal Gyrus

Parahippocampal structures, which are critically implicated in the generation and propagation of seizures in TLE, are also essential for declarative memory. Longitudinal neuropsychological studies have shown that persisting epilepsy is associated with progressive memory impairment. Those who undergo ATLR are at further risk of memory impairment, the nature of which depends on whether surgery is on the dominant or nondominant side. Patients typically show a decline in verbal memory following surgery involving the language-dominant hemisphere and deficits in topographical memory following nondominant temporal lobe resection. Neuropsychological assessment, quantitative MRI, and (more recently) fMRI indicate that the medial temporal lobe structures (MTL) play a role in sustaining material specific memory functions; these studies have also demonstrated that reorganization of memory occurs with TLE and that memory deficits after ATLR are related to the functional integrity of the parahippocampal structures. The strength of connections, or structural connectivity, of the parahippocampal gyrus in TLE has not been evaluated or related to function.

Our group performed tractography in 18 patients with unilateral TLE undergoing presurgical evaluation, and in 10 healthy controls. An anatomically-derived seed region in the anterior parahippocampal gyrus was selected from which to trace the white matter connections of the medial temporal lobe. A correlation analysis was also carried out between volume and mean FA of the connections on the one hand, and preoperative material specific memory performance on the other hand. There was no significant difference between the left- and right-sided connections in controls. In the left TLE patients, the connected regions ipsilateral to the epileptogenic region were found to be significantly reduced in volume and mean FA compared with the contralateral region (**Figure 2(a)**). Significant correlations were found in left TLE patients between left and right FA, and verbal and nonverbal memory, respectively. Though there was a trend towards ipsilateral reduction in volume and FA in right TLE patients, this difference was not significant (**Figure 2(b)**). Similarly, no correlations between left or right FA and memory measures were found in this group of patients. It is unclear why no differences were seen in right TLE patients. It is possible that the speech-dominant hemisphere has greater and denser connectivity to the rest of the brain, and that it is more sensitive to damage than is the nonspeech-dominant hemisphere. The finding of fewer occipital connections in the left TLE group compared with right TLE patients and controls may also be significant in this respect (**Figure 2(a)** and **2(b)**). Animal models suggest that potential roles for these connections may involve the priming of mesial temporal lobe structures

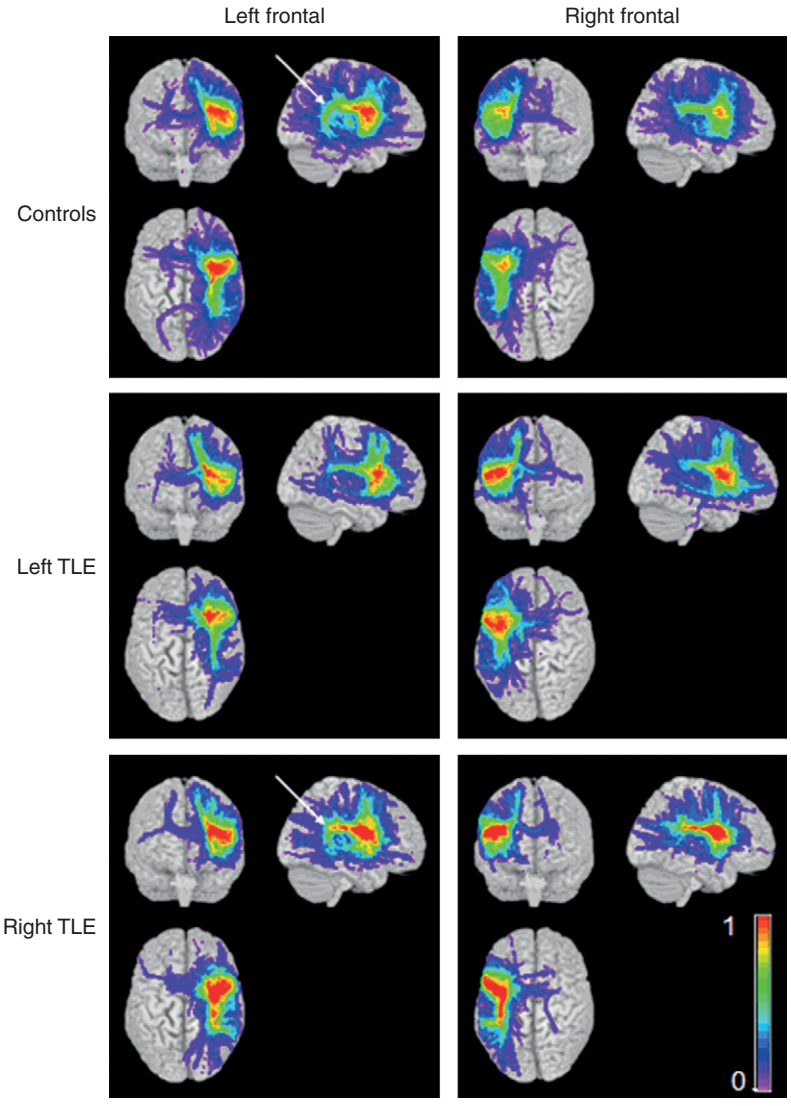


Figure 1 Group variability maps of the connecting paths tracked from left and right functionally defined frontal ROIs for each of the three groups controls, Left TLE and Right TLE patients. Each image shows the maximum intensity of the commonality maps in each plane of view as a brain surface rendering. The color scale indicates the degree of overlap among subjects (expressed as commonality value); for example, a value of 1 (pure red) represents 100% subject overlap (i.e., every subject's identified tract contains the voxel in question). Controls and right TLE patients show a similar pattern of connections with greater SLF connections to the temporal lobe on the left (arrowed) than the right. In the left TLE group the opposite pattern is seen with greater temporal lobe connections on the right. Reprinted from Powell HW, Parker GJ, Alexander DC, Symms MR, Boulby PA, Wheeler-Kingshott CA, Barker GJ, Koepp MJ, Duncan JS (2007) Abnormalities of language networks in temporal lobe epilepsy. *Neuroimage* 36(1): 209–221, with permission from Elsevier.

to facilitate the consolidation of visual memory or to enhance the visual processing of emotionally significant stimuli. Visual cues may be important not only for nonverbal memory but also for verbal memory. This could explain the correlation of FA found with verbal and nonverbal memory in left TLE patients. In the right TLE patients, the occipital connections did not seem to be as severely affected, and hence these patients may have been able to use visual cues to aid both verbal and nonverbal memory.

Other groups have assessed other memory structures within the limbic system. Concha *et al.* found that patients with unilateral TLE have bilateral changes in the fornix and cingulum bundle, characterized by impaired tracking of these pathways, and increased MD and reduced FA along them. This result was thought to be consistent with the degeneration of pathways connecting to the hippocampus (**Figure 3**). Studies by the same group have assessed the progression of Wallerian degeneration in the limbic structures in patients with refractory epilepsy after corpus callostomy and temporal lobe resections.

Taken together, these results suggest that the use of tractography-derived quantitative measures may have a significant role to play in the longitudinal evaluation of the effects of epilepsy on the brain, and on cognitive

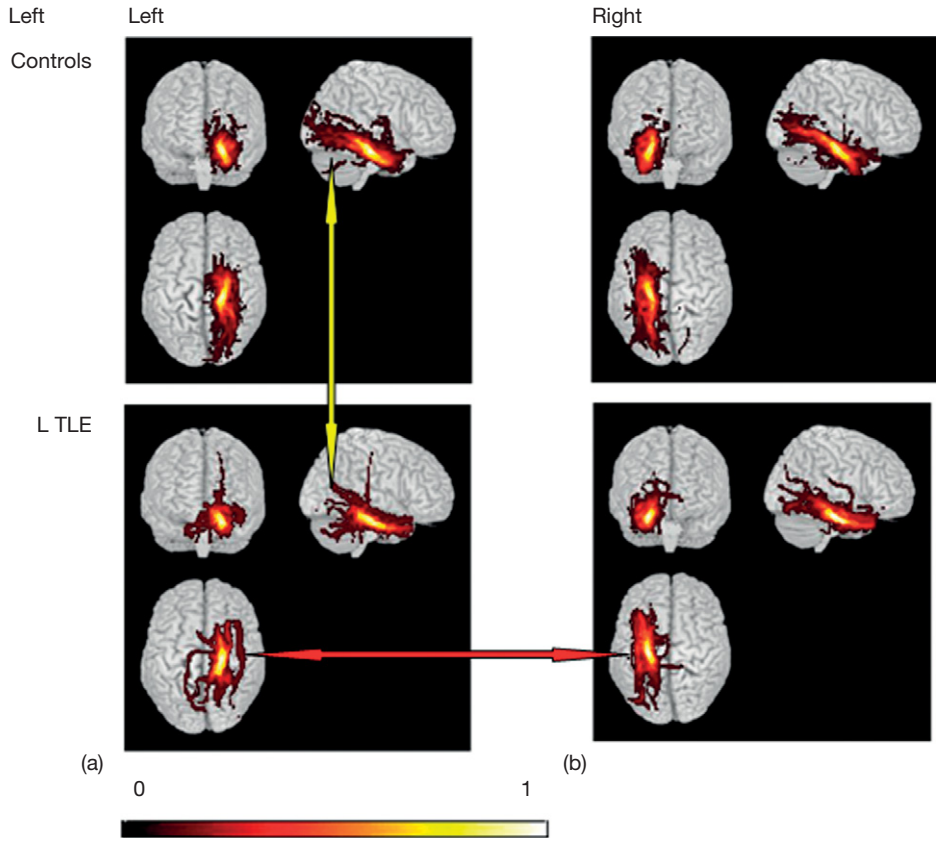


Figure 2 Group maps of the parahippocampal gyrus tracts comparing left (a) and right (b) TLE patients against controls. Tracts are overlaid upon a 3D rendered brain template. A voxel commonality value of 1.0 indicates that each individual had a connection identified in this voxel while a *C* value of 0.0 indicates that none of them did. In controls, PHG connections were symmetric. There is more marked asymmetry of connections in left TLE patients compared with controls and right TLE patients. There is a decrease in ipsilateral compared with contralateral connections in left TLE, such that the body of the PHG appears smaller (red arrow). In addition, fewer connections to the occipital lobe are present in left TLE patients compared with controls and right TLE patients (yellow arrow). Reprinted from Yogarajah M, Powell HW, Parker GJ *et al*. (2008) Tractography of the parahippocampal gyrus and material specific memory impairment *Neuroimage* 40: 1755–1764, with permission from Elsevier.

functions such as memory and language, particularly when correlated with neuropsychological measures.

Preoperative Planning – Visual Pathways

ATLR can also cause visual field defects (VFD) in up to 80% of patients. Indeed, in up to 10% of patients this defect can be severe enough to render the patient ineligible for a driving license, despite the patient's being seizure-free. Typically, VFDs after ATLR occur in the superior homonymous field contralateral to the resection, and are due to disruption of fibers of Meyer's loop. The anterior extent of the Meyer loop is not visualized on conventional imaging and varies from person to person. As a consequence, the occurrence and extent of a postoperative VFD cannot be accurately predicted by conventional MRI, or from the extent of resection performed. Tractography has been used to demonstrate the optic radiation in normal subjects, and has previously been applied to pre and postoperative surgical patients with AV malformations and tumors in and around the visual pathways. A study by our group demonstrated the application of this approach to temporal lobe surgery for epilepsy (**Figure 4**). The optic radiation was visualized before and after ATLR, and disruption of Meyer's loop was clearly demonstrated in a patient who developed a quadrantanopia. More recent studies by other groups have confirmed the utility of this technique.

Future Goals

The development of tractography-based techniques is still at its infancy, and faces several major challenges. The most pressing of the current problems is the lack of a 'gold standard' method of validation. Tractography is essentially a user-defined process, and tracking results may vary according to the algorithm used, size and definition of a ROI, FA threshold for termination of tracking, angular threshold, step length, and numbers of sampling

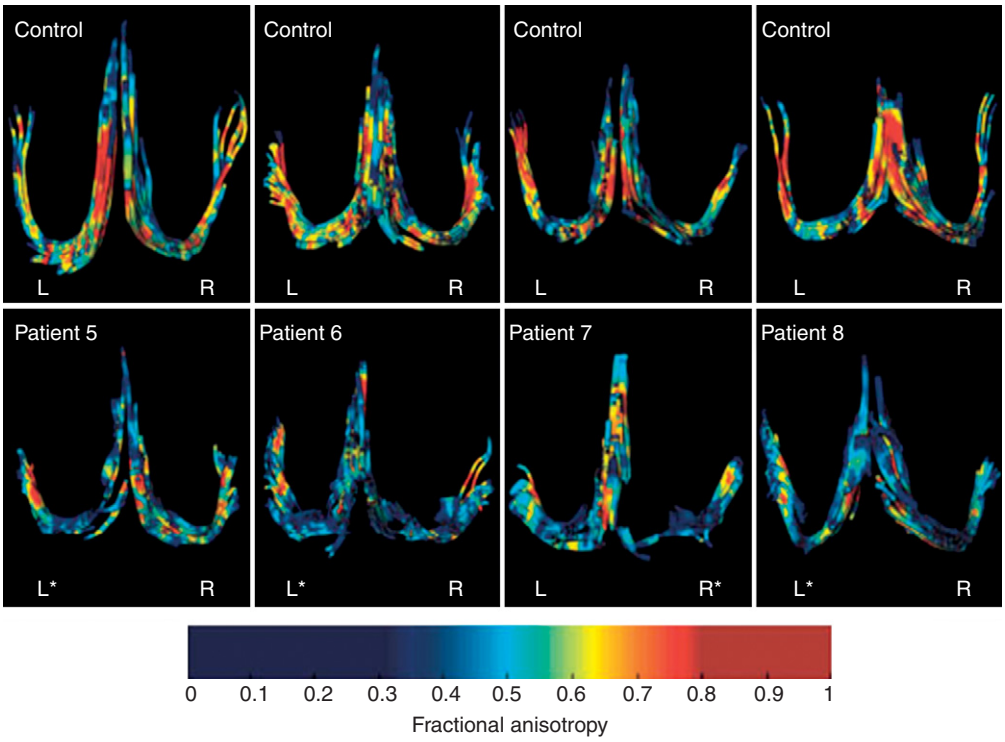


Figure 3 Tractography of the fornix in four control subjects and four patients with unilateral hippocampal sclerosis (HS). Bilateral three-dimensional visualisation of the fornix viewed from above, where fractional anisotropy (FA) values are color-coded for each voxel. The hemisphere with MTS is denoted by an asterisk. Note that patients show overall lower FA values and less continuous tracts bilaterally compared with control subjects. These results imply marked degradation of the fornix in both hemispheres. Reprinted from Concha L, Beaulieu C, and Gross D (2005) Bilateral limbic diffusion abnormalities in unilateral temporal lobe epilepsy. *Annals of Neurology* 57(2): 188–196, with permission from Wiley.

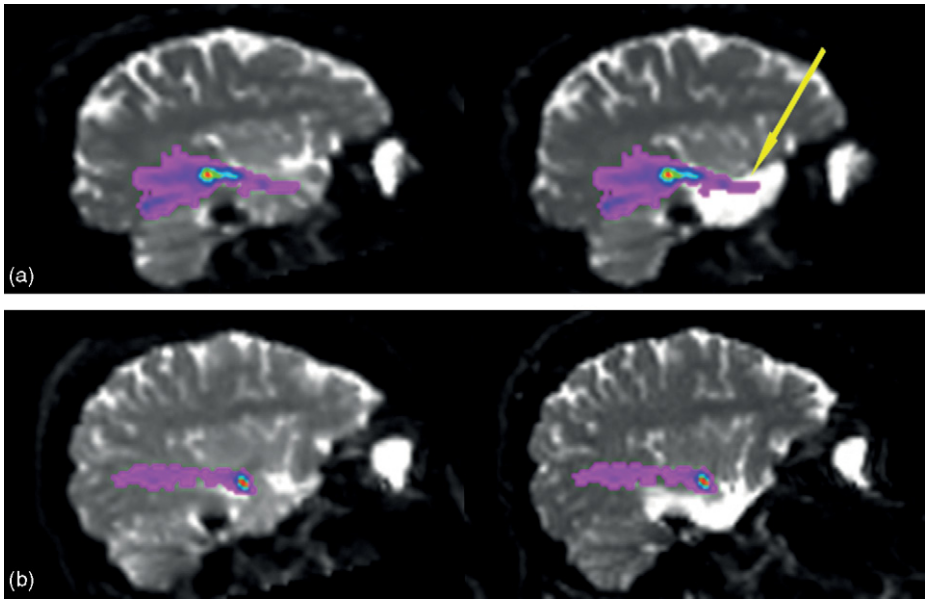


Figure 4 Tractography of the optic radiation in two patients who underwent anterior temporal lobe resections (ATLR), superimposed on sagittal MR images. Preoperative images on the left and postoperative images on the right. Patient A suffered a quadrantic field deficit postoperatively due to surgical interruption of the optic radiation (arrow). The visual fields of patient B remained intact. Reprinted from Powell HW, Parker GJ, Alexander DC *et al*., (2005) MR tractography predicts visual field defects following temporal lobe resection. *Neurology* 65: 188–196, with permission from Wiley.

within a voxel. Despite the concordance of results in studies on language, and studies showing that the technique can reconstruct (and is consistent with) major fiber tract trajectories, there is a need for robust standardized tests to assess the different algorithms and data acquisition techniques. Pre and postoperative tractography studies in epilepsy surgery may be in a prime position to rectify this problem. The use of tractography together with MEG, fMRI, and fMRI–EEG has the potential to provide complementary but equally important information in these patients. Furthermore, concordance of results derived from these techniques in the same patient groups would be very helpful in the validation of tractography-based techniques.

The other challenges of tractography relate to the quality of data acquisition and the tracking algorithms. Current problems include eddy current distortions, gradient nonlinearities, motion artifact, signal loss due to susceptibility variations, and low resolution acquisition. The relatively low signal-to-noise ratio (SNR) of DTI data introduces errors; SNR is inversely proportional to spatial resolution, in that increasing spatial resolution (i.e., decreasing voxel size) leads to a reduction in SNR. Using a higher magnetic field or longer acquisition times can compensate for this loss of SNR. Even at higher spatial resolutions, however, the size of a typical imaging voxel is 2–3 mm cubed, so a single voxel could contain thousands of axons. This limited spatial resolution can lead to false positives, and can lead to the false definition of a tract direction in a voxel, especially in the presence of fibers crossing or touching within individual voxels. In addition, low SNR may also lead to false negatives by ignoring small fiber tracts that may be functionally important.

Despite these limitations tractography is the only technique available for tracing white matter pathways in vivo. Furthermore, as methodological developments occur in orientational and spatial resolution, and in diffusion modeling and tractography algorithms, these limitations should prove less of a problem. By applying tractography both postoperatively and in a longitudinal fashion, it is hoped that tractography-derived data, in synergy with fMRI and neuropsychological data, may provide valuable prognostic information regarding cognitive outcomes in patients with refractory epilepsy. In addition, when the technical challenges of coregistration of preoperative tractography with the T1-weighted MR images are surmounted, preoperative tractography of the optic radiation and other vital white matter connections will be helpful in planning and undertaking surgical procedures. Further, the advent of intra-operative MRI will allow the correction of the movement of tracts caused by craniotomy, and will improve the accuracy of the data used in surgical planning, resulting in a lower risk of postoperative deficits. Finally, it is hoped that this imaging approach may lead to the development of novel approaches that allow us to functionally disconnect the seizure focus from the surrounding brain.

See also: **Cognitive Dysfunction and Other Comorbidities**: Cognitive Function in Temporal Lobe Epilepsy; Language and Communication Disorders; **Imaging**: Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Structural Magnetic Resonance Imaging of Epilepsy.

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NEUROPATHOLOGY

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Neuropathology of Epilepsy

Long Term Epilepsy-Associated Tumors

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Introduction

Among the large spectrum of brain tumors, a distinct group of neoplasias can be identified in patients suffering from chronic focal epilepsies, i.e., long-term epilepsy-associated tumors (LEATs). With few exceptions, these tumors share one or more of the following features: (1) LEATs behave, clinically, in a benign manner and correspond to grade I or II, as defined by the World Health Organization (WHO); (2) LEATs occur predominately in the temporal lobe; (3) LEATs have a histologically variable appearance consisting of neuronal and glial components (glio-neuronal tumors); (4) LEATs are likely to originate during early brain development and are often associated with cortical dysplasias; (5) LEATs do not share those oncogenes or tumor suppressor genes typically observed in common brain tumors, i.e., malignant gliomas. In contrast, neurodevelopmentally regulated genes and signaling pathways are likely to be involved, i.e., reelin and mTOR.

LEATs can be detected in epilepsy patients using modern neuroimaging techniques. However, the characterization of the epileptogenic zone remains challenging and should be examined in specialized centers. It remains to be shown whether the tumor itself or adjacent brain parenchyma, often characterized by structural as well as molecular reorganization patterns, contribute to seizure susceptibility and pharmaco-resistance.

Increasing availability of surgical tumor specimens open the possibility to address, neuropathologically as well as neurobiologically, the origins and epileptogenicity of tumor-associated focal epilepsies. Intriguingly, most studies of these tissues have failed to identify genetic or chromosomal aberrations that are usually associated with neoplastic transformation – i.e., loss of TP53 tumor suppressor gene or EGFR oncogene amplification. In contrast, neurodevelopmentally regulated genes are likely to play a role, such as the insulin growth factor receptor pathway involved in the tuberous sclerosis complex (TSC, mTOR) or reelin signaling associated with neuroblast migration and cortical layering. Research analysis of LEAT opens, therefore, also new avenues to understanding brain development under physiological as well as pathophysiological conditions.

Background

Neuropathological Classification of LEATs

The benefit of tailored resection strategies is increasingly recognized for the treatment of patients with intractable focal seizures and benign brain tumors. Surgical resection strategies that encompass the lesion as well as the epileptogenic area result in seizure freedom in ~80% of cases. The comprehensive database of the German Neuropathological Reference Center for Epilepsy Surgery currently includes 3319 samples, of which 938 were diagnosed as LEATs (Table 1).

Unfortunately, many tumors escape early detection in patients with early seizure onset (duration of epilepsy showed a mean 13.2 years), and the various consequences of long-term seizure disorders on mental as well as social development and behavior are considerable. Histopathological diagnosis of LEAT remains challenging. There is a large spectrum of morphological phenotypes, even within neuropathologically defined entities (Table 2). Neuropathological evaluation should, therefore, be obtained from experienced centers. The need for such expertise is critical, since the variability of histopathological phenotypes may often result in a high-grade tumor diagnosis, whereas the long-term clinical course of LEAT behaves very modestly, without enhanced risk of recurrence and/or malignant transformation.

LEAT and Epileptogenesis

Pathogenic mechanisms underlying focal hyperexcitability in LEAT patients need further clarification. Two major hypotheses should be considered. The

Table 1 Neuropathological findings in focal chronic epilepsies

Diagnosis	*Number*	*Age (years)*	*Onset (years)*	*Duration (years)*
Mesial temporal sclerosis	1109	33.9	10.7	22.7
Tumors	938	28.2	14.8	13.2
Dysplasias	407	18.5	4.7	11.6
No lesions	225	28.3	12.4	15.8
Vascular	195	35.9	20.1	12.3
Scars	167	23.9	9.2	14.7
Dual pathology	150	23.1	8.4	13.1
Inflammation	52	20.7	13.7	8.4

Number = number of cases; age = age at operation; onset = age at onset; duration = duration of epilepsy. Data obtained from the German Neuropathological Reference Center for Epilepsy Surgery (www.epilepsie-register.de).

Table 2 Neuropathological spectrum of brain tumors in epilepsy patients

Diagnosis	*in %*	*Age (years)*	*Onset (years)*	*Duration (years)*
GG (WHO I)	46.0	25.1	25.1	13.7
GG (WHO II)	5.0	26.3	12.2	11.7
GG (WHO III)	0.9	38	29.3	4.7
DNT (WHO I)	16.4	26.6	13.8	12.7
A (ISO)	1.7	28.9	15.3	16.2
A (WHO I)	4.9	25.4	11.9	14.5
NOS	4.8	29.1	16.1	16.0
PXA (WHO II)	2.4	30.8	19.2	15.2
SEGA (WHO I)	1.4	18.8	4.5	14.8
A (WHO II)	4.4	33.8	25.7	6.8
A (WHO III)	2.4	39.5	37.0	10.3
O (WHO II)	2.7	35.6	20.6	13.9
O (WHO II)	0.9	40.7	14.7	22.3
OA (WHO II)	2.6	34.8	18.8	10.7
OA (WHO III)	1.2	43.0	21.0	16.3
Cysts	1.9	33.4	21.0	14.2
Others	0.4	6.3	3.0	3.3

The upper half of the table refers to typical LEAT entities, which represent more than 80% of all brain tumors detected in patients with chronic focal epilepsies: GG, ganglioglioma; DNT, dysembryoplastic neuroepithelial tumor; A (ISO), isomorphic astrocytoma variant; A (WHO I), pilocytic astrocytoma; NOS, these tumors cannot be classified according to the revised WHO classification system (2007); PXA, pleomorphic xanthoastrocytoma; SEGA, subependymal giant-cell astrocytoma. The remaining cases (lower half of table) belong to the group of common glial tumors, although malignant variants are rare: A, astrocytoma; O, oligodendroglioma; OA, mixed oligo-astrocytoma; cysts, epidermoids, dermoids, or arachnoidal cysts. In %, % of the 938 tumor samples obtained from the German Neuropathological Reference Center for Epilepsy Surgery (see **Table 1**), age = age at operation; onset = age at onset; duration = duration of epilepsy.

neuronal component of the tumor itself may contribute to epileptogenic activity. Immunohistochemical studies have identified aberrant expression of neuroactive molecules in dysplastic neurons, such as glutamatergic neurotransmitter receptors. Whether these neurons functionally integrate and excite neuronal pathways remains to be shown. A recent study applying intracerebral recordings from electrodes implanted to localize seizure onset identified early ictogenic discharges from a ganglioglioma (GG). Such findings would support a hyperexcitable neuronal tumor component functionally integrated into excitatory circuitries. An alternative mechanism involves tumor-associated, epileptiform changes in the adjacent brain. Altered expression patterns of neuroactive molecules within the perilesional brain parenchyma, as well as the clinical observation that the epileptogenic area may be substantially larger than the tumor mass itself, would be consistent with this hypothesis. In many patients, seizure relief cannot be achieved if only the lesion is resected. Neuronal hyperexcitability within perifocal brain areas may be evoked by kindling mechanisms in limbic structures such as hippocampus and amygdalae, as has been observed in experimental animal models. Besides molecular reorganization phenomena in adjacent brain tissue, enhanced epileptogenicity may also evolve from architectural disturbances of cortical layering (similar to those observed in focal cortical dysplasia type I). Such alterations are frequently encountered in LEAT patients, in particular with dysembryoplastic neuroepithelial tumors (DNTs). Whether such cortical dysplasias should be considered as 'dual pathology' – i.e., underlying a different pathomechanism compared with adjacent tumorigenesis – awaits further clarification.

Methods: Neuropathology of LEAT Entities

Neuropathological examination and diagnosis relies on microscopical inspection of surgical brain specimens obtained from patients with chronic, focal epilepsies and follows the current WHO classification and grading scale (last revised in 2007). This classification scheme has proven useful for the prediction of the biological behavior of an individual tumor entity. However, the large spectrum of LEATs and their variable histomorphological appearance is not fully reflected by the WHO grading system. The following description will, therefore, summarize the most frequent LEAT entities (see **Table 2**).

Gangliogliomas

Whereas gangliocytomas (i.e., Lhermitte–Duclos disease) are homogeneously composed of a dysplastic neuronal-cell population, GGs present with a broader spectrum of histopathological alterations (**Figure 1(a)–(d)**). Besides the recognition of dysplastic neurons (more than one nucleus, condensed Nissl-substances along the cell membrane, irregular anatomical orientation), an additional

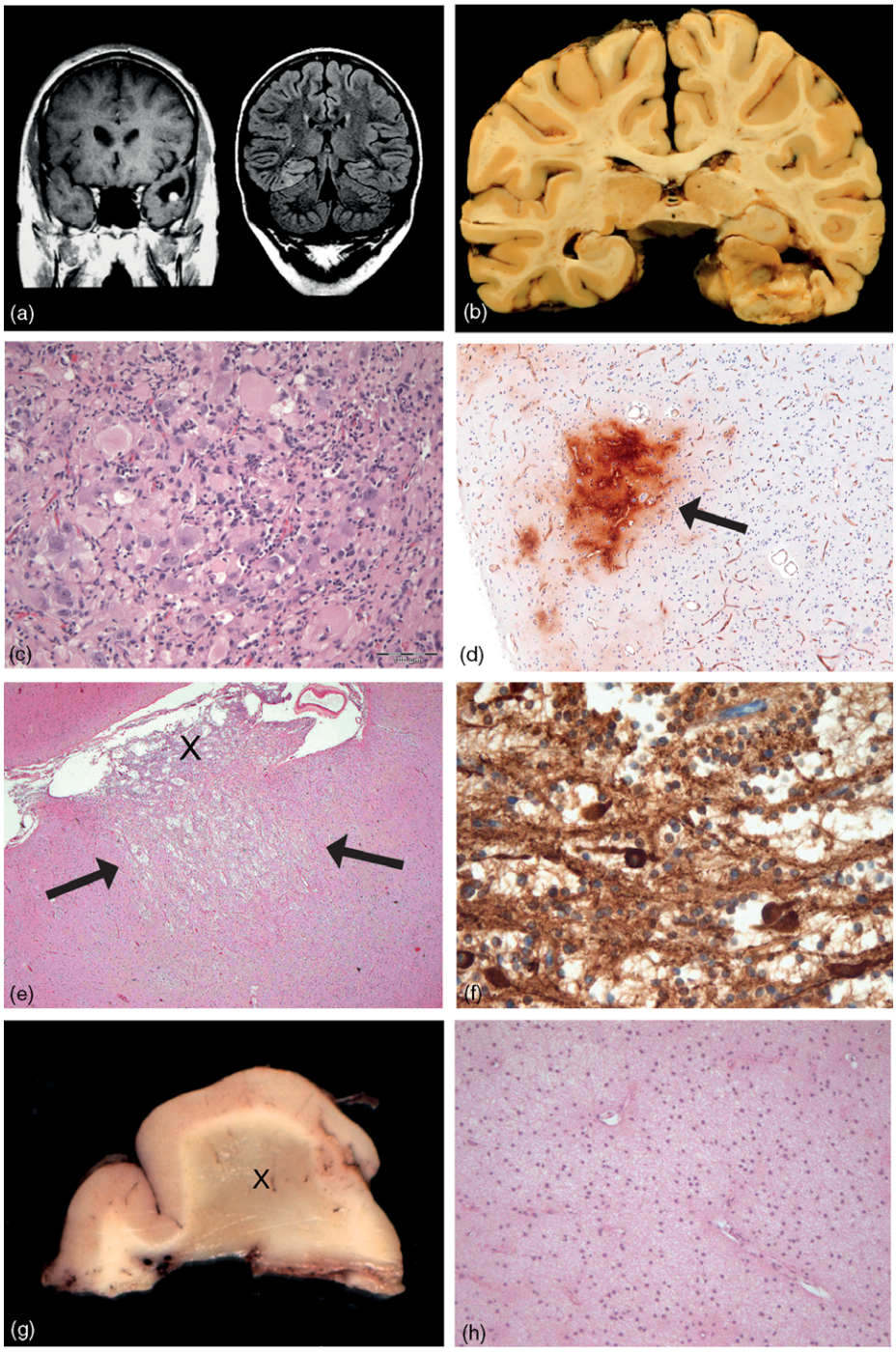


Figure 1 Long-term epilepsy-associated tumors (LEATs). (a) Magnetic resonance imaging in two patients with intractable temporal lobe epilepsies and histopathologically confirmed gangliogliomas (GGs). The different gross appearance of both lesions exemplifies the large spectrum of morphological variants. (b) Autopsy specimen obtained from an elderly patient with a GG located in the right temporal lobe, courtesy of Dr. P Kleihues, Freiburg, Germany (with kind permission). (c) High-power magnification of a GG (WHO grade I). Note the admixture of irregular oriented, dysplastic neurons (larger nuclei and prominent nucleoli) and neoplastic glia cells (200× magnification). (d) Adjacent to the tumor shown in (c), a cluster of CD34-immunoreactive tumor cells was detected in adjacent neocortex. (40× magnification). (e) Dysembryoblastic neuroepithelial tumors (WHO grade I) predominantly occur in superficial neocortical localization (arrows). This tumor also involves also the subarachnoidal space (x) and is characterized by a microcystic appearance (25× magnification). (f) At high-power magnification, mature neurons (MAP2-immunoreactivity) are recognized among tubules of neuropil, which are aligned by oligodendroglial-like cells (specific glio-neuronal element) (200× magnification). (g) Surgical specimen obtained from a young epilepsy patient with a suspicious tumor in the temporal lobe (X), courtesy of Dr. V Hans, Bielefeld-Bethel, Germany. (h) Histopathological examination revealed an isomorphic astrocytoma variant with a benign cellular phenotype (100× magnification).

neoplastic glial-cell population can be identified. This glial component most commonly shows features of astrocytic differentiation, but oligodendroglial-like cells are also recognized. The composition of neuronal and glial cells varies to a considerable extent, with tumors presenting a predominantly neuronal phenotype (in these cases, the differential diagnosis of a gangliocytoma (WHO grade I) should be considered) or predominantly glial phenotype (the differential diagnosis of an astrocytoma (WHO grade II) needs consideration). Additional histopathological features, which can be observed in GGs, are calcification, lymphocytic infiltrates, as well as microcysts. Tumors may also present with an increased vasculature, mimicking cavernomas. Subarachnoidal spread of the tumor can be detected in superficially located tumors and confirm autonomous growth. Additional variants with papillary organization or rosetted neuropil islands have been identified; the latter may present with a less-favorable prognosis.

Immunohistochemistry

Immunohistochemical reactions are mandatory to establish the diagnosis of GGs (Table 3). Almost 80% of tumors are labeled with antibodies against the stem-cell epitope CD34, which does not occur in normal, mature neuroepithelial tissue. In GGs, CD34 immunoreaction product is confined to the outer cellular membrane of glial and neuronal cells, either within the bulk tumor mass or small tumor clusters in adjacent, microscopically unsuspicious cortical regions. Dysplastic neurons accumulate neurofilament proteins or harbor a perisomatic rim of synaptophysin ('corona'). The glial tumor population often expresses glial fibrillary acidic protein (GFAP) whereas glial MAP2-staining is absent. The proliferation index is low (1–2% of cells are labeled with the Ki67 epitope). Higher proliferation activities may result from CD68-positive microglia cell infiltration and should be further investigated. If the glial-cell component contributes to higher proliferation activity, atypical or anaplastic variants of GGs should be considered (see later).

Table 3 Differential diagnosis of LEAT according to their immunohistochemical profile

LEAT	*GFAP*	*MAP2*	*CD34*	*P53*	*Ki67*
GGs	+	+(N)	+	−	1–2%
DNT	+	+(G/N)	−	−	1%
Astrocytoma (isomorphic variant; WHO I)	+	−	−	−	<1%
Astrocytoma (pilocytic; WHO I)	+	+	(+)	−	1–5%
Astrocytoma (WHO II)	+	+	−	(+)	<5%
PXA (WHO II)	+	+	+	−	var.
SEGA (WHO I)	+	+	−	−	var.

DNT, dysembryoplastic neuroepithelial tumor; GG, ganglioglioma; PXA, pleomorphic xanthoastrocytoma; SEGA, subependymal giant-cell astrocytoma; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein; +, epitope is present within the neoplastically transformed glial-cell population; in GG, only the neuronal component (N) is labeled, a few DNT neurons and glia (G) are immunodecorated; CD 34, stem-cell epitope; p53, gene product of the TP53 cell-cycle regulator/tumor suppressor gene; (+), TP53 mutations are present in almost 50% of diffuse astrocytomas; Ki67, proliferation antigen; numbers relate to those areas of the tumor with the highest labeling index.

Differential diagnosis

Since GGs are usually associated with a benign prognosis (WHO grade I), distinction from pilocytic astrocytomas (WHO grade I), diffuse astrocytomas (WHO grade II) and oligodendrogliomas (WHO grade II) should be confirmed. A critical issue to be addressed is the presence of dysplastic neurons within cortical tumor spread, which can also be mimicked by infiltration of diffuse gliomas. However, immunohistochemical reactions for CD34 and MAP2 are most helpful. Distinction from other mixed glio-neuronal tumors (i.e., DNTs) or cortical dysplasia may be difficult to obtain in small surgical specimens.

Molecular pathology

Although characteristic glioma-associated tumor suppressor genes or oncogenes are not affected, neurodevelopmentally regulated signaling cascades appear to play a role. Increased frequency of rare polymorphisms in those genes associated with the tuberin/insulin growth receptor pathway (mTOR) support this hypothesis. In addition, decreased expression of the reelin-signaling cascade has been identified. However, the pathomechanism underlying abundant expression of the stem-cell marker CD34 in GGs remains to be elucidated. Biphasic differentiation patterns in GGs challenge molecular neuropathology and stress the need for cell lineage-specific analysis. Laser-assisted microdissection indeed supports a dysontogenic origin from a glioneuronal precursor lesion with neoplastic, clonal proliferation of the glial-cell component.

Grading

In a large series of 184 patients with supratentorial GGs, a median follow up of 8 years identify tumor recurrence in only 3%. However, malignant progression (2%) and death (1%) have been encountered (Figure 2). Favorable clinical outcome associates with (1) tumor location within the temporal lobe, (2) complete surgical resection, and (3) histopathological classification according to WHO grade I. Although the current WHO classification system 2007 does not specify histological parameter of atypical

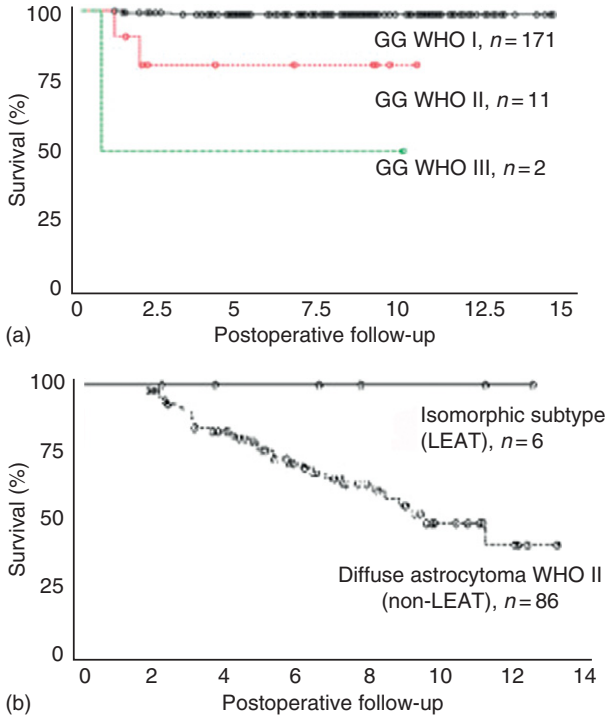


Figure 2 Clinico-pathological correlation in two different LEAT entities. (a) Follow up of 184 gangliogliomas according to the current WHO classification system 2007. Note the favorable survival after complete resection. Modified from Luyken C, Blumcke I, Fimmers R, Urbach H, Wiestler OD, and Schramm J (2004) Supratentorial gangliogliomas: Histopathologic grading and tumor recurrence in 184 patients with a median follow-up of 8 years. *Cancer* 101: 146–155. (b) In contrast to diffusely infiltrating astrocytomas (WHO grade II) obtained in 86 nonepileptic (non-LEAT) patients, the histopathological distinct isomorphic variants associates with a benign long-term outcome. Modified from Schramm J, Luyken C, Urbach H, Fimmers R, and Blumcke I (2004) Evidence for a clinically distinct new subtype of grade II astrocytomas in patients with long-term epilepsy. *Neurosurgery* 55: 340–347.

GGs (WHO grade II) or anaplastic variants (WHO grade III), increased cellularity, nuclear pleomorphy, and proliferation (>5% Ki67 labeling index) within the glial component may indicate a semibenign nature of the tumor. Significantly increased proliferation index above 10% and tumor necrosis should be regarded as signs of anaplastic progression (WHO grade III).

Dysembryoplastic Neuroepithelial Tumors

DNTs manifest with multiple nodules within the neocortex and are associated with intractable seizures (**Figure 1(e)–(f)**). The histopathological hallmark is the 'specific glio-neuronal element,' characterized by axonal tubes lined by oligodendroglia-like cells and floating neurons embedded in a mucoid matrix. However, many tumors display additional histopathological features such as cortical dysplasia or other neoplastic differentiation patterns, resembling pilocytic astrocytomas, oligodendrogliomas, or GGs. Whereas the detection of dysplastic neurons in the vicinity of the tumor should be regarded as component of its dysontogenic origin (and not as 'dual pathology'), variants with additional features of vascular malformations, pilocytic astrocytomas, or GGs are regarded as complex form of DNTs (WHO grade I). There is no hint for unfavorable prognosis in these variants.

Immunohistochemistry

Floating neurons are usually mature and can be immunohistochemically characterized by neurofilament protein, MAP2 or synaptophysin. The glial component of this tumor usually does not stain for GFAP, but expresses S-100. CD34 is absent in neuroepithelial tumor cells. Ki67-positive nuclei are rare (below 1%), supporting the concept of a dysembryoplastic origin of this tumor entity.

Differential diagnosis

Confirmation of the 'specific glio-neuronal element' and multinodular occurrence should be decisive for the diagnosis. However, a prominent clear cell phenotype warrants the differential diagnosis of oligodendrogliomas. In fact, the DNT was recognized almost 20 years ago from a cohort of epilepsy-associated oligodendrogliomas with benign prognosis. The complex form of DNTs may be more challenging for routine histopathology. Since histological features of astrocytomas, oligodendrogliomas, and GGs can be recognized in this tumor entity,

immunohistochemistry using CD34, MAP2, synaptophysin, and S-100 is mandatory for the differential diagnosis.

Molecular pathology

Histomorphological similarities with other clear cell tumors of the nervous system – i.e., oligodendrogliomas, mixed oligoastrocytomas, or neurocytomas – have initiated molecular genetical analysis of specific chromosomal regions. Compared with oligodendrogliomas, however, loss of heterozygosity testing has not revealed alterations at chromosome 1p and 19q, nor have TP53 mutations been identified. Although the molecular pathogenesis of DNT appears to be entirely unknown, a recent report described familial occurrence at same age and cortical location. Thus, genetic analysis of affected family members may provide novel clues to its pathogenesis.

Astrocytomas

Histopathological grading and differential diagnosis of low-grade gliomas are challenging in epilepsy patients, and investigators should consider a large spectrum of morphological variants – i.e., pilocytic astrocytomas (WHO grade I), diffusely infiltrating astrocytomas (WHO grade II or III), or the recently described angiocentric glioma and isomorphic astrocytoma variants. In recent years, molecular–genetic findings emerged as valuable means to support this distinction. In low-grade diffuse astrocytomas, a significant portion of tumors presents with mutations in cell-cycle control genes, i.e., TP53, and associates with a higher risk for anaplastic progression. In contrast, there is still no reliable genetic marker available for the identification of pilocytic astrocytomas or pleomorphic xanthoastrocytomas (PXAs), nor for their risk of tumor recurrence and malignant progression.

Pilocytic astrocytomas

PAs are common tumors of the cerebellum in children and young adults. However, pilocytic astrocytomas may occur at all other sites of the CNS, i.e., as intraspinal tumors or optic pathway gliomas ('optic gliomas') in patients suffering from neurofibromatosis type 1. Since pilocytic astrocytomas can occur in any age group, this tumor has to be always considered in the differential diagnosis of low-grade gliomas and LEAT.

Histopathological hallmarks of pilocytic astrocytomas include prominent 'hairy (piloid)' cellular processes, Rosenthal fibers, and eosinophilic granular bodies. In contrast to diffusely infiltrating astrocytomas, microvascular proliferation and tumor necrosis may occur but are not associated with anaplastic transformation. An increasing number of reports deal with the risk for tumor recurrence and malignant progression in pilocytic astrocytomas. The pilomyxoid variant should, therefore, be differentiated (WHO grade II).

Isomorphic astrocytoma

Recently, we identified a novel astrocytoma variant in a series of 19 patients with chronic seizures and diffuse astrocytomas (**Figure 1(g)–(h)**). Histopathological hallmarks included low cellularity, lack of mitotic activity, and highly differentiated astroglial elements infiltrating into adjacent brain parenchyma. Compared with 'classical' variants of diffuse astrocytomas (WHO grade II), immunohistochemical reactions reveal cellular proliferation below 1%, absence of nuclear p53 accumulation, as well as lack of glial MAP2 and CD34 expression. These features distinguish the isomorphic astrocytoma subtype from GGs, pilocytic astrocytomas, and DNTs as well as from cortical dysplasia or reactive gliosis. Long-term survival and lack of tumor progression after a maximal follow-up time of 13 years further support the distinction of this clinically and histopathologically benign astrocytoma variant, and are consistent with classifying this tumor as WHO grade I (**Figure 2**).

Pleomorphic Xanthoastrocytoma

This glioma variant frequently infiltrates the subarachnoidal space, and shows a histopathological pattern consisting of pleomorphic glial cells, presenting with multinucleated giant cells, and intermingled with vacuolated, xanthochromic cells. The latter results from intracellular accumulation of lipids, and are usually responsible for the macroscopically recognizable yellowish appearance of the tumor. There is an ongoing debate with respect to the malignant behavior of this tumor entity. The WHO consortium assigns PXAs to WHO grade II or III (anaplastic variant). Molecular genetic studies may advance our understanding of this intriguing tumor entity.

Subependymal Giant-Cell Astrocytoma

This rare low-grade glioma is always associated with the TSC. Exophytic tumor growth originating from the ependymal-cell layer extending into the ventricular system is usually observed. Histopathologically, these tumors are composed of ganglioid astrocytes. Clinically, malignant progression has not been encountered and this tumor entity is classified according to WHO grade I. Hence, the presence of prominent cellular atypia in a subependymal giant-cell astrocytoma (SEGA) is not a criterion for anaplastic transformation.

Future Goals

Tumor-associated epilepsies represent a fascinating interface between clinical and basic research. To understand pathogenic mechanisms of neoplastic transformation in neuroepithelial cells remains a major topic in the

neurosciences, but the glio-neuronal phenotype of most LEAT entities challenge modern neurodevelopmental concepts as well. Early disease onset, benign clinical courses, variable pathomorphological patterns, and detection of the onco-fetal marker CD34 point rather towards compromised developmental signaling pathways than enhanced proliferation or apoptosis of genetically transformed tumor progenies (as reasoned in high-grade tumors). With increasing availability of surgical epilepsy specimens, these intriguing issues can be directly addressed in human brain using a large variety of modern anatomical, molecular, genetic, as well as electrophysiological techniques. Recent evidence for the involvement of the reelin-signaling cascade as well as mTOR pathway (associated with insulin growth factor receptor and TSC) support this notion and will further corroborate our understanding of human brain development under physiological as well as pathophysiological conditions.

However, poorly understood epileptogenicity of focal (neoplastic) brain lesions represents another challenging issue in the field of LEATs. Since appropriate animal models are not yet available, the investigator must turn to human brain slices; slices from within the tumor itself or within adjacent brain parenchyma can be examined with respect to cellular and molecular alterations. Recent studies describe different brain 'compartments' to be involved, including neurotransmitter systems, blood–brain-barrier disturbances, altered composition of extracellular matrix molecules, or even aberrant drug transporter expression. Human brain tissue can be directly retrieved from the operation theater and, therefore, successfully used for most experimental studies. Notwithstanding, any alteration detected in individual patients then needs to be confirmed in animal models with respect to its involvement in altered 'epileptogenic' network signaling and/or enhanced seizure susceptibility.

Last but not least, molecular genetic studies addressing brain tumors should be further advanced to determine predictive factors in those patients suffering from increased risk of tumor recurrence or malignant transformation. The proportion of these patients is rather small in LEATs, but will help to improve clinical treatment strategies in patients with chronic focal epilepsies and brain tumors.

Acknowledgements

The Neuropathological Reference Center for Epilepsy Surgery is a consortium of distinguished colleagues from the following German epilepsy centers: (Berlin) H. J. Meencke, M. Merschhemke, N. T. Lehmann; (Bielefeld) A. Ebner, H. W. Pannek, F. Woermann, V. Hans; (Bonn) C. Elger, C. Bien, C. Helmstaedter, J. Schramm, H. Clusmann, H. Urbach, A. Becker, M. Majores; (Erlangen) H. Stefan, B. Kasper, E. Pauli, M. Buchfelder, A. Dorfler, T. Engelhorn, I. Blumcke, M. Hildebrandt; (Freiburg/Kehl-Kork) B. Steinhoff, A. Schulze-Bonhage, S. Fauser, J. Zentner; (Greifswald) S. Vogelgesang; (Marburg) F. Rosenow, S. Knake; (Munich) P. A. Winkler, S. Noachtar; (Stuttgart) P. Winkler; (Ulm) H. Lerche; and (Vogtareuth) H. Holthausen, T. Pieper.

See also: **Development/Malformations**: Focal Malformations of Cortical Development: Pathogenesis and Pathways; **Glia/Astrocytes**: Peritumoral Epilepsy; Tumor-Induced Epilepsy and Epileptogenic Potential of Brain Tumor Treatment; **Neuropathology**: Neuropathology of Epilepsy.

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Neuropathology of Epilepsy

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Introduction and Background

Neuropathology refers to the study of the diseases of the nervous system. The term, however, is traditionally referred to the study of morphological alterations that can be observed by means of macro- and microscopic inspection. The term 'neuropathology' generally evokes the image of a scientist sitting in front of a microscope in a room smelling of paraffin and formalin traditionally the major tools of neuropathologists. Although this basic picture remains valid, numerous tools have been added to the traditional techniques; those diseases determined by morphological and functional alterations of the brain can now be examined through the application of innovative technologies. This advance in the general field is particularly applicable to epilepsy, since epilepsy is basically a functionally defined disorder. In modern clinical practice, in addition to formulating a 'correct' diagnosis based on anatomical alteration(s), the neuropathologist is often also assigned the task of offering insights into the pathogenesis of the primary lesion. In dealing with epilepsy-related lesions, the neuropathologist may be asked to hypothesize about the genesis of the epilepsy, as reflected in the neuropathological findings. Thus, although it remains important to produce a detailed description of the observed morphological alterations, this description is not sufficient. It is also fundamental to provide evidence of the related 'etiopathogenesis,' in order to provide insight into functional features of the epilepsy.

In the last two decades, the increasing development of neuroradiological techniques, particularly the high resolution Magnetic Resonance Imaging (MRI), has provided a dramatic improvement of the 'in vivo' diagnosis of many symptomatic epilepsies, thanks to the early recognition of underlined structural alterations of the brain. The neuroradiologist and the neuropathologist now work together to provide structural observations that help explain the functional abnormality. Furthermore, integrated approaches of different branches of the neurosciences, such as neurophysiology, neurogenetics, and molecular biology, combined with new technologies in neuroimaging and neurosurgery, have given rise to an increasing interest in epilepsy, in order to achieve a better understanding of basic and pathogenetic mechanisms.

Finally, since many symptomatic epilepsies are unresponsive to pharmacological therapies, their treatment frequently relies on the surgical removal of areas thought to be responsible for seizures. As a consequence, the last decade has seen an increase in the number of epileptic patients surgically treated, with a parallel increase in the availability of surgical specimens for neuropathological analysis. This developing new perspective highlights the role of the neuropathologist, who has become a primary actor in an integrated, interdisciplinary, 'non traditional' team that spans both bench and bedside (**Figure 1**).

In the epilepsy field, the neuropathologist is instrumental in characterizing the different types of epilepsies, from the idiopathic to the symptomatic forms, in the wide spectrum of their etiologies. In the past, the neuropathological identification of underlying causes was achieved through autopsy investigations. However, in increasingly available surgical specimens from patients with intractable focal epilepsies have now become the major focus of interest for the neuropathologist. The partial epilepsies from which these specimens derive may be caused by a variety of different lesions, including vascular disorders, head trauma, tumors, malformations of cortical development (MCD), infections, neurodegenerative, and inflammatory disorders. Although the basic cellular mechanisms of epilepsy have a common functional output (reflected in an abrupt, excessive, and synchronous discharge of a certain neuronal population – i.e., a seizure), each one of the above mentioned pathological processes may determine this abnormal discharge through different mechanisms.

Glial tumors, for example, are generally not intrinsically epileptogenic per se, since the tumoral tissue generates paroxysmal discharges only indirectly, by its effect on the surrounding neuronal tissue. In contrast, MCD may be intrinsically epileptogenic, presumably due to an abnormal rearrangement of the intrinsic circuitry. Indeed, in the last decade, there has been a progressively increasing number of publications devoted to epileptogenesis related to cortical dysplasia. In this respect, the in vivo localization and definition of these alterations, greatly improved by neuroimaging techniques, have determined an increasing number of partial epilepsies – previously defined as 'cryptogenetic' – that are now recognized to be associated with malformative cortical lesions. Today, MCDs represent one of the most challenging problems in epilepsy, involving basic issues about both normal and abnormal brain development.

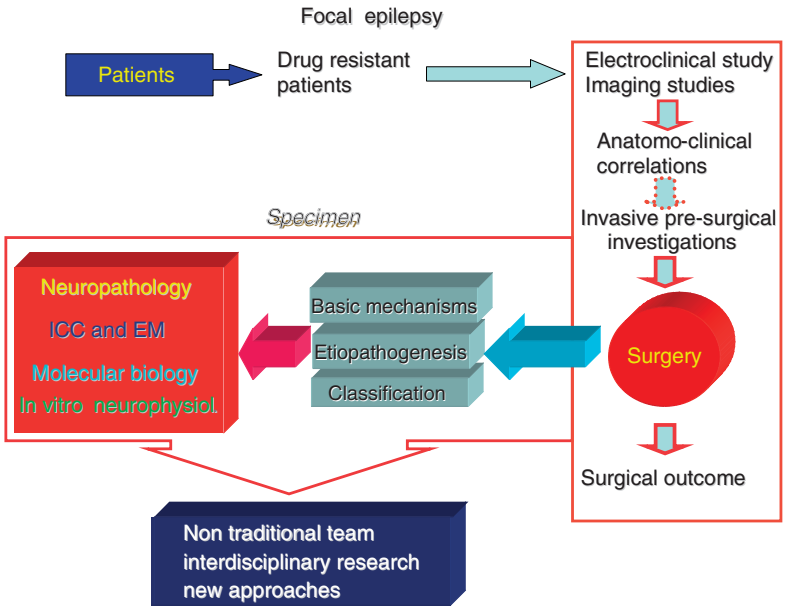


Figure 1 Neuropathological involvement in the diagnosis and treatment of patients with epilepsy.

Relevant to this topic of MCDs, some major questions include:

1. How do malformations develop?
2. Why, in the majority of cases, do they generate epileptic activity?
3. Do the different types of malformations have the same mechanisms for generating epilepsy; in other words, do the different subtypes of malformations have the same pathophysiological mechanism for epileptogenesis?

These questions are fundamental to our understanding of MCD-related epilepsies, and neuropathology plays a key role in interpreting and decoding the content of this black box.

Methodological Caveats

In addition to the traditional routine histological methods, neuropathologists have in their hands powerful 'new' techniques such as immunocytochemistry using different antibodies against structural and functional proteins (such as neurotransmitters and receptors) and in situ hybridization using several mRNA probes that can be fruitfully used on human tissue obtained from surgical specimens. It is, however, of fundamental importance that the neuropathologist be present in the surgical theater during surgery. When a specimen containing the malformation is resected, it is mandatory to obtain an accurate record of the orientation of the piece of ablated cortex to determine precisely, from the surgeon and from comparison with imaging data, the exact position of a given sample. Moreover, when (as frequently it happens) the surgery is performed after pre-surgical invasive recordings, it is important to know not only the exact location of the lesion but also that of the epileptogenic zone and of the surrounding areas (as identified by electrophysiological recordings), in order to establish the morphofunctional correlations of the different ablated regions.

Recent Results

To provide a realistic picture of the role of the neuropathologist in the study of epileptic lesions, let us consider in detail the above mentioned questions about MCDs. This exercise will depict the framework in which we are moving, explain the methodological possibilities, and provide a perspective for future developments in this field.

How Do Malformations Develop?

The mechanism of outward displacement of neurons in the developing cortex remained unknown for decades. It was only in the early 1970s that active radial migration was finally recognized as the basic mechanism for building the cerebral cortex. In addition, early reports concerning the existence of cells with tangential orientation were revisited, providing evidence for distinct, non-radial routes of some cell cohorts in which migration travels parallel to the pial surface. Lastly, the history of radial glia has been recently enriched by novel exciting data indicating that these cells are capable, by asymmetric division, of giving

rise to neurons and glial cells, and that radial glial cells are, therefore, neurogenic.

Thus, two main trajectories – a radial and a tangential route – were identified for neuronal migration. The process of radial migration implies that new neuroblasts, generated from the ventricular proliferative zone, migrate to the cortical plate, climbing along radially oriented fibers that extend from the ventricle to the pial surface. Radially migrating neurons give rise primarily to pyramidal neurons (i.e., projection neurons), which are excitatory and utilize glutamate as neurotransmitter. In contrast, non-radially migrating neurons, generated in the ganglionic eminence – the primordium of the future basal ganglia located in the ventral forebrain – follow a long tangential migratory route to reach the cortical plate and form the majority of non-pyramidal neurons (i.e., cortical interneurons), which are GABAergic.

This brief summary suggests that during cortical development, a spatial and ordered sequence of migratory events is necessary to construct the cerebral cortex. These mechanisms become extremely complex in the formation of the large neocortex of primates. Both intrinsic (genetic) and environmental mechanisms are involved in corticogenesis, although the intimate processes leading to the mature cortical structure are not yet completely understood. Disturbances of these processes can lead to a wide range of alterations, from diffuse and severe brain malformations to local disruption of cortical structure.

MCD, recognized by neuropathologists since the end of the 19th century, and now attributed to a defect of neuronal migration during a crucial period of cortical development, are a heterogeneous group of focal or diffuse anatomical derangements. The pathological features largely depend on the timing of the defect in the developmental process and to a lesser extent on its cause. The advent and development of imaging techniques has had a great diagnostic impact in this area of neuropathology, allowing the investigator to recognize MCDs in vivo. Since most of these malformations are epileptogenic, the advances in imaging have also been helpful in providing correlations between imaging features and electroclinical findings. The cortical malformations previously grouped under the general term of 'neuronal migration disorders' (NMDs) have now been reclassified, in order to reflect our improved knowledge of their pathological substrates, their possible etiological factors, and the relationship between the structural features of the malformation and the type of epilepsy. Indeed, not all the so-called NMDs have been shown to be due directly to impaired neuronal migration; other mechanisms are thought to be involved. And despite many efforts, it is widely recognized that the classification of these disorders is far from satisfactory, and there is no general consensus on these complex structural abnormalities. While it can be argued that classification is an old-fashioned problem, it appears to be a fundamental issue, as stated by Sarnat: 'Classification of simple concepts is almost reflexive but, at high level of abstraction, classification requires the greatest creativity that the human mind can muster and in no categories of disorders is classification more complex than in the field of diverse malformation of the nervous system.' Neuropathology thus has much to do with this aspect of the research. Furthermore, the etiology of malformative disorders is often uncertain and the mechanisms by which they generate epilepsy are unclear.

Over the past decade, molecular biologic and genetic studies of cortical development have greatly expanded our knowledge of the mammalian brain's normal development and derangements. Several disorders of cortical development have been recognized, and for some of them specific causative genetic defects have been identified. For example, periventricular nodular heterotopia (PNH) can be causally related to mutation of specific genes, such as Filamin A and adenosine dipphosfate-ribosylation factor guanine exchange factor 2 (ARFGEF2). However, other nodular heterotopia, presenting single or multiple unilateral or bilateral nodules, sometimes associated with other brain malformations and extending from the periventricular region to involve the adjacent overlying cortex, are not genetically determined. In these cases, the presence of risk factors for prenatal brain damage and the absence of obvious genetic causes of the PNH suggest that extrinsic factors (damaging more or less limited regions of the developing brain) may be involve. Such cases provide evidence that nodular heterotopia represent a heterogeneous disorder. Neuropathological investigations performed on autopsy cases with genetically determined PNH and on surgical specimens from patients without genetically determined forms, have shown great similarities between genetic and non-genetic forms. The neuropathological data, obtained using immunocytochemical procedures to reveal the different subpopulations of excitatory and inhibitory neurons, suggested that the final structure and intrinsic organization of the nodules may be independent of their etiology and unrelated to whether the heterotopia is unilateral or bilateral, subependymal or subcortical. Nodular heterotopia are currently considered to be the result of defective neuronal migration in early development. However, some authors have suggested that the defective gene product linked to one genetic form – an actin-binding protein necessary for the movement of certain cells – is required to signal the end of neuroblast division, and that lack of (or abnormality in) this product results in the neuroblast over-proliferation that characterizes nodular heterotopia. This possibility would explain the neuropathological similarities between the different forms of nodular heterotopia and why an exuberant number of neurons are clustered between the ventricles and the neocortex. Moreover, neuropathological investigations

revealed that the cortex overlying the nodules is frequently disorganized, and is characterized by an alteration of excitatory–inhibitory circuits that gives morphological and functional support to the electrophysiological data showing that in some cases seizures start from this nearby cortex and not only from the heterotopic nodules.

However, nodular heterotopia are quite rare. Recently, particular attention has been given to the most frequent cortical disorganization, represented by the so-called focal cortical dysplasia (FCD), some of which have specific electroclinical, neuroradiological, and histopathological characteristics. Although there is a general consensus that FCD can be highly epileptogenic, this feature of the disorder does not per se imply a correlation between the lesion and the epileptogenic area. The possibility of such dissociation has led to a continuing debate concerning the relative advantages of selective lesionectomy versus less conservative surgical approaches. One crucial issue is the extent of the surgical excision in relation to the lesion revealed by MRI. It has been reported that the extent of the resection of cortical structural abnormalities is the most important determinant of surgical outcome, but it has also been suggested that the epileptogenic areas may exceed the MRI-visible lesion and that, in many cases, surgical resection should be guided by the results of pre-surgical invasive recordings. Neuropathology could help to resolve this issue by supplying pertinent data, not only in order to estimate the extent and type of the malformation, but also to establish the extent of the epileptogenic zone. This task could be accomplished by studying, in homogeneous and selected populations of epileptic patients with FCD, the morphological aspects and the basic pathological mechanisms in the surgically excised tissue that correlate with the imaging signals, and with the electrophysiologically identified epileptogenic zone.

Once again, the problem of terminology and classification appears of fundamental importance, not only for establishing a diagnosis but particularly for providing a useful prognosis for the patients who undergo epilepsy surgery.

Thanks to accurate neuropathological investigations, two main forms of FCD, type I and type II, have been recognized. Both type I and II FCD have as a common feature a disruption of the cortical lamination, but they differ in intrinsic cellular organization, in alterations of the GABAergic system, and in cytological aspect (**Figure 2** and **3**).

Recent data provided evidences that type I FCDs are mainly found in temporal regions while type II are detected in extra-temporal areas. Furthermore, a more favorable post-surgical outcome is reported in type II FCD, probably because this type of dysplasia, intrinsically epileptogenic, is more localized than type I and thus accessible for complete resection. We still do not know the etiology of these malformations; the two forms may have different pathological mechanisms at their origin. One of the future challenges will be to determine if the two types of FCD have common origin. For this type of investigation, new tools are now available for molecular classification of cortical cells, and to reveal and map neuronal position within cortical layer in normal and malformed cortex. A common pathogenetic mechanism has been suggested, by the presence of histopathologic similarities, between FCD and dysplastic areas in autosomal inherited phacomatosis such as tuberous sclerosis. The determination of molecular genetic alterations in specific epilepsy-associated malformations would help to identify molecular pathways with potential pathogenetic relevance. The correlation between clinical, imaging, neuropathologic, and molecular analysis will help to further develop comprehensive classification systems and treatment strategies in epileptic patients with MCD.

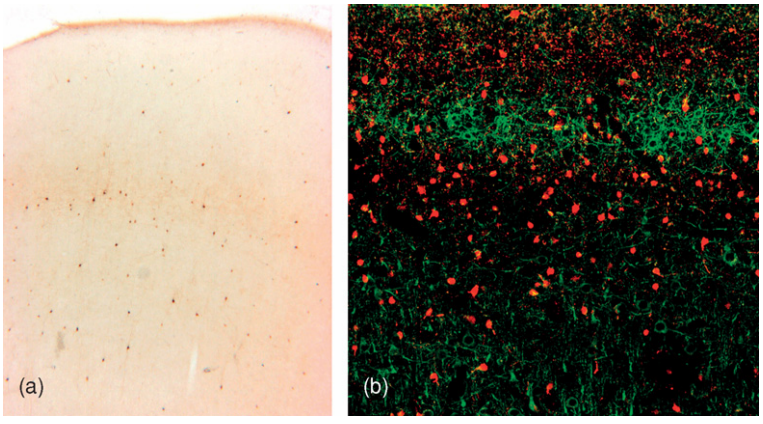


Figure 2 (a) Reduced immunoreactive staining for Parvalbumine (PV; a calcium binding protein specifically staining a subpopulation of GABergic interneurons) suggestive for an impairment of GABA circuitry in type 1 FCD. (b) Architectural abnormality in type I FCD as observed at confocal microscope. A double fluorescent staining using MAP-2 (green signal) and Calbindine (red signal).

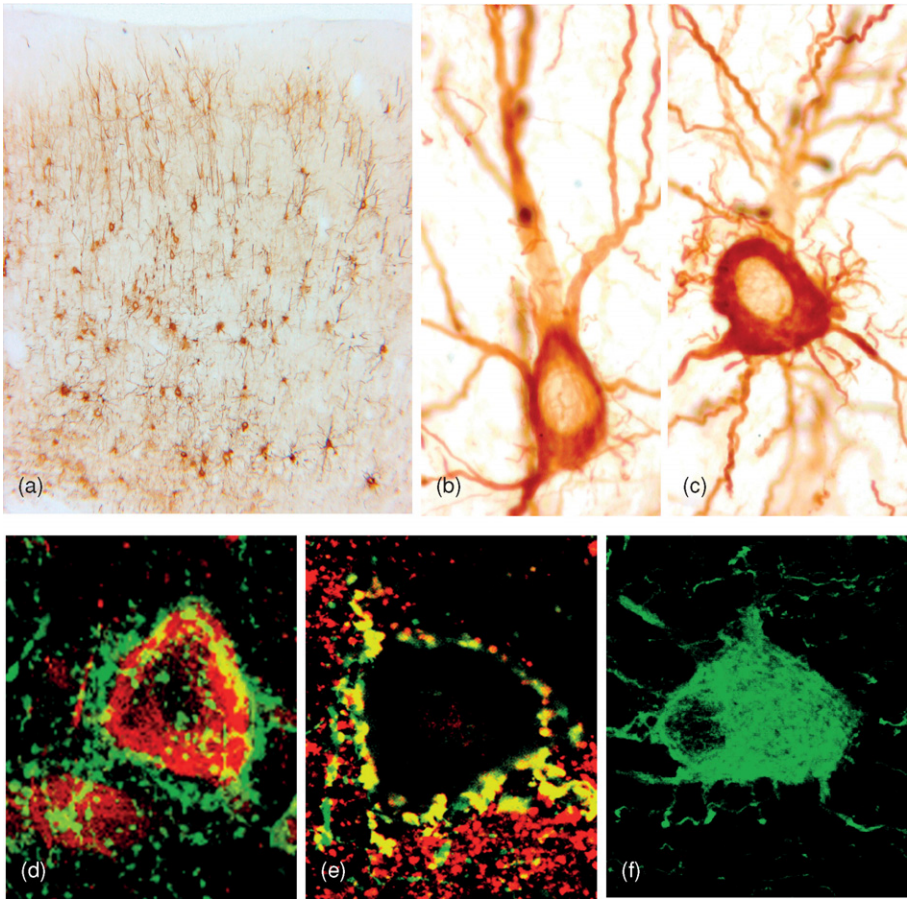


Figure 3 (a) Cortical disorganization in type II FCD as observed using an antibody against neurofilaments; note the presence of giant and dismorphic neurons (at high magnification in (b) and (c)) representing typical elements in this dysplasia. (d) Confocal image showing an increased concentration of PV terminals (green signal) surrounding the dimorphic neuron labeled with an antibody against neurofilaments (red signal). These terminals are double labeled (yellow signal) with PV and GAT1 (a transporter of GABA) demonstrating that they are inhibitory terminals (e). Another typical cellular element of this type of FCD is represented by balloon cells specifically labeled by Vimentin (f).

Why, in the Majority of Cases, do these MCDs Generate Epilepsy? Do the Different Types of Malformations Employ the Same Mechanisms for Generating Epileptiform Discharge/Epilepsy?

Human epileptic dysplasia exhibit a spectrum of abnormal cell aggregation and orientation patterns, as well as a variety of and laminar disruptions. It is, therefore, likely that many 'plastic' axodendritic and cellular changes occur during development. For example, intrinsic circuitry alterations and remodeling of receptor assemblies could be responsible for neuronal hyperexcitability – giving rise to imbalanced excitatory and inhibitory mechanisms leading to seizures. Despite considerable research, the exact mechanisms by which seizures are generated in dysplastic brain remain largely unknown. Combined morphologic and molecular approaches, investigating the neurotransmitters and related receptors, have been developed. One line of research suggests that NMDA impairment is associated with altered molecular structure of postsynaptic membrane, and that different malformations may be associated with different alterations of these receptors, contributing to the genesis of epileptic phenomena. A second line of research, devoted to the study of the inhibitory circuitry, suggests a critical role for GABA-mediated inhibition in the overall control of neuronal excitability in cortical dysplasia. That the aberrant expression of NMDA receptors play a role in the genesis of seizures is not surprising, since a large body of data is consistent with the conclusion that glutamate-mediated excitation is enhanced in epileptogenic cortical malformations. It should be noted, however, that the sophisticated functional analysis performed on glutamate-mediated excitation in dysplastic brain has not yet been applied to the GABAergic system. The impairment of the two

systems is not mutually exclusive, and perhaps coexists at different levels of efficacy in the diverse forms of malformations.

Many mechanisms may be implicated in the epileptogenesis involving both neurons and glial elements and several lines of evidence link glial cells to modulation of synaptic transmission showing that functional alterations of specific glial membrane channels receptors and transporters are involved in epilepsy.

Although intercellular communication in the mammalian cortex is generally thought to take place through chemical transmission, recent studies showed that intercellular communication via gap junctions represents an important pathway through which cells exchange ions, second messengers and low molecular weight metabolites to maintain tissue homeostasis and to control growth and differentiation. Gap junctions are the morphological correlates of electrical coupling (low resistance pathways), and have been implicated in the genesis of several diseases of the nervous system, including epilepsy. New lines of research, based on RNA blot analysis and on morphological studies using specific antibodies against connexins, have focused on the relationship between gap junctions and epilepsy; such studies have suggested that increased intercellular coupling may be implicated in contributing to the synchronization of discharges in epileptic foci. This relatively new line of research has also generated new interest in pharmacology approaches – i.e., gap junction blockers that might act as potent antiepileptic drugs.

Challenges for the Future

The complex pathology of focal epilepsies, and particularly those related to MCDs, should now give rise to functional genomic investigations. The recent development of new technologies, such as microarray and gene-expression profiling techniques, can identify large numbers of genes with potential involvement in these structural abnormalities. Thus, the integration and interpretation of the data from neuropathologically characterized tissue or specific cellular populations (e.g., using laser-microdissection approaches) represent a future challenge that may provide new, important insights into the pathogenesis of many forms of epilepsy.

See also: **Genetics**: Genetic Contributions to Brain Structure and Epileptogenesis; Single Gene Mutations Causing Epileptogenic Malformations of the Cerebral Cortex; **Pediatric Epilepsy**: Animal Models of Infant-Onset Epileptic Encephalopathy; Mechanisms of Epileptogenesis in Models of Pediatric Epilepsy Syndromes Associated with Brain Malformation; **Neuropathology**: Long Term Epilepsy-Associated Tumors; **Non-Synaptic Mechanisms**: Role of Gap Junctions in Epileptic Synchronizations; **Temporal Lobe Epilepsy**: Genetic Determinants of Temporal Lobe Epilepsy.

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NEUROPHYSIOLOGY

Contents

Seizure Detection and Prediction
Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings

Seizure Detection and Prediction

J Gotman, Montreal Neurological Institute, McGill University, Montreal, QC, Canada

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Introduction

Seizure detection usually refers to the detection of the electroencephalographic (EEG) correlate of an epileptic seizure, which is primarily an event generated in/by a large group of neurons. Seizure prediction similarly refers to the possibility of predicting, before its clinical or encephalographic onset, the occurrence of an epileptic seizure from analysis of the EEG signal. Towards these ends, it is conceivable that measurements other than EEG could be analyzed (e.g., electrocardiogram, electromyogram, movement, oxygen saturation), but these measures have not retained the attention of many researchers. Therefore, our discussion here will review the problems of detection and prediction from the point of view of EEG analysis.

Seizure Detection

Why Detect Epileptic Seizures?

Patients who are investigated for severe epilepsy are often subjected to long-term EEG/video monitoring, a procedure that can last from a few hours to 2 or 3 weeks. Its purpose is to record the EEG and observe behavioral manifestations during seizures, in order to determine the type and frequency of seizures to better adjust medication, or in order to localize the seizure onset when surgical treatment is considered. During such a procedure, the patients are usually asked to press a button when they feel a seizure coming. If patients could always feel their seizure coming, there would be no need for automatic seizure detection. It is frequently the case, however, that patients are not aware of their seizures, and observers are not always present and/or not always able to recognize a seizure. Furthermore, some seizures have very minimal clinical signs – and sometimes no visible signs at all. Finding all seizures may require the review of days and days of EEG, a process that is long and tedious – and not always effective inasmuch as short seizures can be missed, particularly if there are many channels, as in intracerebral recordings. Automatic seizure detection can be helpful in this context, as it provides a means for marking the EEG sections that are likely to include seizure patterns. For this purpose, the detection can take place at any time during the seizure (not necessarily early) because the EEG will be reviewed a posteriori.

Seizure detection could also be used in real time, to warn the patient or observer that a seizure has just started. In this case, the detection must occur early to allow the patient to take protective measures (if he or she is still able to do so), to assist an observer in providing protective measures in a timely fashion, and to facilitate the observer's attempts to question the patient so as to improve our understanding of clinical signs (e.g., can the patient follow commands, talk, remember?). Such an early seizure detection system can be used during monitoring in the clinic,

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but could also be incorporated in an implantable device that would provide a warning (e.g., auditory) to the patient during normal everyday activities.

Seizure Warning vs. Seizure Prediction

Although the terminology is not obvious and not necessarily universally recognized, there is a large difference between what we will call 'seizure warning' and 'seizure prediction.' Seizure warning refers to a signal given *because a seizure has just started* – as discussed above. Seizure prediction refers to the ability to forecast the *future occurrence of a seizure*. The difference is large from the point of view of signal analysis: in the first case, the objective is to detect the seizure as soon as it has started; in the second case, it is necessary to search for what may be taking place in the EEG that precedes a seizure, to search for a change that has not been identified by visual analysis – and that may not always exist in the EEG (see below). For a method to be effective for seizure warning, the following requirements must be met: high sensitivity (detect most seizures), early detection (the warning is only useful if it arrives within a few seconds of seizure onset), and low false alarm rate (a warning is likely to be ignored if it occurs erroneously too often). By providing a warning only for seizures that resemble a specific template (and not for any type of seizure), it is possible to reach a good 'compromise' regarding these requirements. Recent efforts have also made it possible to provide a warning with good reliability without relying on a specific template. Seizure warning methods have been developed separately for scalp and for intracerebral EEG.

Methods and Results

Seizure Detection Methods

In epileptic patients and in experimental animals, the EEG is recorded either on the scalp, on the surface of the brain (epidural or subdural), or inside the brain (intracerebral). Our current understanding is that seizures consist of paroxysmal rhythmic activity generated by a large number of synchronized neurons. This activity usually evolves, with changes in spatial distribution, frequency, and amplitude, although there are exceptions (e.g., the spike and wave bursts typical of generalized epilepsy present with very little evolution). The patterns that are actually recorded depend not only on the discharge itself, but also on the respective position of the electrodes and the generator. In some cases, no seizure discharge may be visible because the electrodes are too far from the generator. For the same reason, the seizure discharge may appear only as a mild modification of the ongoing EEG. It is therefore important to distinguish the seizure itself from its manifestations (i.e., from the vantage point of the recording electrodes). As seizure detection is performed from analyzing the EEG, it is only the seizures that have a clear manifestation in the EEG that can be detected. **Figures 1** and **2** show examples of seizures recorded from the scalp, and **Figure 3** shows a seizure recorded from intracerebral electrodes. In **Figures 1** and **3**, the specific moment of onset is not easily identified. In **Figure 2**, the onset is clear, consisting of a few seconds of rhythmic discharge followed by irregular slow waves.

For the reasons given above (at least in part), there is no formal definition of what a seizure consist of in the EEG. Qualitative definitions usually include the terms 'paroxysmal, rhythmic, evolving' – but an unambiguous definition has not been formulated. Automatic detection methods have therefore relied on trying to encode these qualitative characteristics into algorithms, and improving the algorithms by trial and error in an effort to increase detection sensitivity and decrease the false seizure detection rate. The first general-purpose seizure detection method that was validated on a large data sample, characterized each two-second epoch by measures of frequency, rhythmicity, and amplitude change and compared these measures with a constantly updated background. A set of empirical rules then combined the findings in multiple epochs and multiple channels to reach a decision on detection. The concept of a 'constantly updated background' plays an important role in the analysis of long-term data, as the background changes considerably with different states of vigilance of the subject. It is also important that the results of detection not be dependant on the selection of a particular background epoch, a dependence that would introduce a significant degree of arbitrariness into the process.

A method specifically designed for intracerebral recordings was based on the detection of a repetitive spiking pattern, as well as of possible flattening at seizure onset. Another approach for seizure detection consisted of using leaky integrators functioning as simulated neurons to assess increases in amplitude and slope over a certain time (the simulated neurons fired 'action potentials' as a function of the composition of the EEG). Because the patterns and the artifacts are quite different in scalp and intracerebral EEGs, recent wavelet-based methods have been developed separately for scalp EEG and for intracerebral EEG, with performance much better than that of the older common methods.

Artificial neural networks (ANNs) have found broad applications in many areas, including seizure detection. Early work showed their utility in detecting bursts of spike-and-wave in experimental models of epilepsy. Several methods have been published that involve various EEG features as inputs to an ANN. The AAN is presented with a large number of training examples which comprise both seizure and nonseizure data, and gradually learns to differentiate seizures from nonseizure EEG. A set of features characterizing the EEG needs to be specified in



Figure 1 Scalp-recorded right temporal seizure occurring in the context of muscle and eye movement artifacts. This early part of the seizure consists in a discharge in the theta range of frequencies, commonly seen in temporal lobe seizures.

advance (e.g., amplitude, rhythmicity, dominant frequency, etc.), but it is not necessary to describe seizure patterns explicitly. The ANN automatically determines which combinations of features are characteristic of seizures by adapting its internal structure to reflect the training data. The performance of ANN methods depends on its being given an appropriate set of features and on its exposure to a sufficiently large training set containing a variety of seizure patterns. It is also necessary to specify a priori the structure of the ANN; large networks can handle complex classification tasks, but risk overfitting the training data so that it is unable to generalize other types of seizures. It is also difficult to investigate the operation of a large ANN; the network acts as a black box that provides output regarding classification values, depending on the features it receives as inputs. To circumvent this black box issue, some researchers have used an algorithm based on a set of rules, each rule being implemented using a small ANN; this approach makes it easier to analyze the operation of the system.

Performance of a detection system can be improved by incorporating a wide context in detection algorithms. Rather than defining the event only locally (i.e., comparing the characteristics of a 5-s epoch in one channel with the 30-s epoch that precedes it), it is helpful to include measurements of spatial context (activity in other channels) and of temporal context (state of the subject: awake, stages of sleep, previously recorded events). This approach has been used to reduce false detections by allowing a detection method to remember the patterns that caused frequent false detections in each subject. Another detection method uses spatial and temporal clustering of elementary detections made on individual channels and epochs, thus resulting in a global representation of seizures. This approach reduced false detections by only detecting a seizure with a number of elementary detections adjacent in space and time.

Wavelet methods allow for the analysis of the temporal evolution of frequencies; these methods are thus well suited for the identification of paroxysmal rhythmic activity. However, intracerebral EEGs also include frequent rhythmic bursts as part of normal background activity. Therefore, it may be necessary for an algorithm to adapt automatically to the background of each patient by deactivating the

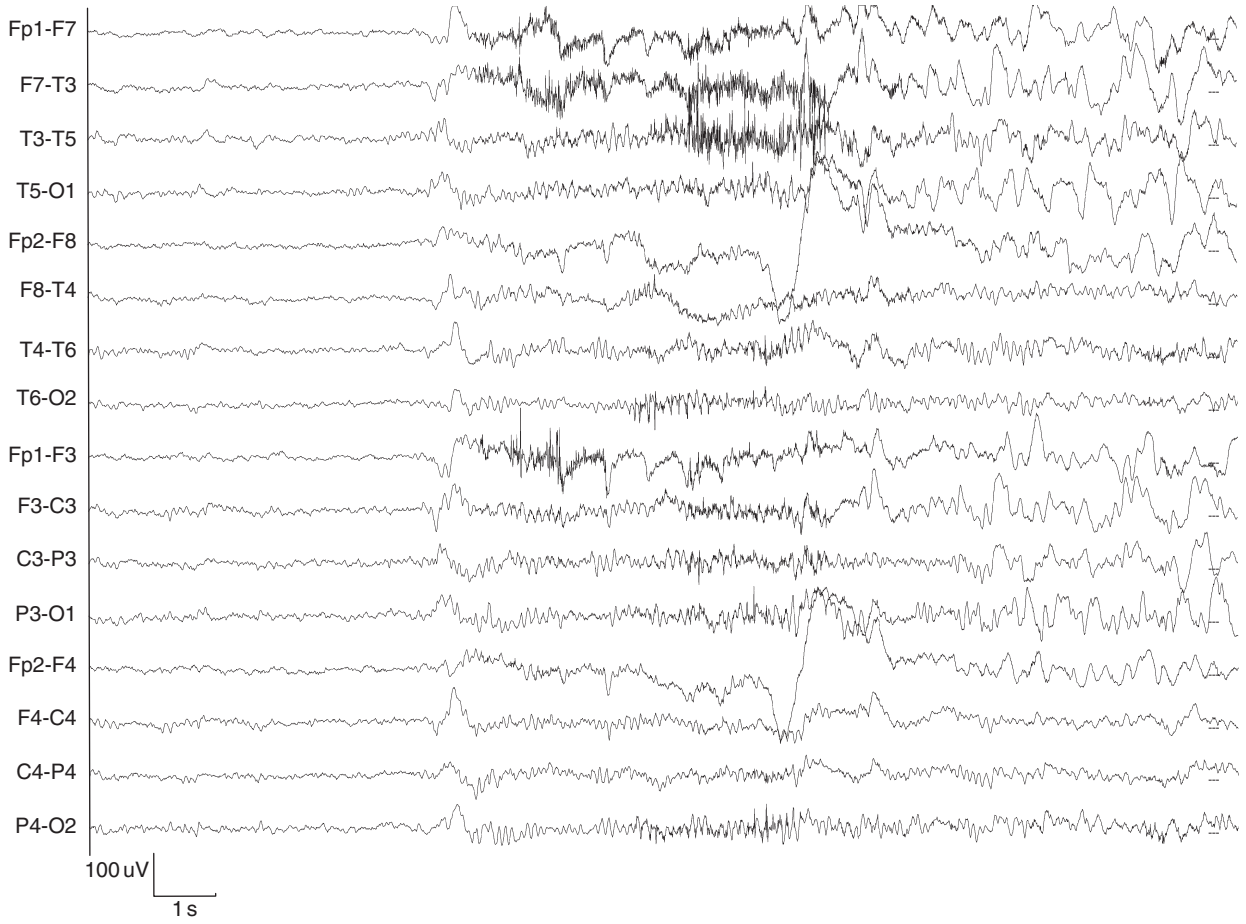


Figure 2 Scalp-recorded seizure with a generalized and abrupt onset. A few seconds of rhythmic theta frequency activity are followed by irregular slow waves in the delta range.

detection of paroxysmal activity that occurs consistently in the same channels and at the same frequencies. This deactivation results in an important decrease in false detections. It has also been shown that better performance can be obtained when analyzing the scalp EEG with a midline reference rather than with a bipolar montage (which has been most commonly used in other methods) or with an average reference.

An extension to the above concept of context is to incorporate a particular patient's seizure characteristics in the context. In this case, the method is aimed at detecting seizures in that particular patient, and only seizures that look like the sample seizures (that have been incorporated in the context) are detected. The method can then be labeled 'patient-specific' – or more correctly, 'seizure-specific' – as a patient can have several types of seizure. Better detection performance can thus be obtained, if it is acceptable to detect only a specific seizure pattern in a given patient.

Newborns have an incidence of seizures much higher than older children and adults. When they occur, seizures are also more frequent. For these reasons, it is often necessary to monitor newborns, but the monitoring does not require such prolonged observation periods (usually a few hours to a day or two is sufficient). Seizure patterns are quite different in the newborn EEG than in older children and adults: the discharges are often much slower and sometimes very focal (limited to one electrode). For these reasons, methods specific to the newborn have been developed, with the particular aim of detecting the very slow discharges often present during newborn seizures. Recent methods have proposed a neuronal model of EEG generation, for both seizures and background activity. Fitting the model to the EEG gives a reflection of how much of the EEG may be due to seizure activity. Interchannel synchronization, an approach that has been rarely used in seizure detection, has also shown promising results in newborns, as has time–frequency analysis to detect low-frequency rhythmic activity, as well as high-frequency bursts of spikes. Another approach has been to design a multistage system based on a large number of features, and then retaining only the most appropriate of these features for use in an ANN to classify seizure and nonseizure EEG.

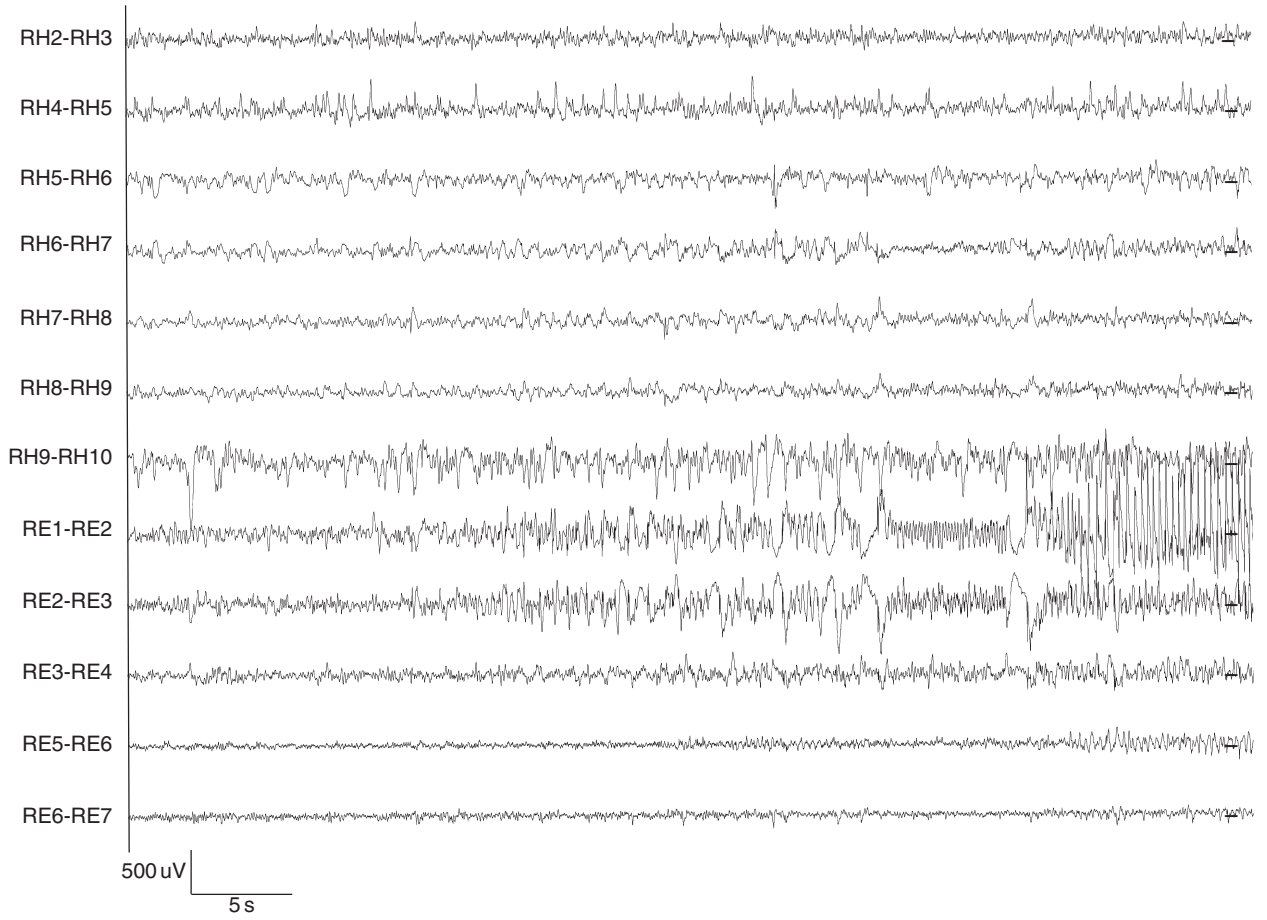


Figure 3 Seizure recorded from intracerebral electrodes placed on the temporal lobe. The RH contacts are along an orthogonal multicontact depth electrode having its deepest contacts (lower numbers) in the hippocampus and most superficial contacts in the second temporal convolution. The RE electrodes are epidural electrodes placed along the first temporal convolution. The seizure onset consists of mixed frequency activity, followed by a more rhythmic discharge. Note compressed time scale. For the purpose of clarity, this EEG illustrates only a fraction of the electrodes implanted in this patient's brain.

Validation

Validation of seizure detection and seizure warning methods requires particular care if one is to obtain results that are predictive of future performance in a realistic clinical context. Whereas early studies often did not follow legitimate validation protocols, recent methods have usually been validated in an appropriate context. Most importantly, the method must be tested on a set of data completely independent from that on which it was developed. It is critical, then, that the testing data be representative of the context in which the method will be used: it should include a large variety of seizures, and a much larger amount of nonseizure data than of seizure data (to ensure that all types of interictal data – quiet and active wakefulness, different sleep stages, and different types of artifacts common during long-term monitoring – are well represented). The best way to avoid selection bias is probably to select all data between two given dates.

An important issue in validating a method is the definition of a seizure itself. Although there is no ambiguity with a clear seizure lasting 30 s or 1 min, significant questions arise for short events: What is the minimum duration of a seizure? Should a seizure having clinical manifestations, but no change in the EEG, be counted as an event to detect? What if the EEG changes are very minor, such as a brief flattening or a short burst of slow waves? When presenting validation results it is imperative that these issues be addressed.

Methods that were evaluated on extensive data sets (hundreds or thousands of hours, tens of patients) report detection sensitivities varying from 75% to 90%. Sensitivity is often lower in newborns than in adults, as some newborn seizure patterns are very subtle. The false detection rate, the most often quoted measure of failure, was ~1–3 per hour in the early methods, and is more in the range 0.3–1 per hour in more recent studies. Few studies

report results with intracerebral electrodes, but these types of recordings have important distinguishing characteristics: the most prominent scalp artifacts (EMG, movement, eye blinks) are absent, thus reducing false detections; the dynamic range of the spontaneous fluctuations in background is much larger in intracerebral than in scalp EEG – thus resulting in more false detections; the intracerebral EEG often includes many paroxysmal bursts of uncertain significance, which can also contribute to false detections; and seizure patterns are often more prominent in intracerebral EEG, thus resulting in better sensitivity.

Seizure Prediction

Why Predict Epileptic Seizures?

Seizures can be, in themselves, harmful but most are not, and the main difficulty encountered by patients with epilepsy is the unpredictability of seizure occurrence. A loss of consciousness and automatisms lasting 30–60 seconds would be only an annoyance if the patient could be warned a few minutes ahead of time that it will happen. In addition to simply providing a warning, it is possible to consider interventions that could prevent the seizure from occurring, or at least make its occurrence less likely. When such a warning is generated, one could consider the use of chronically implanted devices that could inject an antiepileptic agent or generate electrical currents capable of preventing seizure occurrence. Although it has not been demonstrated at this point that any such intervention is efficacious, the aim of predicting a seizure is clearly of interest even if there is no possible intervention, simply to alert the patient.

When discussing seizure prediction, a methodological issue must first be addressed. One can only talk about prediction if the seizure onset is unambiguously defined. If the onset is not well defined, it becomes impossible to separate what may be a preictal change (that could be used as a warning that a seizure *will* occur) from an early sign indicating that a seizure *has just started*. Such an unambiguous definition of seizure onset is difficult. Seizures are mostly phenomena that are observed through clinical manifestations and EEG. Given these methods of observation, one could define the seizure onset as the first clear change which leads to an unambiguous seizure – either in the internal state of the patient (as reported by the patient), the patient's behavior, or the EEG. One has to be aware that such a definition may lead to different results, depending on what we call 'the patient's behavior' and 'the EEG.' Simple examples illustrate the issues: If the first clinical sign of a seizure is that the patient stops talking, this 'change' will only be observed if the patient is talking or if someone is asking a question; if the patient is not talking, this sign will be missed and the next clinical sign will be interpreted as the first. With respect to the EEG, the earliest change may vary greatly, depending on where the electrodes are. The first change may occur at time T with scalp electrodes, at time T-10s with intracerebral temporal electrodes, and at T-20s with intracerebral orbitofrontal electrodes. It is not trivial to define the seizure onset, and this question remains an important issue when evaluating the performance of seizure prediction algorithms (see below).

Methods and Results

Seizure Prediction Methods and Performance

A few patients are able to predict their seizure either a long time ahead of time ('I feel like I will have a seizure today') or just a few seconds ahead of time (as a result of a particular sensory or experiential phenomenon). In the latter case, it is most likely that the patient is actually aware of the onset of the seizure, and that the so-called premonitory sign is simply the first clinical sign of the seizure. One cannot, then, talk about seizure prediction per se. Studies of patients' abilities to predict their seizure have shown that about 20% of patients are quite capable of predicting their seizures (one day in advance); stress and lack of sleep are important precipitants that patients can incorporate into their predictions. Dogs able to predict seizures have often been mentioned, but controlled studies are required to determine if this phenomenon is more than simply anecdotal.

A few observations have indicated that there may be changes in metabolism prior to seizures. In one study, changes in the BOLD (blood oxygenation level dependent) signal several minutes prior to seizure were described, but there has been no further report on such changes, despite their obvious interest. A change in the SPECT (single photon emission computed tomography) signal, recorded by chance prior to a seizure, has also been reported, as has a change in deoxyhemoglobin and blood volume about 20 s prior to seizure onset (as defined in the electrocorticogram). These observations are interesting, and may indicate that sometimes something is changing prior to seizures. However, there is no simple way to monitor continuously oxygen concentration or metabolism in patients, and there has consequently been no further effort to predict seizures with such measurements.

The scalp and intracranial EEG have been the main tools used for attempting to predict seizures. The EEG has the obvious advantages of being recorded relatively simply and continuously, and of showing clear changes at the time of seizures. Because the EEG changes so dramatically during seizures, one might hope that preictal changes – if they exist – are present in the EEG. It is sometimes thought that interictal epileptic activity (spikes and sharp waves) increases in frequency prior to seizures.

Long-term studies have shown that this is not the case, so increased frequency of such events has not been pursued as a predictive measure.

Most analyses aimed at seizure prediction have examined changes in the EEG background. Early reports were very optimistic that seizures could be predicted, particularly using nonlinear analysis methods, but also using simple linear methods. These reports indicated that changes occurred several minutes prior to most seizures. Most work was done with intracerebral EEGs, but widespread preictal changes were also observed in the scalp EEG. Synchronization between EEG signals from intracerebral channels was also used as a measure for predicting seizures. An important weakness of several of these studies was the fact that they analyzed mostly relatively short sections of EEG, including seizures and a short preictal interval (e.g., 1 h). When more extensive recordings were analyzed, it became clear that the changes that were observed prior to seizures were also observed at other times, independently of seizure occurrence. It was shown that most false prediction occurred during sleep, probably as a result of the frequent changes in EEG patterns corresponding to the changes in stages of sleep. Preictal changes, therefore, appeared relatively sensitive but lacked specificity.

Much of the early work on seizure prediction relied on nonlinear methods (e.g., Lyapunov exponent, correlation dimension), but it later became clear that more traditional linear methods did not necessarily perform less well. It was shown that multivariate methods (those analyzing simultaneously several channels) tended to perform better than univariate methods (analyzing one channel at a time).

The methodology appropriate to evaluate seizure prediction methods has now been well established. One approach that is promising is that of seizure time surrogates: to test if prediction results are significantly better than chance in a long recording including several seizures, results are compared with those obtained after randomly shuffling seizure times in the recording. There have been no recent reports using this methodology and indicating a reliable seizure prediction performance, although promising preliminary results have been reported.

Conclusions and Directions for Future Research

Although seizures are most often very prominent events in the EEG, automatic detection methods remain imperfect, in large part because of the difficulty of capturing within an algorithm the many features that constitute a seizure and differentiate it from a background that is changing continuously – and that sometimes changes in ways that resemble a seizure. Sensitivities of 80–90% can be reached but average false detection rates remain in the vicinity of one false detection per one or two hours of recording, with much higher rates in a few patients. Progress has been incremental in the last few years; there has not been any new method that has led to a quantum jump in performance.

The possibility to warn a patient (or observer) that a seizure has just started has become a reality, with current methods able to give a warning a few seconds after the seizure onset in the EEG. The performance of such methods can be greatly enhanced if they aim at warning only of a seizure of which a template is available (as opposed to any seizure). Because most patients have only one seizure type, and its pattern is relatively stable, this is a reasonable approach.

Seizure prediction, in contrast, remains more a dream than a reality at this point. Early optimism has been tempered by the subsequent understanding of the many issues involved in making a reliable prediction algorithm. Now that the validation methodology is well in place, the stage is set for the realization of this dream. Interestingly, it is often stated that focal seizures are more likely to be predictable than primarily generalized seizures, although no evidence has been presented to support this view. As most results point to pre-ictal changes that are not limited to the region of seizure onset, but encompass widespread regions, it is not clear why such changes could not precede generalized seizures.

See also: **Neurophysiology**: Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; Seizure Prediction.

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Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings

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Introduction

Epileptic seizures are transient events that produce clinical symptoms ranging from brief sensory phenomena to loss of consciousness and convulsive activity. Seizures can be focal (partial) or generalized (bilateral), and the clinical manifestations reflect the regions of brain involved by the seizure activity. Although the average seizure lasts less than 2 min, this short period can be enough to have a significant impact on quality of life for the patient with uncontrolled epilepsy, especially if the seizures produce altered consciousness.

Only about 50% of partial onset seizures (the most common seizure type) can be controlled with antiepileptic drugs (AEDs), even with the approval of a number of newer medications in the last decade. With the development of digital video and EEG recordings, there has been a corresponding growth in epilepsy monitoring units to record and analyze actual seizures. Patients may be admitted to these units for a variety of reasons, but common indications are for seizures that have not been controlled with AEDs and for presurgical evaluations. Presurgical evaluations include ictal scalp monitoring, and may in some patients require further intracranial monitoring if seizures cannot be adequately localized with scalp recordings or if the focal onset is close to eloquent cerebral regions. Appropriately selected patients with medically intractable partial epilepsy may have ~70% chance of becoming seizure free after surgery (e.g., temporal lobectomy for mesial temporal sclerosis) compared to ~5% with additional AED trials.

In order for seizure surgery to be successful, the seizure focus must be identified and the region to be resected must be determined. Visual analysis of ictal and interictal EEG can provide considerable useful information. Interictal recordings can reveal focal or multifocal spikes, while ictal recordings (the current gold standard for presurgical evaluations) can reveal the localized dramatic changes that occur with seizure onset. With the introduction of computational methods, many epilepsy centers have begun implementing signal processing methods for analysis of epileptic scalp and intracranial EEG (ICEEG). In particular, with the introduction of digital EEG machines, it has become easier to employ digital analytic methods. Understandably, much of the interest in clinical assessment is in seizure detection and in determining the location (focus) of seizure onset. Several centers have focused on spike and seizure detection, and have developed methods to that end. It is recognized that seizures that originated from a single focus in a given patient often have similar patterns of onset; this reproducible pattern has allowed for the tuning of detection algorithms for improved sensitivity and specificity.

In some instances, eloquent areas (e.g., motor or language) may limit the extent of safe resection; in other patients more extensive resections can be made. While intracranial electrode arrays (e.g., depth electrodes, subdural strips and grids) can provide considerable information regarding seizure onset and propagation, seizure onset (particularly in neocortical foci) may at times be regional or ill-defined. The concept that there is not just

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Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings

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Introduction

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In some instances, eloquent areas (e.g., motor or language) may limit the extent of safe resection; in other patients more extensive resections can be made. While intracranial electrode arrays (e.g., depth electrodes, subdural strips and grids) can provide considerable information regarding seizure onset and propagation, seizure onset (particularly in neocortical foci) may at times be regional or ill-defined. The concept that there is not just

an epileptic focus but an epileptic zone has evolved from these observations.

Background

Epileptic seizure evolution is characterized by rapidly changing dynamics at the seizure focus, as well as in regions involved in seizure propagation. While surgical treatment is directed to the seizure focus and not remote regions of propagation, patterns of seizure propagation can determine the clinical manifestations and provide insights into the self-limited nature of epileptic seizures. Early approaches to seizure analysis employed mostly single channel linear signal processing methods, including spectral analysis (e.g., fast Fourier transformation, FFT) or autoregressive methods. Changes in spectral content of the signal could be observed at seizure onset and during seizure evolution. Analyses of interactions between different brain regions involved during seizure propagation were performed using multichannel analysis based on correlation and coherence. More recently, nonlinear methods of analysis have been applied to understand the underlying dynamics of the systems that generate epileptic activities, with the ultimate goal of being able to predict the occurrence of the seizures (if that, indeed, is possible). Current intracranial recording arrays employ high sampling rates, have more extensive coverage, and use special electrodes to better define seizure dynamics, particularly the involvement of high frequency activity. Still, there is a need for additional methods of analysis to assist in the presurgical clinical analysis and to better understand the mechanisms of seizure generation and termination.

No single technique has proven to be able to fully describe the dynamics of the epileptic seizure generation, onset, and propagation – because of the complexity and variability of the problem encountered. Two methods for signal analysis that have recently been applied to intracranial recordings of ictal events are the Gabor atom density (GAD), based on the matching pursuit (MP) time–frequency decomposition, and the short-time direct transfer function. These two methods will be discussed further as examples of advanced techniques that can provide additional information about seizure dynamics, propagation, and patterns of the flow of activity. These techniques can provide complimentary information for clinicians to more effectively assess the onset and propagation of partial seizures.

Methods

ICEEG monitoring is performed with subdural grid or strip arrays, or depth electrodes. Depth electrodes and subdural strips are often used (with bilateral implantation) for seizure lateralization when there is not sufficient evidence from ictal scalp recordings. Subdural grid arrays are typically employed when seizure lateralization has been determined, but better localization of the seizure focus is needed. In all instances of intracranial recordings, the observations made are limited by the spatial sampling done by electrodes. For depth electrode recordings, the goal is usually to assess the involvement of certain structures in seizure onset (e.g., amydgala, hippocampus); for subdural strips and grids, the participation of neocortical regions in seizure onset is commonly sought. Compared to scalp recordings, ICEEG electrodes provide a much better localization of brain activity, often allowing a more precise assessment of the seizure onset zone. Nevertheless, the spatial resolution remains low in comparison to the underlying neural networks.

The interpretation of the recordings obtained in the epilepsy monitoring unit is done visually by neurologists who are trained to recognize patterns of onset and evolution of epileptic events. Electrographic changes are correlated with clinical manifestations. Most of the time, the evaluation is done visually – with little or no quantification of the pattern beyond the approximate frequency of the activity at seizure onset (when it can be determined). Determining the region(s) of focal seizure onset is the goal of the presurgical evaluation. Considering the importance of stereotyped patterns in the evaluation of focal seizure onset, there are potential benefits in being able to quantify these onset patterns in a more deterministic way.

Matching Pursuit and Gabor Atom Density

MP is a decomposition algorithm recently introduced to advance the analysis of spectral content. One of its advantages is that this algorithm does not have a requirement for signal stationarity – a signal property criterion that often limits the applicability of traditional analysis as it requires that the mean and variance of the signal do not change over the time period analyzed. MP also has the advantage that the time–frequency reconstructions that it allows do not have cross-term components. Cross-terms components are spectral components that do not really exist in the signal, but are often produced by other spectral analysis methods – a problem that has hindered previous methods of representation in the time–frequency space.

The original MP method was described in 1993 by Mallat and Zhang. The algorithm decomposes the signal on a nonorthogonal base of functions called atoms; i.e., the $(m+1)$th atom in the decomposition depends on the previous m atoms. However MP still maintains conservation of energy, so the time-frequency decomposition obtained has the same total energy than the original signal.

These atoms are mathematically defined by Gabor functions, which are modulated Gaussian functions:

$$g_\gamma(t) = \frac{1}{\sqrt{s}}\, g\left(\frac{t-u}{s}\right) e^{i\xi} \quad \text{with} \quad g(t) = 2^{1/4} e^{-\pi t^2} \quad \text{and} \quad \gamma = (s, u, \xi)$$

where $\frac{1}{\sqrt{s}}$ normalizes the norm of g_γ to 1, which allows the conservation of energy of the decomposition.

The signal f can be written as the sum of m atoms g_n and a residue.

$$f = \sum_{n=0}^{m=1} \langle R^n f, g_n \rangle g_n + R^m f$$

Several methods exist to determine the number m of atoms needed to obtain a sufficiently precise representation. One highly effective method for the analysis of epileptic seizures was introduced in 2003, and limits the decomposition based on a threshold of the energy of the last atom added to the decomposition.

A composite index, called the GAD, has been introduced to describe the complexity of the signal based on that number of atoms. GAD is used to assess the complexity of the EEG signal by normalizing the number of atoms to the time–frequency region analyzed:

$$\text{GAD} = \frac{2\text{m}}{N}$$

where N is the number of points in the time frame considered. When applied to all channels, the composite information gathered can also be used to assess the propagation of epileptic activity to other brain regions remote from the seizure focus.

Multichannel Analysis of the Flow of Brain Activity

One important feature of epileptic seizures is the pattern of regional and remote seizure propagation to different parts of the brain. Indeed, this pattern is (at least in part) what produces the clinical manifestation of a seizure. Single channel analyses, either alone or combined with multichannel composites, provide tools only for quantifying patterns of spread, but do not provide information regarding the actual flow of activity.

Late in the last century, methods for analysis of flow – based on multichannel autoregressive models – were developed and first applied to analysis of EEG. The directed transfer function method (DTF) uses a multivariate autoregressive process (MVAR) to evaluate the flow of activity patterns in a set of several time series. The model assumes that the value of the signal $\boldsymbol{x}$ at a time t depends on its p previous values and the random component $\boldsymbol{e}$. Analyzed signals (from K different channels) are treated as realizations of one multivariate stochastic process. The MVAR process can be expressed as:

$$\boldsymbol{x}(t) = -\sum_{j=1}^{p} \boldsymbol{A}_j \boldsymbol{x}(t-j) + \boldsymbol{e}(t)$$

where $\boldsymbol{A}_j$ is a $K \times K$ MVAR coefficients matrix, $\boldsymbol{e}(t)$ is a zero mean uncorrelated residual noise vector with covariance matrix $\boldsymbol{V}$, and p is the model order. Estimation of the $\boldsymbol{A}_j$ coefficients and matrix $\boldsymbol{V}$ can be obtained from estimated covariance matrix $\boldsymbol{R}(n)$ by solving the Yule–Walker equation:

$$\sum_{j=0}^{p} \boldsymbol{A}_j \boldsymbol{R}(-r+j) = 0$$

Where $\boldsymbol{A}_0$ is equal to the $K \times K$ identity matrix. To determine the correct value of model order p, the Akaike Information Criterion is used.

Because EEG activity may be understood in terms of rhythms and oscillations, it is useful to describe the spectral properties of their signals. For this purpose the MVAR equation may be transformed to the frequency domain. Then,

$$\boldsymbol{X}(f) = \boldsymbol{H}(f)\boldsymbol{E}(f) \quad \boldsymbol{H}(f) = \left(\sum_{j=0}^{p} \boldsymbol{A}_j e^{-i2\pi f f \Delta t}\right)^{-1}$$

where $\boldsymbol{H}(f)$ is the transfer function of the multichannel system, f is frequency, and Δt is the sampling interval.

The element h_{kl} of the matrix $\boldsymbol{H}(f)$ describes the transfer function between the kth output and the lth input of the system. If the element h_{kl} of $\boldsymbol{H}$ is equal to zero, the hypothesis that $\boldsymbol{x}_k(t)$ causes $\boldsymbol{x}_l(t)$ can be rejected. The matrix is not symmetric, meaning that transfer from channel k to channel l is different from that in the opposite direction. In this way, the directional properties of a multichannel system may be obtained and interpreted as causal relationships, signal flows, or activity transfers. The $\boldsymbol{H}$ matrix has been used to calculate DTF which is a normalized $|H_{kl}(f)|^2$.

The first applications to EEG from epileptic patients showed flows of activity from the epileptic focus not only at the seizure onset but also during later phases of seizure evolution. The limitation of the original DTF method is that it requires at least weak stationarity of data during analysis period; too short periods of analysis lead to overestimation of parameters. This constraint limits applicability of the DTF method in analysis of seizure activity to relatively few periods during the seizure when at least 5 s of data can be regarded as quasistationary.

Recently, the DTF method has been applied to the analysis of event-related potentials. The new method, short-time DTF, takes advantage of multiple trials available for analysis to improve the data-to-parameter ratio in short analysis windows. Since it is common in individual patients for seizures originating from the same focus to produce very similar EEG signals (especially when recorded intracranially), the same method was thought

to be applicable to epileptic seizures. In the short-time DTF, the estimator of covariance matrix $\boldsymbol{R}(n)$ is computed from all data epochs which are assumed to be realizations of the same stochastic process. However, this method does not distinguish between direct and indirect flow. To address that problem, the short directed DTF (SdDTF), which multiplies the original DTF values $|H_{kl}(f)|^2$ by the partial coherence function, was introduced to allow for identification of direct flows between two channels.

The SdDTF provides information of flow for each frequency separately. Usually these values are integrated in frequency bands of interest to represent average flow within a specific band. To better visualize the flow of activity in brain, we represent them as arrows between relevant electrode sites projected on an MRI image (see Figure 3); the color and width of each arrow represent relative strength of the flow.

Results

Seizure Complexity

There are misperceptions that rhythmic seizure activity like the organized activity (theta–alpha) often seen in mesial temporal lobe seizures is a low complexity signal. This belief seems to originate from early work on chaos theory. However, several definitions of complexity exist – and many references often only use it as a concept without proper mathematical definition of the quantity. The mathematical definition of a single measure to quantify the complexity of the EEG signal is a necessary step to assess the pattern of complexity associated with epileptic seizures. The GAD was therefore introduced to provide this quantitative measure of signal complexity. GAD computation relies on the MP decomposition to obtain a description of the time–frequency content of the signal which can be quantified. Both MP reconstruction and GAD complexity are represented together as a convenient way to assess the frequency and dynamical properties of a seizure simultaneously. GAD reveals that there is often an increase of signal complexity associated with the onset of epileptic seizures and associated with the dynamical changes of each seizure.

Figure 1 shows an example of such a representation, with the time–frequency reconstruction of a complex partial seizure and the GAD complexity superimposed on it. The original ICEEG signal of the epileptic seizure recorded from a contact near the seizure focus is plotted (a) above the reconstruction. Two different zoom levels are added below to show a more detailed view of the dynamic changes in the EEG during the seizure (b) and at the seizure onset (c).

Intrinsic dynamics of a seizure vary during seizure evolution but typically include various phases with different levels of complexity corresponding to different phases of the seizure. The seizure onset period is associated with a dramatic increased complexity. Later in the seizure, the relatively lower complexity of a clearly rhythmic pattern is often observed; the complexity at this point, however, remains much greater that the default baseline complexity. The termination pattern is associated with higher complexity also, and in this example is followed by a common low complexity (but high amplitude) postictal pattern. The complexity in this example dropped below preictal levels and returns to normal baseline level quasiexponentially.

Seizure Onset

The stereotyped nature of epileptic seizures in a given patient is a characteristic that clinicians rely on to help assess the origin of epileptic seizures. Even a sophisticated measure of complexity (e.g., GAD) reveals epileptic seizures of the same type, which often follows a similar time course in a given patient. This similarity of complexity pattern is observed for mesial temporal onset seizures as well as for neocortical onset seizures. This similarity of pattern of onset is even seen with partial seizures and partial seizures with secondary generalization. Again, for both mesial temporal and neocortical onset seizures, the early period of the seizure evolution has similar dynamics, regardless of the subsequent electrical and clinical evolution of the seizure.

The link between EEG patterns of seizure onset and region of onset – which has been previously explored by traditional means of analysis – requires further study to assess the localization potential of epileptic seizures onset based on the focal ictal increase in GAD. The time–frequency decomposition and GAD appear to be suitable for detecting the occurrence of high-frequency activity at the onset of seizure. Such high frequency activity is often regarded as a strong indicator of epileptogenesis in the corresponding area.

Seizure Propagation

Despite the similarity of onset pattern within a given patient, seizures can vary in degree of propagation. The clinical definition of simple partial seizures and complex partial seizures, with or without secondary generalization, fail to distinguish the whole spectrum of propagation patterns that can be observed.

When GAD information is computed for all channels, multichannel propagation patterns can be displayed for each seizure. Figure 2 represents three different complex partial seizures from the same patient with left temporal neocortical onset and variable degrees of propagation to the frontal lobes. The stereotyped nature of epileptic

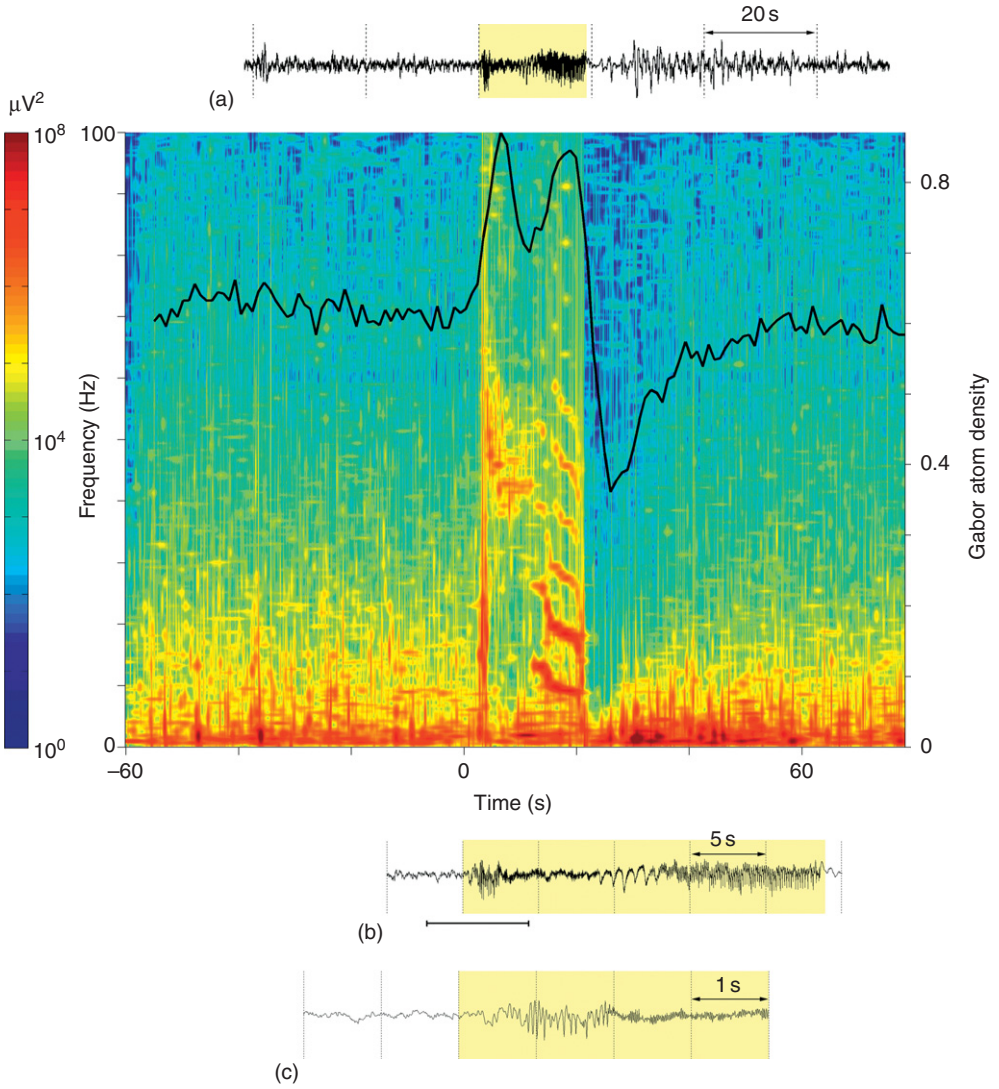


Figure 1 Time–frequency reconstruction and GAD complexity for a single channel intracranial EEG (ICEEG) recorded from a neocortical onset seizure. The time–frequency decompostion is obtained by matching pursuit analysis and then reconstructed in time–frequency. Black trace represents the GAD measure of complexity of the signal. Above (a) is the full EEG signal analyzed for this decomposition. Below the EEG of the seizure is zoomed over the entire seizure (b) and over the onset (c).

events is remarkable in the early period of the seizure, with almost identical timing and patterns of propagation of the epileptic activity over the temporal lobe channels. However, the extent and duration of that propagation is variable within the same clinical definition of a complex partial seizure. The existing seizure classification is currently under review, with much discussion regarding links to clinical symptoms; for example, alteration of consciousness is a variable which is at times difficult to quantify (e.g., with frontal lobe partial seizures). While it appears that a certain degree of partial seizure propagation is needed to affect consciousness, there has not yet been any clear determination of the extent and duration required to produce these alterations in awareness.

Analysis of Flow

While GAD can clearly show the patterns and extent of seizure propagation, its single channel basis does not reveal the direction of flow of activity that produces these patterns of propagation. SdDTF and other methods based on multichannel autoregressive modeling derive all information from cross-correlation between channels. Thus, SdDTF complements the analysis of the seizure dynamics from a single channel by providing additional information regarding flow of activity that underlies the pattern of seizure propagation. Figure 3 shows such example of determination, with the application of the SdDTF method to intracranial recordings during several

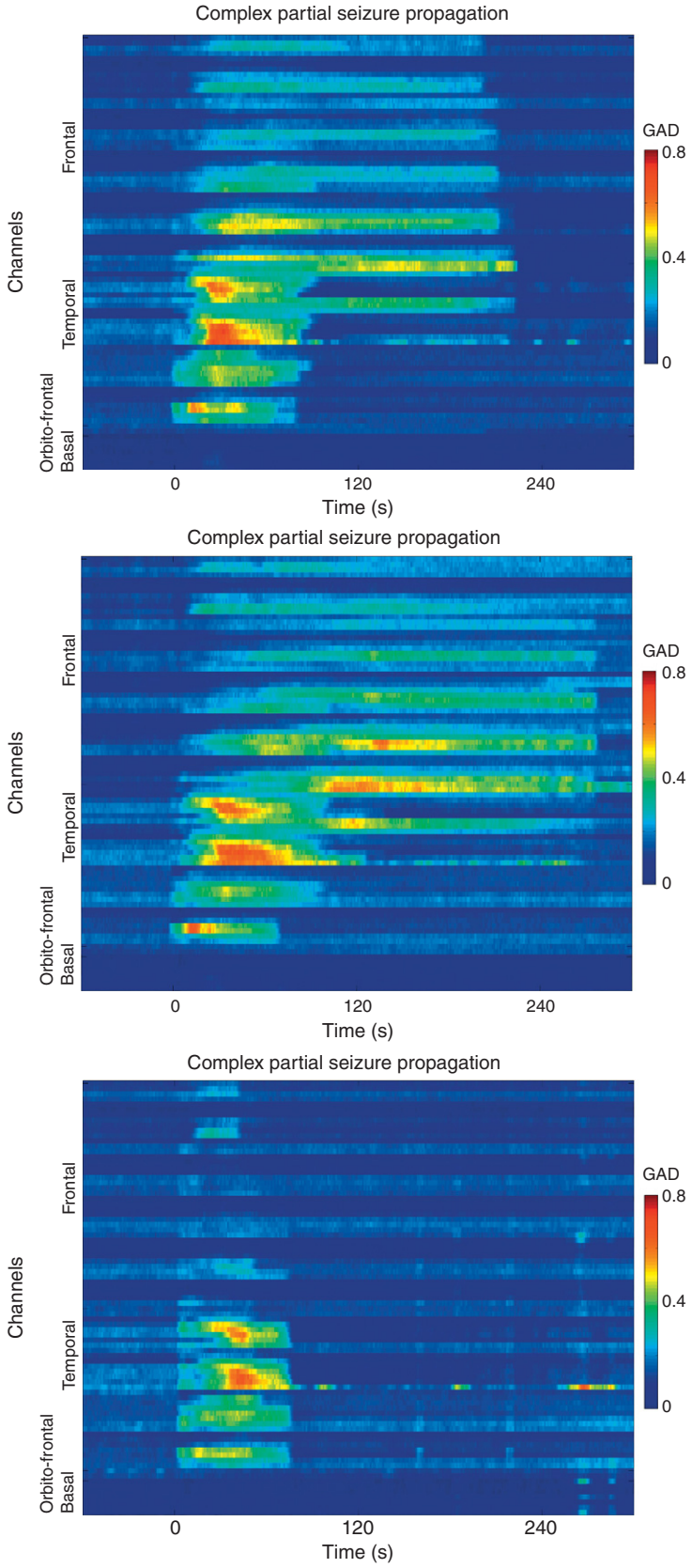


Figure 2 GAD-based propagation maps for three complex partial seizures from a given patient. All three seizures present similar pattern of onset and two of them a similar pattern of propagation.

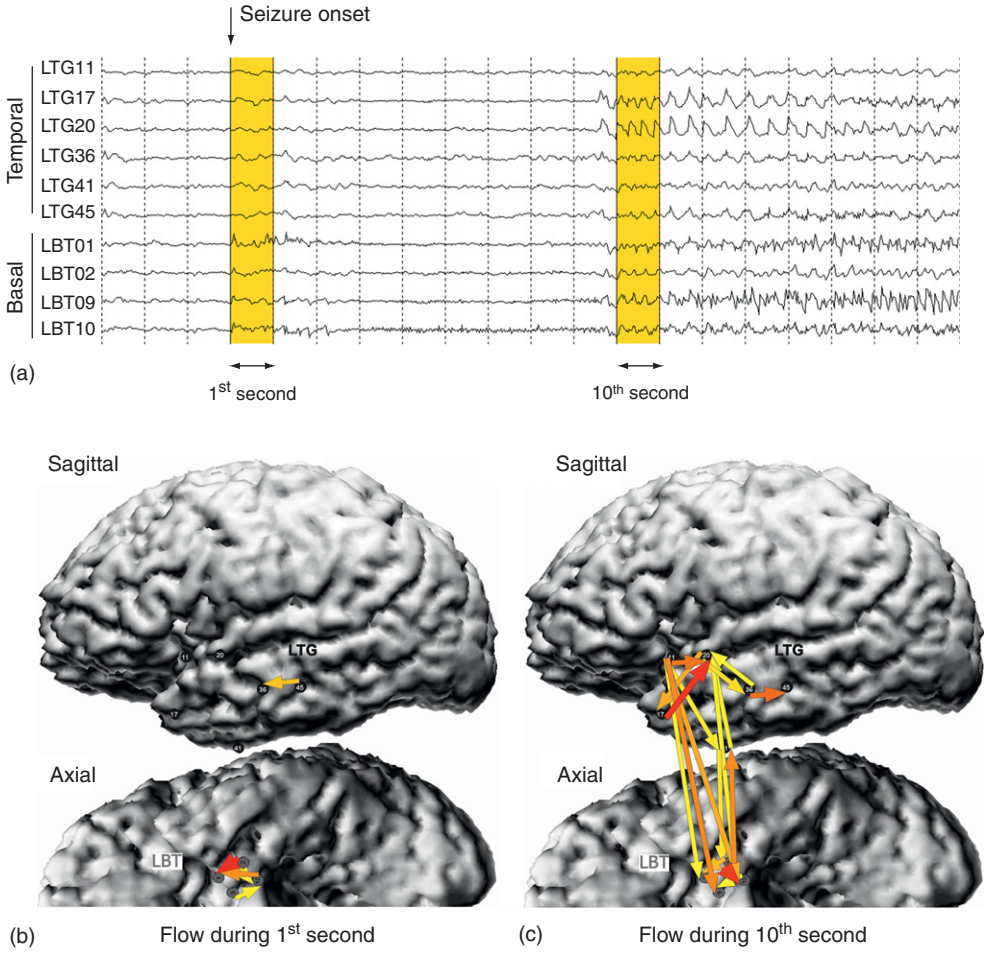


Figure 3 Illustration of changes in the pattern of flow of activity during seizure integrated from SdDTF in 0.5–25 Hz frequency band. The arrows projected on MRI image represent flow of activity computed from 10 similar seizures in same patient during first (b) and tenth (c) second after onset. The width and color of arrows are proportional to the integrated value of SdDTF in the 0.5–25 Hz band (low: yellow; high: red). For clarity, only flows with values greater than 75% of maximum flow during the whole seizure interval are shown. (a) Intracranial EEG of one of the seizures used in the analysis with the first and tenth second shaded.

complex partial seizures. The analysis was performed using 10 similar seizures. Panel (a) shows a 20 s recording of 10 channels during one of a series of temporal lobe onset seizures from a given patient. The results of the SdDTF analysis of the first and tenth second after onset (shown as shaded in panel (a)) are projected on an MRI of the brain (panels (b) and (c), respectively). The size of each arrow represents the strength of the flow. During the first second, the significant flows are visible only in the focal area (left mesial temporal lobe channels LBT), with the most prominent flow from LBT02 toward LBT01. During the tenth second after the onset, activity spread to other areas; the flow pattern clearly shows that there is now feedback between the basal temporal region LBT and the upper temporal grid area LTG. There is still strong flow within the basal area (red arrowhead LBT10 → LBT02), but there is also a strong flow within the temporal grid LTG17 → LTG20. There are also multiple flows between both regions, suggesting a possible feedback to the original seizure focus.

Future Applications

Both techniques described here are complementary and attempt to give a more detailed understanding of the dynamics of epileptic seizures. From seizure dynamics at the focus, to patterns of flow of activity and regional or global seizure propagation, these elements are offering new ways to examine epileptic seizures, particularly for seizure epochs after seizure onset.

The GAD and SdDTF methods allow for analysis of different characteristics of seizure dynamics and propagation. On the one hand, the GAD method allows for analysis of each seizure separately and provides excellent description of dynamical properties of the recorded signal

with very limited assumptions. In particular it does not rely on assumptions about linearity or stationarity. It gives clear information about changing seizure patterns over time for signals recorded from different brain regions. On the other hand, the SdDTF analysis provides information about interactions between regions – i.e., how the activity in one region may influence other regions. However, there are more limitations associated with this method than with the MP or GAD analysis. SdDTF requires multiple seizures for analysis, and as a result it shows only flow patterns which are common for all seizures. Also, the analysis can only optimally be performed on a limited number of channels, which leads to less spatial resolution than the available multichannel ICEEG recordings. However, both methods can be used in parallel to complement each other. The initial analysis with GAD method may suggest which channels are most involved in seizure propagation, and these channels can then be included in SdDTF analysis. Then SdDTF analysis may help in interpretation of the spread of activity shown by GAD. For example, it can show if there is significant feedback from areas of spread to the original focal area later in the seizure.

These methods of analysis can be applied to any electrical activity. Application to other modalities – for instance magnetoencephalographic (MEG) signals – could allow for more complete description of other activity during seizures.

Current recording techniques are employing increased frequency ranges, both high frequency and DC, requiring higher sampling rates and larger storage capability. Collection of larger amounts of data will provide more information, but in addition to requiring larger storage capability, also requires development of more efficient software for managing and processing these data.

Challenges remain for seizure localization. There is considerable interest in looking at high frequency and ultra-high frequency activity at the time of seizure onset, in the hope that these discharges may assist in defining the seizure focus. Recording this activity requires specialized high-density electrodes and appropriate sampling rates. Whether seizures, particularly neocortical onset seizures, originate from focal or broader regional areas is still not resolved. While there are patterns of ictal onset activity that are readily acknowledged, less clear is whether there are earlier preictal changes that can be detected and could assist in seizure localization. Indeed understanding what causes the transition from the interictal to the ictal state, both at the network and cellular levels, could lead to improved strategies to reduce these transitions. The stereotyped ictal patterns of onset and evolution seen in seizures from a given patient suggest that there are potential interventions (e.g., neurostimulation) that could interfere with this evolution and the number of disabling events experienced by the patient.

See also: **Models**: Computer Models of Seizures and Epilepsy: Understanding Epileptogenesis using Complex Large-Scale Models; **Neurophysiology**: Seizure Detection and Prediction; **Seizures**: A New Look into Epilepsy as a Dynamical Disorder: Seizure Prediction, Resetting and Control; Seizure Prediction.

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PHARMACORESISTANCE

Contents
Multidrug Resistance

Multidrug Resistance

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Introduction

Resistance to treatment with drugs is a problem across human disease, from cancers to infections. In epilepsy, a wide range of drugs is available for the control of seizures. These antiepileptic drugs (AEDs) have, putatively, a range of mechanisms of action, though in practice most AEDs share many mechanisms, and act primarily either on sodium channels or GABAergic processes. Most patients (70%) with epilepsy respond to AEDs and become seizure free – although the questions of how much of this response is intrinsic to the natural history of epilepsy and how much is caused by treatment with AEDs remain an issue. But in common with those with other diseases, there are patients who do not respond to treatment. Their epilepsy continues despite treatment with AEDs; they continue to have seizures despite using AEDs that work in other patients with the same type of epilepsy, and despite taking as much of a given drug or drugs as they can tolerate. This is a real clinical problem in need of a solution. For these patients, drug-resistant epilepsy is responsible for increased morbidity, and increased risk of premature mortality. Apart from the pervasive consequences for the patients with drug-resistant epilepsy themselves, drug resistance in epilepsy accounts for much of the costs of epilepsy to health service providers, not least through AED budgets. This article considers some of the latest developments and research in this area. The potential gains from advances in understanding drug resistance in epilepsy are enormous, and relate directly to other central nervous system diseases which may share similar mechanisms of resistance. Understanding the mechanisms of resistance might also cast light on the basic processes and mechanisms in epilepsy.

Background

There are many conceptual approaches to drug resistance in epilepsy. For example, one could apply a 'bottom-up' approach, attempting first to understand the mechanisms of the epilepsies, and related or relevant pathways and processes, and then from this knowledge attempt to predict or explore mechanisms of drug resistance. Enormous amounts of work have been undertaken to elucidate the basic processes that may underlie the generation of epileptic seizures. Of necessity, much of this work has been done in animal models. Undoubtedly, there has been tremendous progress, but it is still the case that for any single seizure type, we do not yet have a complete depiction of the underlying mechanisms at any level, from the genetic to the whole organism. Any 'bottom-up' approach is further complicated by the spectrum of epilepsies that exist, although of course there may be shared mechanistic final common pathways in the generation of seizures. With respect to drug resistance specifically, the 'bottom-up' approach has received comparatively little attention, although it is relatively easy to generate hypotheses from such an approach. For example, one could propose that there are likely to be epilepsies in some people caused by inherited abnormalities of the neuropeptide Y neurotransmitter system (which has been demonstrated to be antiepileptic); as far as we understand currently, there are no AEDs that act on this system, and therefore one might predict that patients whose epilepsy is due to a defect in the neuropeptide Y neurotransmitter system would have epilepsy that would currently be classed as refractory or drug-resistant – either because none of our drugs works directly on that system, and/or because drugs working on other systems might not be able to compensate sufficiently for defects in the neuropeptide Y system.

At the other end of the spectrum, one might consider what could be called a 'top-down' approach. One could consider the key agents, the AEDs themselves, examine in detail how they work, and thus propose hypotheses based on disruptions of the processes normally involved in their activity. This approach has been much more widely taken up in research of drug resistance in epilepsy.

One can consider the actions of AEDs on the body as pharmacodynamic effects, and the actions of the body on

AEDs as pharmacokinetic effects. Pharmacokinetic effects are likely to take place at several levels, including systemic absorption, metabolism in various tissues (including the liver), and also possibly in the brain itself. Drug distribution depends not only on intestinal and hepatic processes, but also on microdistributive processes occurring across the blood–brain barrier and in the immediate vicinity of neuronal and glial cells within the brain. Pharmacokinetic factors also include excretory processes, again occurring at various tissue levels in various organs. Pharmacodynamic effects, in contrast, occur at a much finer level of resolution, in or on neurons and glia in the brain. A parallel formalization of these two classes of events (pharmacokinetic, pharmacodynamic), alterations in which might contribute to drug resistance, effectively leads to the 'transporter' and 'target' hypotheses of drug resistance in epilepsy.

The target hypothesis states that drug resistance in epilepsy results from an altered interaction between drugs and their targets, when the concentration of the drug at the target would normally be sufficient to bring about the desired effect of those AEDs. The transporter hypothesis states that drug resistance in epilepsy comes about as a result of the inadequate local concentration of AEDs at their target(s), resulting from the pathological overactivity of multidrug transporter mechanisms.

One conceptual framework for considering candidates for either mechanistic hypothesis is as follows and any mechanism postulated to be involved must satisfy the following criteria:

1. mechanisms must be detectable in epileptogenic brain tissue
2. mechanisms must have appropriate functionality
3. mechanisms must actually be active in drug resistance
4. overcoming the mechanisms should affect drug resistance

Most researchers consider that if either of these classes of mechanism turns out to be relevant, combinations of these (and possibly other) mechanisms will contribute to drug resistance to varying degrees in individual patients. It must be mentioned that other possible mechanisms have also been proposed, but have received less research attention.

Methods

In our laboratory, two main methods of investigation have been used to address the transporter and target hypotheses. These methods are neuropathological studies, and population genetic studies. Neuropathological studies address the fundamental issue of the presence, distribution, and quantity of multidrug transporters, and other entities, within and beyond epileptogenic tissue. No hypothesis based on the activity of a protein putatively involved in drug resistance in epilepsy could be sustainable if that protein were truly not detectable within relevant areas of human epileptogenic brain tissue. Beyond this issue of detectability, the specific distribution, orientation, and quantity of such a protein would have implications for finer structures of the hypotheses – for example, proposed mechanisms of activity of transporter proteins in mediating resistance.

Determining the activity of any detected proteins, particularly given possible complexities of the distribution of the proteins, is very difficult in living human brain tissue, whether in vivo or ex vivo. Whilst microdialysis experiments are occasionally undertaken in living human beings, usually during epilepsy surgery, the precise positioning of a microdialysis probe can never be fully known, and is unlikely ever to achieve the degree of resolution required to formally test the potential role of proteins with specific microscopic distributions (see **Figure 1** for an example of the microscopic distribution of multidrug transporters). However, surrogate estimations of the role and activity of particular proteins, and associated mechanisms, might be achieved through population genetic studies. In principle, if a protein has a role in a given process, then genetic variation in the encoding gene that affects the function of that protein might have an association with a relevant clinical phenotype, thus providing supportive – if not more definitive – evidence for a role for that protein in the proposed process. Both of these areas of study are be described below.

Tissue Studies

Research in epilepsy benefits uniquely from the availability of freshly resected brain tissue, which of course must first be studied for clinical neuropathological diagnostic purposes; however, any surplus tissue may be used for detailed experimentation. This tissue is a valuable resource, which we must use as carefully and as comprehensively as possible. With the evolving 'omic' methodologies (genomic, transcriptomic, proteomic, and metabolomic), truly comprehensive tissue profiling at several levels has become impossible. There remains, nevertheless, an important position for detailed neuropathological studies of the distribution and quantity of proteins, for example using immunohistochemistry.

We have used tissue resected for therapeutic purposes from patients with drug-resistant epilepsy. Surgery is undertaken according to clinical imperatives, usually in the context of a comprehensive multidisciplinary assessment, when the benefits of focal resective surgery considerably outweigh the risks. The most common procedure undertaken is the resection of the mesial temporal structures, including the amygdala and hippocampus. This surgery is undertaken by different centres in different ways; in our own centre, removal of the mesial temporal structures follows the en bloc removal of the anterior

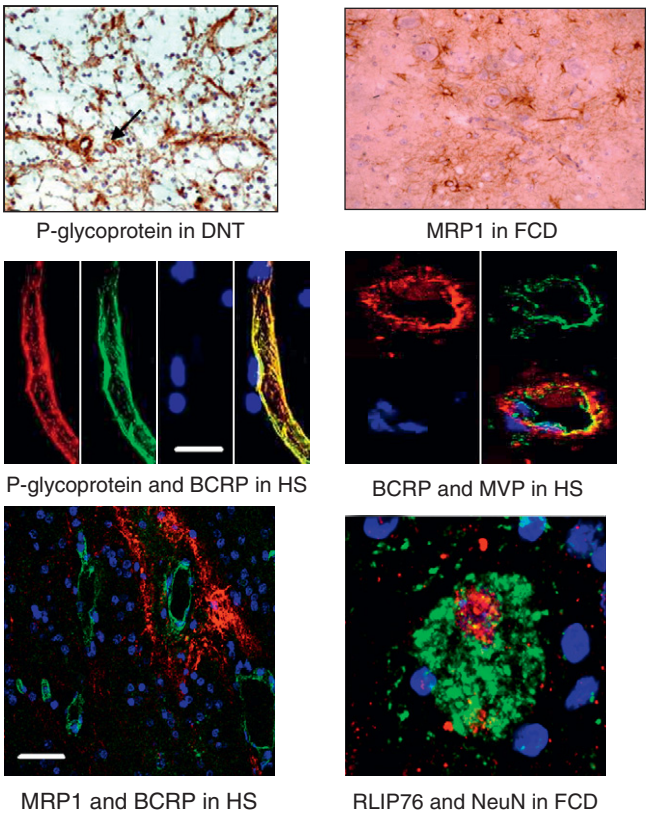


Figure 1 Examples of immunohistochemical studies in resected human epileptogenic tissue of putative multidrug transporters or related proteins. Reproduced from Sisodiya SM, Lin WR, Harding BN, Squier MV and Thom M (2002) Drug resistance in epilepsy: expression of drug resistance proteins in common causes of refractory epilepsy. *Brain* 125: 22–31; and Sisodiya SM, Matinian L, Scheffer GL, *et al*. (2006) Vascular colocalization of P-glycoprotein, multidrug-resistance associated protein 1, breast cancer resistance protein and major vault protein in human epileptogenic pathologies. Neuropathology and Applied Neurobiology 32: 51–63.

temporal lobe. A spin-off of this standard procedure is the availability of hippocampal and temporal neocortical tissue for clinical neuropathological, as well as research, purposes.

A major hurdle in such neuropathological studies is the lack of availability of completely normal control brain tissue from comparable regions. Histologically normal material does rarely become available, for example, when otherwise normal tissue has to be resected for purposes of access to lesions or relief of pressure following trauma. Histologically normal post mortem tissue is occasionally also available, but of course would often have suffered from agonal and post mortem changes, which to some extent limits its utility for various purposes. Temporal neocortical tissue, when histologically normal, can provide a class of comparative tissue for control purposes, having the advantages that it will be from the same individual, with the same germline genotype, the same history of epilepsy, the same exposure to AEDs, and the same post resection treatment. However, temporal neocortex is clearly anatomically different from mesial temporal (i.e., hippocampal) structures, and there are some limitations in possible comparisons. It is also likely to be the case that at the gene expression level, there are normal, and disease-related, differences between the hippocampus and temporal neocortex. Ideally, a variety of control tissues should be employed in such investigations.

Several immunohistochemical methods have been used in our own laboratory, ranging from routine single antibody studies in formalin-fixed, paraffin-embedded tissue, to semiquantitative analysis using immunofluorescently labeled antigens – all with preservation of anatomical specificity and the ability to provide some degree of quantitative information.

Population genetic studies offer a conceptually simple and powerful approach to testing mechanistic hypotheses. The principle is to compare distributions of genetic measures, such as genotype or allelic frequencies, between clinical cases (that have the trait in question) and controls (that do not). In such case–control association studies, critical aspects are a robust definition and application of phenotypes (traits) of interest, sufficient numbers of patients to be able to test a given hypothesis with sufficient power to produce a definitive result, comprehensive coverage of the variation-bearing structure in question (e.g., the gene encoding the protein of interest) and a competent genotype–phenotype association statistical framework. Subsequently, replication of association findings is often required, as well as functional work to identify the action of the variant.

Results

Tissue Studies

We have used standard immunohistochemical methods, in epileptogenic brain tissue containing a variety of pathologies and in comparator tissue, to study a range of transporters considered to be potential candidates for mediating resistance to AEDs. We have studied P-glycoprotein (also known as MDR1 or ABCB1), multidrug-resistance associated protein 1 (MRP1 or ABCC1), breast cancer resistance protein (BCRP, also known as MXR or ABCG2), major vault protein (MVP), and RLIP76. At various times and in various publications, these proteins have been implicated in the causation of drug resistance in various cancer cell lines and human tumours, or in other experimental studies of epilepsy. Other groups have also looked at these and other proteins. In general, the results tend to be concordant, although with some important differences with respect to the cell specificity of expression. In this article, we present only our own results (in summary form). Neuropathological findings from other groups are detailed extensively in the excellent review articles listed in Further Reading.

We have identified expression of P-glycoprotein in cells in which it is not normally or commonly detected (such as glial cells), within epileptogenic regions, at much greater levels than in comparator control regions. In our studies, we have not detected P-glycoprotein in normal or abnormal neurons from normal or epileptogenic brain regions. Other researchers *have* detected the presence of P-glycoprotein in neurons. This discrepancy raises an important point, which is that the specificity and sensitivity of antibodies and experimental protocols are known to vary between laboratories and tissues, making standardized procedures important.

We have also noted that MRP1 is detectable in glia, again with a much greater emphasis in epileptogenic regions compared with control regions. We also detected MRP1 in dysplastic neurons in regions of focal cortical dysplasia (FCD). In contrast to P-glycoprotein, which can be detected in the microvasculature, we have not detected MRP1 in the microvasculature in the brain. We have identified BCRP only in the microvasculature, and not in glia or neurons, from epileptogenic or other regions. We also noted the strong expression of major vault protein within dysplastic neurons and within blood vessels, but not within glia. Normal neurons did not appear to express major vault protein. We did not find RLIP76 expression within microvasculature or glial cells, but detected it only in neurons, especially within dysplastic neurons from areas of FCD.

More recently, we have explored P-glycoprotein expression at a quantitative level. In contrast to our expectations, quantitative immunofluorescence showed that there was less signal in the microvasculature from epileptogenic regions in comparison with comparator control regions. This finding was surprising. It clearly requires replication, but were it to be correct, this finding could suggest that there is less P-glycoprotein quantity, and possibly less P-glycoprotein activity, in epileptogenic brain region microvasculature compared to control regions. Most workers in the field have assumed, although no one has formally tested in situ (in vivo or ex vivo) that there is *more* P-glycoprotein within the microvasculature in epileptogenic regions compared to control regions. Our findings, although in contrast to expectations, nevertheless are in keeping with other work that suggests that the blood–brain barrier may in fact be impaired in epileptogenic brain regions in chronic epilepsy, both in animal models and human epileptogenic brain tissue. It also supports the view that overexpression of P-glycoprotein, in particular, in epileptogenic brain regions in glia, might represent a 'second' blood–brain barrier, as a pathophysiological response to impairment of the normal blood–brain barrier at the level of the microvasculature.

Some of these immunohistochemical findings are illustrated in **Figure 1**.

Population Genetic Association Studies

In cohorts of patients with drug-resistance or drug-responsive epilepsy (with specific definitions), we examined the distribution of a particular variant in the gene *ABCB1*, encoding P-glycoprotein. This study was undertaken some time ago, but remains a robust study. We demonstrated an over-representation of the CC genotype (in comparison to the TT genotype) at the 3435 locus in *ABCB1* in patients with drug-resistant epilepsy, in comparison with patients with drug-responsive epilepsy. There is a considerable body of evidence demonstrating that the C allele is associated in western European populations with an increased quantity and activity of P-glycoprotein. These initial association findings were therefore in keeping with the hypothesis that an increased quantity and activity of P-glycoprotein were associated with the drug-resistant phenotype in epilepsy.

However, this initial finding was not replicated in the only published study structured exactly as our study. The second study utilized a larger cohort, and concluded that this polymorphism in *ABCB1* could not have a major role to play in the drug-resistant phenotype. Several other association studies have examined the same problem, but all have used different phenotypic definitions and varied in their coverage of genotypic variation. None can be considered a formal replication of our study. Formal comparison of the studies is therefore difficult, but it would be reasonable to consider that it is unlikely that the 3435 variant has a major role to play in mediating drug resistance in all patients with epilepsy. Currently, studies of larger sample sizes and more comprehensive

representation of variation within *ABCB1* would be deemed appropriate.

It is of great interest that in almost every other human disease phenotype in which an association with this polymorphism in *ABCB1* has been sought, there has been equal ambiguity. Whilst some disagreement between studies might be attributed to methodological differences, very recent work suggests that: (1) this synonymous polymorphism might affect mRNA stability, leading to allelic imbalance for expression despite the polymorphism being noncoding and (2) even more interestingly, any allelic influences on expression might additionally depend on rates of gene expression. One implication of these findings is that very careful matching of replication cohorts is necessary, as the impact of the polymorphism on protein levels and substrate specificity might depend on expression demand – as influenced, for example, by seizures, drug treatment, or hypoxia. There is clearly much complexity yet to be explored, with exciting possibilities still in play for P-glycoprotein and *ABCB1*. For example, it seems that substrate-specificity changes may be plastic, and themselves influenced by the presence of various factors, including functional inhibitors of P-glycoprotein. Another implication of this complexity is that we are still some way from human trials of P-glycoprotein inhibitors.

In conclusion, whilst association studies to-date would seem to rule out a major role for the C3435T variant in *ABCB1* in the causation of drug resistance overall, a role for this variant, particularly in patients with more resistant epilepsy, has not yet been excluded.

We have also employed association studies to consider the target hypothesis of drug resistance, focusing particularly on variants in *SCN1A*, encoding the shared target of many AEDs, the brain-expressed sodium channel alpha-subunit. Whilst we have not been able to demonstrate any direct association with response phenotypes, such as seizure freedom or drug resistance as defined in the *ABCB1* study, we have shown that a common variant in *SCN1A* associates with maximum clinical dose of the alpha-subunit binding AEDs, carbamazepine, and phenytoin, used in individual patients. We have gone on to show that the associated polymorphism is itself likely to be causal, directly influencing alternative splicing of *SCN1A*. This work illustrates the power of pharmacogenetics not only to explain drug-related parameters such as drug dosing, but also to contribute to an understanding of disease biology – in this case, throwing new light on the role of alternative splicing of *SCN1A* in neuronal biology (its control and disruption).

Finally, others and we have shown alterations at a neuropathological level of the expression of cation-chloride transporters, NKCC1, and KCC2. Such expression changes can alter the transmembrane chloride gradient and thus the action of GABAergic neurotransmission on neuronal function – in turn, affecting drug targets.

As we see more and more influences on drug targets and transporters, mechanisms of drug resistance can be seen to be increasingly complex and interdependent. Candidate gene studies become obsolete in this setting and in the context of rapidly falling costs of genotyping. It is now possible to undertake population genetic association studies at the level of genome-wide assessment of variation, for example typing 550 000 single nucleotide polymorphisms (SNPs) in thousands of individual patients. We have started using these methods which, although complex, will permit comprehensive evaluation of genetic influences and mechanisms in drug resistance.

Future Goals

A detailed definition and description of mechanisms of drug resistance in epilepsy is an important goal for those involved in caring for patients with epilepsy. Drug resistance remains a major problem. It increases risks at all levels for patients who are affected. Understanding mechanisms of such resistance might lead to new therapeutic approaches, or to better use of current treatments. With significant advances in technology, including advances in 'omic' methodologies, and with increasing cohort sizes being assembled at all levels (ranging from the tissue to DNA data banks), the potential now exists for definitive and comprehensive studies of drug resistance. With the possibility of thorough coverage of the entire human genome, and with the possibility of sophisticated postgenomic studies at the level of individual cell populations, for example using laser cell microdissection, a thorough mechanistic explanation of drug resistance should be possible. For any such studies, however, careful phenotype definition, robust statistical analysis, replication, and continued testing of the implications of such research will be necessary if proper progress is to be made. The potential benefits, both for understanding and treatment, are great. It remains important to learn from other fields that may be more advanced, especially from the field of oncology, with respect to drug resistance. It remains critical to be clear about potential mechanisms and the consequences of their manipulation before treatment trials are attempted in humans.

Acknowledgements

Work in the author's group has been supported by the UK Medical Research Council (grant G69520) and the UK National Society for Epilepsy. The work undertaken at UCLH/UCL received a proportion of funding from the UK Department of Health's NIHR Biomedical Research Centres funding Scheme.

See also: **Antiepileptic Drugs**: Pathways of Drug Diffusion into the Epileptic Brain; **Pharmacoresistance**: Animal Models of Drug-Resistant Epilepsy; Brain Imaging of P-glycoprotein Function in Drug-Resistant Epilepsy; Cellular and Molecular Mechanisms Contributing to Pharmacoresistance in Human Neocortical Tissue; Mechanisms of Pharmacoresistance.

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SURGERY

Contents

Approaches for Individually Optimized Surgical Intervention

Approaches for Individually Optimized Surgical Intervention

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Introduction

Despite the use of anticonvulsant drugs, a considerable number of epilepsy patients still suffer from pharmacoresistant seizures. Of these, 2000–5000 patients per year may be candidates for epilepsy surgery.

Epilepsies can be differentiated into different epileptic disorders with individually varying focal, regional, or generalized involvement of neuronal networks. For the optimal selection of suitable candidates for epilepsy surgery, presurgical evaluation strategies have been developed aiming at (1) localizing the focal epileptic activity responsible for the seizure generation; (2) establishing the relationship of the epileptic network to functionally-important brain areas; and (3) determining the 'reserve capacity' of the nonresected brain tissue.

Because of the existence of a large number of pharmacoresistant epilepsies, and the growing clinical awareness that delayed surgical intervention can result in a severe impact on quality of life (injuries, sudden unexpected death, school performance impairment, driving license, loss of employment, and indirect economic consequences for the society), early surgical intervention is required.

Presurgical Evaluation Techniques

For the detection of the networks responsible for generating epileptic activity, as well as for determining functionally-eloquent regions in the brain that are not readily accessible for surgery, invasive surgical recordings using subdural or intracerebral electrodes are still used. These approaches

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provide excellent information from neocortical and subcortical brain regions. But there are also disadvantages – e.g., distortions produced by these recording approaches, and risks of hemorrhage and infection – that have led to a more and more restricted use of invasive electrodes. High resolution imaging (MRI, fMRI, PET, SPECT) has improved the detection of epileptogenic lesions and functional changes associated with those lesions. Although fMRI, SPECT, and PET provide indirect measurements of brain function by changes of perfusion (interictually or ictally), direct measurements of epileptic activity and function is obtained by EEG and MEG. Long-term video-EEG monitoring proves the epileptic nature of seizures, and in addition is necessary for a detailed classification of seizure types. MEG recording has been used predominately to document interictal activity. With MEG techniques, the differential conductivities of different tissues in the head (e.g., skull, meninges, cerebral spinal fluid, large lesions, asymmetries from previous surgery) are less influential in determining localization of epileptic networks, as compared to EEG. Both techniques are complementary for presurgical evaluation. Magnetic source imaging (MEG source localization visualized within brain anatomy) is helpful in correlating the localization of the epileptic activity with lesion or function (somatosensory, motor, visual, auditory, and language areas). Although this source localization does not strictly provide a physical point, the MEG approximation of the center of focal activity can provide important information for early optimization – i.e., classification of epilepsies with regard to differentiation into lobar categories.

In addition, the noninvasive and safe screening of patients for epilepsy surgery has improved. These noninvasive data can then be used to guide invasive electrode implantations – or even help determine that electrode implantation should be avoided. These presurgical procedures, and additional neuropsychological investigations of material specific memory and other cognitive functions – before and after surgery – have improved the basis for our understanding of brain function. In a dialectic process, they also critically participate in developing the strategies for presurgical evaluation and surgical interventions (Figure 1).

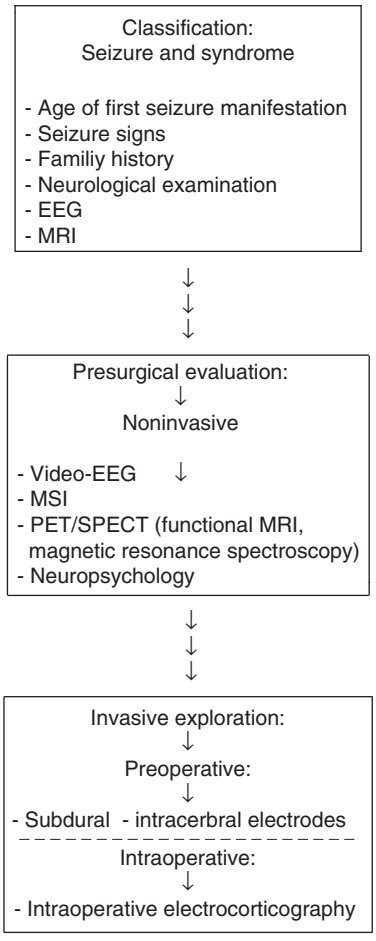


Figure 1 Diagnostic strategies in different phases of presurgical evaluation.

Surgical Interventions

Resective Surgery

A first single systematic approach for resective epilepsy surgery was performed by Victor Horsley and Ferrier. Later, in specialized epilepsy centers like those in Montreal, Paris, Zurich, and Cleveland, epilepsy surgery was performed in large numbers, particularly for temporal lobe and extra temporal lobe epilepsies. Currently, epilepsy surgery is carried out world-wide. The aim of successful epilepsy surgery is twofold: to provide seizure control, and to preserve normal brain function. Therefore, tailoring the resection to the individual patient should be carried out. Compared with drug treatment, resective surgery can control seizures more effective if a careful selection of candidates is provided. The profile of efficacy and side effects of epilepsy and resective epilepsy surgery are shown in Table 1.

Undercutting

If no resection can be performed – e.g., because of a major risk of considerable functional deficit – the microsurgical cutting of horizontal fibers (cuts spaced at ~5 mm) was suggested by Morrell. Although this procedure can lead to improvement, most clinical experience suggests that only a reduction of the intensity of seizures can be obtained by this technique. As a simplified model for the interpretation of such effects, the nucleus shell structure model can be used. In this model, the onset region of the seizure is viewed as the 'nucleus,' and phases of seizure propagation (during the temporal evolution of the

Table 1 Profile of efficacy

Surgery	*Seizure free (%)*	*Improved (%)*	*Complication quote (%)*
Temporal lobe resections			
Antero-mesial standard resection	50–70	20–30	3–10
Selective amygdala-hippocampectomy	50–70	20–30	3–10
Extratemporal neocortical resection	30–50	30–40	3–10
Lesionectomy	40–60	10–25	3–10
Hemispherectomy	70–90	5–15	5–20
Multilobar resection	40–50	30–40	5–20
Callosotomy	0–10	60–70	5–15
Multiple subpial transection	20–30	40–50	5–15

seizure) viewed as 'shells.' If only the networks belonging to the shell are disconnected (by this subpial cutting procedure), only a reduction of seizure intensity may occur. If the nucleus is extinguished, the whole seizure is controlled. Multiple subpial transection appears to influence only the shell networks.

Callosotomy

In multifocal generalized epilepsies (e.g., Lennox–Gastaut syndrome), parts of the corpus callosum can be selectively transected in order to prevent rapid propagation of seizure activity from the side of origin to the contralateral hemisphere. There are anterior and posterior variants of this procedure, with the anterior two-third section of the corpus callosum being the most frequently used. Callosotomy leads to control of predominantly severe tonic seizures. In rare cases, as side effect, a 'split brain' defect can occur.

Brain Stimulation

Electrical stimulation of different brain structures, such as paleocerebellum, centromedian thalamic nuclei, hippocampus, and cortex involving a focal epileptic region, may result in varying effects with regard to seizure control. Seizure reduction up to 96% has been reported in single cases, but other studies report no differences comparing stimulation on and off periods. The pathophysiological mechanisms for a possible deep brain stimulation antiepileptic effect remain to be clarified. Mechanisms that have been postulated include effects on synaptic transmission, long-term potentiation, kindling, quenching, and depotentation.

Focal Radio Surgery

In cases where no resection or transection can be performed, an alternative treatment approach involves precise focal irradiation by sterotactic linear accelerator radiation (LINAC/NOVALIS systems) or ^{60}Co sources (gamma knife). Although the first strategy aims to modulate focal epileptic activity by inducing functional changes, the gamma knife can be used for destroying tissue – e.g., in pharmacoresistant mesial temporal lobe epilepsies – as well as for callosal radiosurgery. Seizure reduction or control has been observed in 40–70% of cases, using the gamma knife or linear accelerator radiation. Seizure control often occurs at a long latency – as much as one and a half years after radiation.

Implantation of Anticonvulsants

The implantation of pumps containing anticonvulsant agents, triggered by seizure activity, is another novel, and still experimental, therapeutic approach that is under development.

Newer Integrative and Pre- and Intraoperative Approaches for Tailored Surgical Interventions

A large array of noninvasive and invasive pre- and intraoperative procedures can be used within a neuronavigation system. After completing a tailored resection, resected brain volume can be determined by intraoperative MRI to determine the adequacy of the resection. An example of this approach is shown in a patient with pharmacoresistent focal epilepsy and periventricular nodular heterotopia (**Figure 2**). Because invasive presurgical long-term monitoring was not possible in this case, MSI (magnetic source imaging) was used together with fMRI/DTI to correlate the localization of the focal epileptic activity with the course of the visual pathways (MR tractography), neuronavigation, intraoperative ECoG and MRI. The periventricular heterotopia in **Figure 2** (marked by white arrow) and a radial band extending to the thinner temporal lateral cortex (marked by red arrow) were the suspected anatomical substrates of the functional abnormality. The relationship of two zones of focal epileptic activity (localized by means of MSI) to the optic radiation is shown in **Figure 3**.

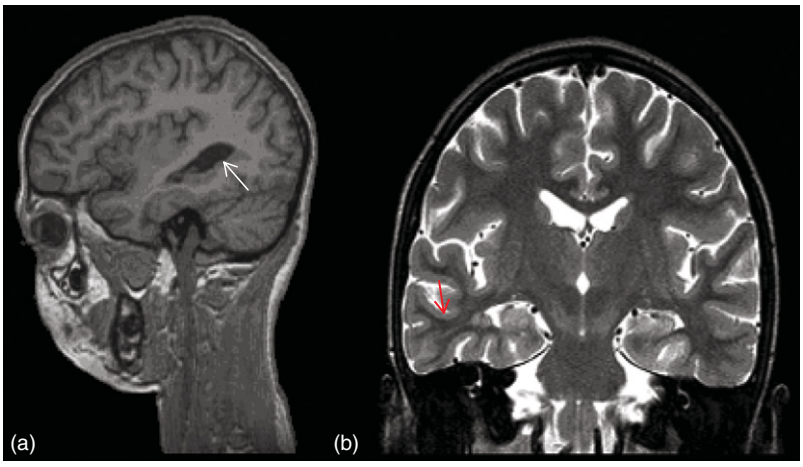


Figure 2 T2-weighted (axial) and MPRAGE (sagittal) MR imaging show periventricular heterotopia in the right basal temporo-occipital area (marked by arrow).

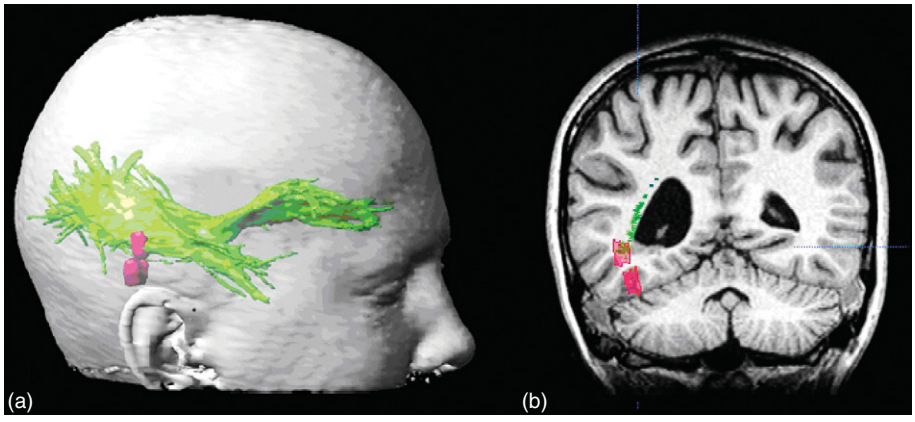


Figure 3 (a) Streamtube visualization of the right optic radiation based on diffusion tensor imaging. For navigation, a 3-D object representing the optic radiation (wrapping the individual fibers) and two distinct MSI foci (red) are generated. (b) Relation of optic radiation (visualized as streamlines) to MSI foci.

Intraoperative MSI-guided electrode implantation (intracerebral depth and subdural) confirmed the MSI localization and the predominance of the laterobasal focus, which was resected without functional deficit (Figure 4).

This case gives rise to several important questions. For example, what is the large mesial periventricular lesion, and what are the functional consequence of the abnormal shape of the hippocampus and the thinner laterobasal temporal cortex? In this case, the epileptic neuronal network extends from the heterotopia to the temporal neocortex, with predominance of epileptic activity in the laterobasal neocortex. It is known that developmental lesions in mesial periventricular structures can produce pathological wiring with distant lateral neocortical areas, which may explain why epileptic activity was found in the laterobasal cortical area. This latter area was resected, but focal activity close to the heterotopia was not resected because of a significant risk of a visual disturbance (as indicated by fiber tract imaging in the neuronavigation). Within the first month after surgery, there were three complex partial seizures (two provoked by fever); in the following 18 months, no more seizure occurred. Therefore, we conclude that important epileptic networks must have been resected or disconnected from the major epileptic lesion.

Clearly, consideration of these results must stimulate discussion concerning the definition of the epileptic 'zone.'

Although Penfield and Jasper required resection of the epileptic zone in their early work, and recommended in addition the elimination of the 'early propagation area,' the above case suggests that severing subnets of epileptic networks may lead to seizure control, leaving even larger lesions untouched. It should be noted that the early use of the term 'epileptic zone' was derived from a basically

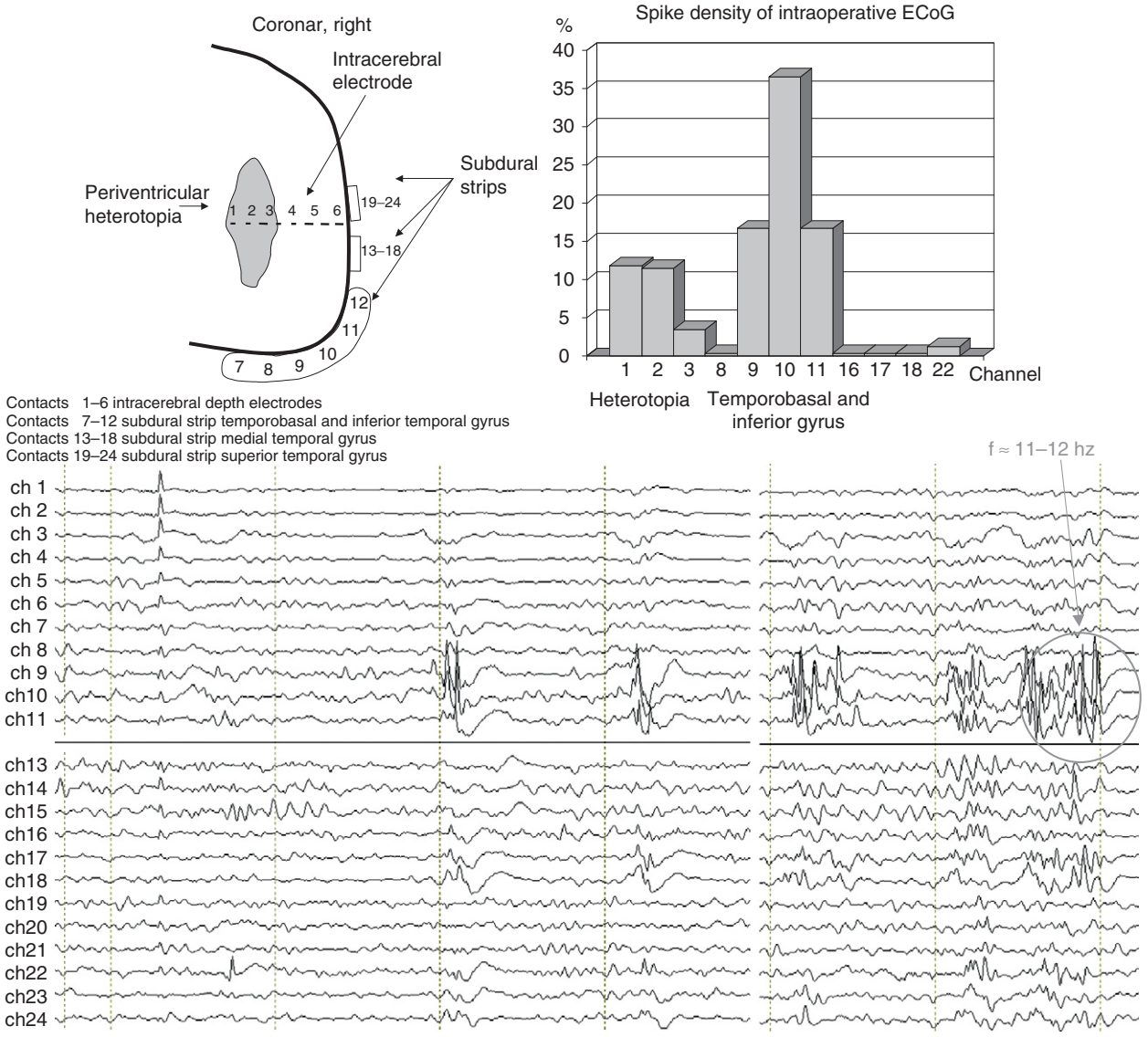


Figure 4 Intraoperative electroencephalographic recordings with platinum electrodes close to the two suspected spike foci as suggested by MSI (white arrows: temporobasal strip, thin white arrow: depth electrode to heterotopia). Additional measurements were picked up from the lateral cortical surface. The electrode position was confirmed by intraoperative T1- and T2-weighted high-field-MR imaging. MSI-guided electrode implantation of intracerebral depth and subdural electrodes; spike activity in lateral cortex and periventricular heterotopia. The neocortex shows predominant spike wave activity and 11–12 s^{-1} polyspikes during intraoperative ECoG.

two-dimensional view to the brain. In the future, a more realistic view of 'epileptic networks' should be developed, based on a three-dimensional approach.

See also: **Antiepileptic Drugs**: Local Delivery of Antiepileptic Drugs; **Imaging**: Magnetic Resonance Imaging in Epilepsy Research: Recent and Upcoming Developments; Structural Imaging in Epilepsy; **Networks**: Network Interactions in the Limbic System and Epileptiform Synchronization; **Neurophysiology**: Seizure Onset and Propagation: Application of Advanced Signal Analysis to Intracranial Recordings.

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